



Autoimmune Disorders

*Symptoms, Diagnosis
and Treatment*

Maria E. Petrov
Editor



IMMUNOLOGY AND IMMUNE SYSTEM DISORDERS

AUTOIMMUNE DISORDERS: SYMPTOMS, DIAGNOSIS AND TREATMENT

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**MARIA E. PETROV
EDITOR**



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Preface

Autoimmune diseases arise from an overactive immune response of the body against substances and tissues normally present in the body. In other words, the body actually attacks its own cells. The immune system mistakes some part of the body as a pathogen and attacks it. The treatment of autoimmune diseases is typically with immunosuppression—medication which decreases the immune response. This book presents current research in the study of autoimmune disorders with a focus on autoimmune liver diseases; Graves hyperthyroidism; system lupus erythematosus; Behcet's disease; and Sjogren's syndrome.

Chapter 1 - Autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are three distinct autoimmune liver diseases. Immunoglobulin G4 (IgG4) associated cholangitis is another immune disorder which mimics PSC and is characterized by formation of biliary strictures responsive to steroid therapy. The simultaneous or successive coexistence of PBC and AIH or PSC and AIH is called overlap syndrome. AIH is a chronic inflammatory immune-mediated liver disease characterized by elevated transaminase levels, hypergammaglobulinemia and histological features of interface hepatitis. The diagnosis of AIH is based on the scoring systems of the International Autoimmune Hepatitis Group (IAIHG) after exclusion of other causes of chronic liver disease. AIH is treated with prednisone alone or in combination with azathioprine and both strategies are equally effective.

PBC and PSC are both chronic cholestatic liver diseases with slowly progressive courses. Fatigue and pruritus are the most common symptoms but the majority of the patients at first presentation are asymptomatic. The diagnostic criteria for PBC include serum alkaline phosphatase levels at least twice the upper limit of normal and a positive test for serum antimitochondrial antibodies. A liver biopsy specimen showing inflammatory changes involving the bile duct supports the diagnosis but is not mandatory. Ursodeoxycholic acid is the only FDA-approved therapy for PBC in a dose of 13-15mg/kg/day. PSC is most commonly diagnosed with endoscopic retrograde cholangiopancreatography (ERCP), although magnetic resonance cholangiography (MRC) is rapidly emerging as the first-choice diagnostic test. There is no effective medical treatment for PSC and liver transplantation is the only life extending therapy for end-stage disease.

IgG4 associated cholangitis is an immune disorder characterized by formation of biliary strictures and frequently involves extrahepatic bile ducts. It is often associated with other autoimmune disorders such as autoimmune pancreatitis. The serum level of IgG4 is usually elevated and there is infiltration of IgG4 positive plasma cells in bile ducts. Clinically,

patients present with abrupt onset jaundice which responds to steroid therapy. Pathogenesis and standard diagnostic criteria for the overlap syndrome have not yet been established. In this review we discuss in detail the clinical presentation, pathogenesis, diagnosis and treatment of AIH, PBC, PSC, IgG4 associated cholangitis and overlap syndromes.

Chapter 2 - For some time now Barabas and colleagues have been describing the term autoimmunity as encompassing two beneficial and two harmful processes. The beneficial processes aim (a) to maintain tolerance to self by eliminating cellular waste and (b) to discriminate properly against abnormal self resulting in the destruction of emerging abnormal cell lines. The harmful processes are the opposite of the beneficial ones, i.e. autoimmune disease and cancer.

In the case of autoimmune diseases, pathogenic immune responses directed against one or more target antigens in the system can cause cell mediated or pathogenic autoantibody initiated injuries.

In the case of cancer, groups of abnormal cells invade previously normal organs, tissues, etc., causing harm or even death because the immune system does not receive the information necessary to recognize them and produce lytic autoantibodies to kill them.

In this chapter we highlight the pathogenic IgG autoantibody mediated immune events observed in an experimental membranous glomerulonephritis called Heymann nephritis which cause the chronic progressive autoimmune kidney disease. We describe how the disease is initiated and maintained, and how it can be diagnosed and terminated by the application of a new vaccination method we have developed and call modified vaccination technique. We also touch on how the same technique may be used to stimulate or upregulate immune response against exogenous antigens and cancer.

Chapter 3 - Two different sets of investigations are at the origin of hematopoietic stem cell transplantation (HSCT) for severe autoimmune diseases (SADs). The experimental evidence consisted in the transfer/cure of animal SADs as murine lupus by means of HSCT, allogeneic but also, almost paradoxically, autologous. The clinical one came from serendipitous reports of patients allotransplanted for coincidental diseases, and finally cured of both conditions. The encouraging results of autologous HSCT (ASCT) in experimental ADs were enthusiastically translated into human therapy by clinicians hoping to achieve great results without incurring into the rigors associated with the allogeneic procedure.

Allogeneic STC has elicited great expectations, but the burden of higher mortality and morbidity, with GVHD in the first place, that it may elicitely, must be considered, even when making recourse to reduced conditioning regimens (RIC). Paradoxical relapses notwithstanding complete donor chimerism have been reported. Further experience is clearly needed, but the early enthusiasm for an attractive one shot therapy must be tempered with a realistic evaluation, at least until new significant breakthroughs will be made.

Well over 1000 ASCT for SADs have been performed worldwide at this time, with multiple sclerosis (MS) and connective tissue diseases in the foreground. Transplant-related mortality (TRM) and morbidity have decreased to well under 5%. A dramatic disease-arresting effect is a constant benefit, but the whole course of the disease appears to be influenced favorably. Profound changes of the autoimmune circuitry have been demonstrated, but no authentical eradication of disease (cure?) should realistically be expected. Important multicentric prospective trials are ongoing to compare ASCT to the best available non-transplant therapies, but it may be argued that in the end both approaches will be integrated for single patients, and that new agents will possibly alter present strategies.

Chapter 4 - The first successful thyroidectomy on record using endovascular interventional techniques to embolize the thyroid arteris was reported by Russian authors, EV Galkin et al, in 1994 in the journal of Vestn Rentgenol Radiol. In order to suppress thyroid pathologic activity in diffuse toxic goitre, these authors have for the first time resorted to roentgenoendovascular functional thyroiedctomy in 32 patients with stages III-IV diffuse toxic goitre. Following superselective catheterization of the left and right thyroid arteris, they embolized these arteries with embolic materials which consisted of nonlyzed synthetic, organic and inorganic substances. Followed up for over 1.5 years after endovascular thyroid arterial embolization, a stable clincial and hormonal remission and reduction of thyroid size to the first degree were observed in all the patients. However, in their report, they did not describe which thyroid arteries were occluded with those embolic materials. Have all the superior and inferior thyroid arteries been embolized? Have any arteries been spared embolization? Have they performed animal experiments of thyroid arterial embolization before they tried this embolization technique in human subjects? No records regarding the embolization details can be found in the English literature.

Chapter 5 - The pathogenesis of autoimmune diseases is multifaceted, and the complexity of symptoms, diagnosis and treatment options associated with these diseases reflects this. Sjogren's syndrome (SS) is an autoimmune disorder affecting up to 4 million Americans, and results from autoimmune reactions in secretory glands (primary SS) and other tissues (secondary SS). Current treatment options for SS are aimed largely at amelioration of symptoms, but without functional restoration of the secretory glands. Prescribed medications for salivary hypofunction or xerostomia are often associated with severe side effects, typically due to the effects of systemically administered muscarinic cholinergic receptor agonists.

Chapter 6 - Systemic lupus erythematosus (SLE) is a multisystemic disease characterized by profound alterations of the immune system that contribute to inflammation and tissue damage. The diverse presentations of SLE range from rash and arthritis through anemia and thrombocytopenia to serositis, nephritis, seizures, and psychosis. SLE should be part of the differential diagnosis in virtually any patient presenting with one of these clinical problems, especially in female patients between 15 and 50 years of age. Since 90% of patients with SLE are female, an important role for sex hormones seems likely, but a protective role for male hormones is also possible. Pathogenic auto-antibodies are the primary cause of tissue damage in patients with SLE. The production of these antibodies arises by means of complex mechanisms involving every key facet of the immune system. There are no diagnostic criteria, only classification criteria. In order to consider SLE for research practice, the patient must present at least four of 11 criteria established by the American College of Rheumatology (ACR). Many different elements of the system are potential targets for therapeutic drugs in SLE. The treatment involves immunosuppressive medications like high-dose of corticosteroids, azathioprine, and cyclophosphamide. Mycophenolate mofetil and rituximab have been used in association with corticosteroids (oral or intravenous pulse therapy) in SLE patients. Besides, anti-malarial medications are used not only to control disease, but also to improve survival and to reduce the risk of thrombosis. The aim of this review is to describe symptoms of SLE that may vary from rash and arthritis through seizures, and psychosis. We will also include a review on current treatment and new medications.

Chapter 7 - Neuropsychiatric syndromes of systemic lupus erythematosus (NPSLE) is a life-threatening disorder and early diagnosis and proper treatment are critical in the management of this neuropsychiatric manifestation in lupus. Symptoms of NPSLE are

extremely diverse, ranging from depression, psychosis, and seizures to stroke. The origin of minor clinical symptoms, such as headaches and mood swings are not specific to NPSLE. In fact, SLE patients may be under the influence of other conditions capable of causing neuropsychiatric symptoms, such as infections, severe hypertension, metabolic complications, steroid psychosis, and other drug toxicities. Without proper treatment, neuropsychiatric involvement in SLE is known to increase morbidity and mortality. The availability of beneficial treatments increases the need for the early recognition of neuropsychiatric manifestations in lupus. Currently, tests for diagnosing NPSLE include brain magnetic resonance imaging (MRI), electroencephalogram (EEG), neuropsychological tests, and lumbar puncture. In addition to the conventional diagnostic tools, increased levels of proinflammatory cytokines and chemokines have been reported in the cerebral spinal fluids (CSF) of patients with NPSLE, and some reports have shown cytokines such as interleukin-6 (IL-6), IL-1, IL-8, IL-10, tumor necrosis factor (TNF)- α , interferon (IFN)- α , monocyte chemotactic protein 1 (MCP-1)/CCL2, interferon-gamma inducible protein-10 (IP-10)/CXCL10 and Fractalkine/CX3CL1 to be elevated intrathecally, thereby allowing these cytokines to be used as diagnostic tools. Cytokines and chemokines are also considered to be therapeutic targets in NPSLE. Based on the number of recently published studies, this review focuses on the diagnosis, pathophysiology and therapeutic strategies for NPSLE.

Chapter 8 - Human cytomegalovirus (HCMV) is a ubiquitous pathogen that causes severe infections in immunocompromised patients. During active infection, the virus is able to modulate the host immune system in immunocompetent as well as immunocompromised individuals. HCMV-infected patients often develop signs of immune dysfunction, such as autoimmune phenomena. Furthermore, case reports suggest a link between primary HCMV infection and onset of autoimmune disorders. Signs of active viral infection have also been identified in a number of autoimmune diseases, which further highlights the potential role of HCMV in the genesis and/or maintenance of immunopathological phenomena. Mechanisms by which HCMV could induce host immunopathology, inflammation and autoimmunity will be discussed as well as the opportunity to administer antivirals in selected patients.

Chapter 9 - Behçet's disease (BD) is a chronic, relapsing, systemic vasculitis of unknown etiology with the clinical features of mucocutaneous lesions, ocular, vascular, articular, gastrointestinal, urogenital, pulmonary, and neurologic involvement. It is believed to be due to an auto-immune process triggered by an infectious or environmental agent in a genetically predisposed individual. HLA-B51 is the most strongly associated risk factor. The disease usually starts around the third decade of life. Mucocutaneous lesions figure prominently in the presentation and diagnosis, and may be considered the hallmarks of BD. Therefore, their recognition may permit earlier diagnosis and treatment. Although, the treatment has become much more effective in recent years, BD is still associated with severe morbidity and considerable mortality. The main aim of the treatment should be the prevention of irreversible organ damage. Therefore, close monitoring, early and appropriate treatment is mandatory to reduce morbidity and mortality. We will review symptoms, diagnosis and the current state of knowledge regarding the therapeutic approaches for BD.

Chapter 10 - Immunomodulation and immunosuppression are important strategies for monitoring autoimmune disorders. As imbalances in immune function affect other physiological processes, immunomodulators may have an important role in restoring and maintaining regular neuroimmune activities. In recent years these agents have demonstrated important benefits in controlling the mechanisms associated with deteriorating central

nervous system pathologies such as Multiple Sclerosis (MS), where central and peripheral nervous system immune regulation is impaired. MS is characterized by severe compromises to neuroimmune processes involving changes in immune cell function, soluble proteins and modulation of inflammatory processes. The introduction of therapeutic agents in the form of immunomodulators; interferon, phosphodiesterase inhibitors and Glatiramer acetate have proven to be useful to some extent in reducing the severity of MS. Herein the implications and effects of these molecules on the immune system in MS are reviewed. Additionally, the available evidence on the mode of action of neuropeptides in MS, their effectiveness on clinical measures, and current knowledge are also reviewed.

Chapter 11 - Premenstrual syndrome (PMS) is a disorder characterized by depressed mood, anxiety, affective lability, irritability, decreased interest in usual activities, difficulty concentrating, low energy, changes in appetite, sleep disturbances, a sense of being overwhelmed or out of control, headaches, joint or muscle pain, breast tenderness, and abdominal bloating.[1] Women with fewer than five of these symptoms are typically diagnosed with premenstrual syndrome. Women who experience more of the symptoms, or fewer symptoms at a debilitating level, meet the diagnostic criteria for premenstrual dysphoric disorder (PMDD). To meet the PMDD criteria, symptoms should be specific to the premenstrual period and at least one of the symptoms should directly relate to mood disturbances.[1] PMS is a phenomenon which impacts the majority of adult women on some level, with millions of women affected severely enough to disrupt daily life. Nevertheless, it is an under-investigated disorder that is still without a definitive etiology. The pronounced gender discrepancy in the prevalence of autoimmune diseases strongly implicates estrogen and/or progesterone in their development; it is known that the hormonal fluctuations of the menstrual cycle cause exacerbation of the symptoms of autoimmune diseases, particularly those with cutaneous manifestations. The demonstration of a dramatic comorbidity of premenstrual exacerbations of cutaneous allergic and autoimmune disorders with PMS, the presence of hypersensitivity reactions to estrogen and progesterone in PMS patients but not in normal controls, and the ability of desensitization therapy to improve symptoms in PMS patients suggests that autoimmunity may play a role in the origin of PMS symptoms.

Chapter 12 - Following the induction of Heymann nephritis or slowly progressive Heymann nephritis immunopathological events are primarily directed against the renal proximal tubules' brush border associated zone of cells by the developing pathogenic immunoglobulin G autoantibodies. Pathogenic immunoglobulin G autoantibodies making their way from the circulation through the glomeruli into the renal tubular environment get absorbed and attack the target nephritogenic autoantigens which reside in the brush border region of the renal proximal tubules.

Due to damage by the pathogenic immunoglobulin G autoantibody directed against the nephritogenic autoantigen the release of autoantigens into the urine and circulation will occur. If the immunopathological attack is continuous then the absorption/retention of vitally important components of the glomerular filtrate will be disturbed resulting in electrolyte imbalance.

If the immunological attack continues into a chronic progressive phase then additional secondary/tertiary injuries will take place to the kidney's glomeruli and renal proximal tubules. Nephritogenic autoantigen, pathogenic immunoglobulin G autoantibody against the nephritogenic antigen and complement components settle in the form of immune complexes on the epithelial side of the glomerular basement membrane. Immune complex glomerular

nephritis further complicates the functional integrity of the kidney by leaky glomeruli causing non-selective proteinuria.

Since the primary target is the nephritogenic autoantigen of the renal proximal tubules' brush border zone of cells, the elimination of modified and native nephritogenic autoantigens from the circulation would halt the production of autoimmune disease causing pathogenic immunoglobulin G autoantibodies against the nephritogenic autoantigens. It has been demonstrated that prevention and/or termination of Heymann nephritis can be achieved by the implementation of a new vaccination technique that we have developed and call modified vaccination technique.

Chapter 13 - Silicosis patients suffer not only from respiratory disorders, but also from autoimmune diseases. To clarify the mechanisms involved in the dysregulation of autoimmunity found in silicosis patients, we have been focusing on Fas and Fas-related molecules in the Fas-mediated apoptotic pathway because Fas is one of the most important molecules regulating autoimmunity in T cells. Our findings have shown that in comparison to healthy donors, silicosis patients exhibit elevated serum soluble Fas levels, an increased relative expression of the soluble fas and dcr3 genes in peripheral blood mononuclear cells, other highly detectable variant messages of the fas transcript, a relatively decreased expression of several physiological inhibitors (survivin and toso), and a dominancy of lower membrane Fas expressers in lymphocytes, which transcribe soluble fas dominantly. These findings are consistent with immunological factors such as serum immunoglobulin G levels and the titer of anti-nuclear autoantibodies. In addition, anti-caspase 8 autoantibody and anti-Fas autoantibody were detected in serum from silicosis patients, and a functional assay showed that anti-Fas antibody stimulated Fas-mediated apoptosis. We hypothesize that there are two subpopulations of silicosis lymphocytes. One is a long-term survival fraction including a self-recognizing fraction showing lower levels of membrane Fas and inhibition of Fas/Fas ligand binding in the extracellular spaces. The other is a fraction exhibiting apoptosis caused by silica/silicates, recruitment from bone marrow, higher levels of membrane Fas, and sensitivity to anti-Fas autoantibody. Further investigations should be performed to confirm the effects of silica/silicates on the human immune system.

In addition, results concerning whether serum soluble interleukin-2 receptor and soluble CD40 ligand levels should be considered immunological markers in silicosis patients are discussed.

Furthermore, based on our previous reports showing *in vitro* activation of peripheral T cells by silica and reduced function of the CD4+CD25+ fraction in which FoxP3+ regulatory T cells (Treg) are located, the reconstitution of the CD4+CD25+ fraction in silicosis patients (SILs) was analyzed. Since T cells in peripheral CD4+CD25+ as well as CD4+CD25-fractions from SILs showed higher expression of pd-1 (marker gene for T-cell activation) compared to that of healthy donors (HDs), chronic T-cell activation is thought to have occurred in SILs. In addition, surface Fas expression of Treg was higher in SILs than HDs. The *ex vivo* experiments using freshly isolated peripheral blood mononuclear cells (PBMCs) from SILs and HDs cultured with or without silica showed loss of Treg by Fas-mediated apoptosis and an increase of activated CD25+ T cells in PBMCs from SILs as well as HDs. Although T cells in SILs are thought to have been exposed to low-dose silica for a long time, their effector T cells (Teff) and Treg still possess the capacity to be activated by silica. These activations of both Teff and Treg cause re-constitution of the peripheral Treg fraction, loss of Treg and contamination of activated Teff, resulting in a reduction of the size and function of

Treg. These results might contribute to an elucidation of the development of autoimmune diseases found in silicosis patients.

Chapter 14 - A rare case of a patient with Sjögren's syndrome is described associated with proteinuria, microscopic hematuria, and gradually deteriorating kidney function. Renal biopsy has shown interstitial nephritis and vasculitis. The patient also had recurrent chronic genital herpes and was positive to human papilloma virus. The patient was treated with high dose corticosteroids. Due to the worsening renal function caused by the progressing vasculitis, cyclophosphamide therapy was inevitable even though the viral infection was a contraindicating factor. Therefore, along with antiviral therapy (acyclovir), we initiated cyclophosphamide therapy whereby the patient's kidney functions improved without progression of the infection. In patients with Sjögren's syndrome the deterioration of the renal function may raise the possibility of ongoing renal vasculitis, therefore thorough nephrological investigations are necessary and if the diagnosis is proven adequate immunosuppressive therapy needs to be initiated immediately, despite the risk of activating any associated infectious diseases.

Chapter 1

Autoimmune Liver Diseases

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Abstract

Autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC) are three distinct autoimmune liver diseases. Immunoglobulin G4 (IgG4) associated cholangitis is another immune disorder which mimics PSC and is characterized by formation of biliary strictures responsive to steroid therapy. The simultaneous or successive coexistence of PBC and AIH or PSC and AIH is called overlap syndrome. AIH is a chronic inflammatory immune-mediated liver disease characterized by elevated transaminase levels, hypergammaglobulinemia and histological features of interface hepatitis. The diagnosis of AIH is based on the scoring systems of the International Autoimmune Hepatitis Group (IAIHG) after exclusion of other causes of chronic liver disease. AIH is treated with prednisone alone or in combination with azathioprine and both strategies are equally effective.

PBC and PSC are both chronic cholestatic liver diseases with slowly progressive courses. Fatigue and pruritus are the most common symptoms but the majority of the patients at first presentation are asymptomatic. The diagnostic criteria for PBC include serum alkaline phosphatase levels at least twice the upper limit of normal and a positive test for serum antimitochondrial antibodies. A liver biopsy specimen showing inflammatory changes involving the bile duct supports the diagnosis but is not mandatory. Ursodeoxycholic acid is the only FDA-approved therapy for PBC in a dose of 13-15mg/kg/day. PSC is most commonly diagnosed with endoscopic retrograde cholangiopancreatography (ERCP), although magnetic resonance cholangiography (MRC) is rapidly emerging as the first-choice diagnostic test. There is no effective medical treatment for PSC and liver transplantation is the only life extending therapy for end-stage disease.

IgG4 associated cholangitis is an immune disorder characterized by formation of biliary strictures and frequently involves extrahepatic bile ducts. It is often associated

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with other autoimmune disorders such as autoimmune pancreatitis. The serum level of IgG4 is usually elevated and there is infiltration of IgG4 positive plasma cells in bile ducts. Clinically, patients present with abrupt onset jaundice which responds to steroid therapy. Pathogenesis and standard diagnostic criteria for the overlap syndrome have not yet been established. In this review we discuss in detail the clinical presentation, pathogenesis, diagnosis and treatment of AIH, PBC, PSC, IgG4 associated cholangitis and overlap syndromes.

Keywords: Autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, Immunuglogulin G4 associated cholangitis, overlap syndrome

Autoimmune Hepatitis

Introduction

Autoimmune hepatitis (AIH) is an idiopathic disease characterized by inflammation of the liver, presence of autoantibodies, and evidence of increased gamma globulins in the serum. AIH like other autoimmune diseases affects women more frequently than men with a gender ratio of 3:1 [1, 2]. Based on limited epidemiological data, the prevalence is estimated to range between 50 and 200 cases per 1 million in Western Europe and North America among the white population [3, 4]. Autoantibodies are important tools for the diagnosis and classification of AIH. According to this approach, AIH type 1 is characterized by the presence of antinuclear antibody (ANA) and /or anti-smooth muscle antibodies (SMA). Type 2 AIH is characterized by antibodies to liver-kidney microsome type1 (anti-LKM1) and AIH type 3 is characterized by autoantibodies against a soluble liver antigen/liver pancreas (SLA/LP) [5-8]. After exclusion of other causes of liver disease the diagnosis of AIH is based on the scoring systems of the International Autoimmune Hepatitis Group (IAIHG) [9, 10]. Only patients who have an established diagnosis of AIH, elevations of aminotransferase activities (ALT, AST), an elevation of serum immunoglobulin (Ig) G, and histological evidence of interface hepatitis or necroinflammatory activity are treated. AIH is treated with prednisone alone or in combination with azathioprine and both strategies are equally effective [11-13].

Clinical Presentation

Onset of AIH is acute in about 20% of cases and rare cases of fulminant AIH have also been reported. The clinical presentation is not spectacular, as could be expected in a chronic disease, but is characterized by fatigue, right upper quadrant pain, jaundice, and occasionally by palmar erythema and spider nevi. In latter stages, the consequences of portal hypertension dominate, including ascites, bleeding esophageal varices, and encephalopathy. A specific feature of AIH is the occasional association of extrahepatic immune-mediated syndromes, including autoimmune thyroiditis, vitiligo, alopecia, nail dystrophy, ulcerative colitis, rheumatoid arthritis, diabetes mellitus, and glomerulonephritis.

Pathogenesis

Experimental evidence supports both innate and adaptive immune responses in the pathogenesis of AIH. The disease process usually starts in genetically susceptible individuals and may be triggered by environmental factors such as hepatotropic viruses or drugs [14-20]. This concept is supported by a strong association between the human leucocyte antigen allotypes DRB1 0301 and DRB1 0401 and the development of AIH [21, 22]. Clinical experiences have linked AIH with protracted hepatitis A virus infection [23]. Laboratory studies have supported this hypothesis by demonstrating homologies between various viral genomes and the cytochrome monooxygenase, CYP2D6, which is the antigen trigger of type 2 AIH [24].

In AIH, T regulatory cells are defective in number and function and this impairment is more marked at disease presentation. Therapy with drugs usually induce remission and a partial T regulatory cell restoration is also observed [25, 26]. There is a similar reduction in the natural killer T cell sub-population indicating a wide immunoregulatory deficit in AIH. T regulatory (CD4+CD25+) cells constitute 10% of normal CD4+ T helper lymphocytes and they are essential in blunting the actions and proliferation of autoreactive T cells through direct contact or modification of cytokine pathways[27]. The transcription factor, forkhead box P3, is specifically expressed by T regulatory cells and it is required for their development and regulatory function [27, 28].

In mice when naturally occurring T regulatory cells are removed by neonatal thymectomy, programmed cell death, antinuclear antibodies and massive hepatic necrosis occur spontaneously[28]. The fatal hepatitis in mice can be prevented by adoptive transfer of T regulatory cells 4-days after thymectomy or it can be suppressed if the T regulatory cells are given in sufficient numbers after the disease has begun.

Diagnosis

There is no single specific test for the diagnosis of AIH. Classically, AIH affects women at any age group and is characterized by marked elevations of serum aminotransferases (ALT/AST), detectable autoantibodies (ANA, SMA, LKM-1 and SLA/LP) and increased serum levels of globulins, particularly gamma globulins. Histologically, AIH is characterized by interface hepatitis, with plasma cells and lymphocytes predominating but there is no specific histological feature that can be used to prove the diagnosis [17].

In 1993, the IAIHG proposed a scoring system to establish diagnostic criteria for AIH [10]. The specificity of this scoring system was insufficient, although the sensitivity was more than 90%. Thus, in 1999, a revised scoring system (hereafter referred to as the original criteria) with sufficient specificity was proposed [9]. Even though the criteria were improved in this revision still the original criteria are complex and have a variety of parameters of questionable value. Table 1 lists all parameters and the cumulative scores of the revised scoring system used to classify patients as having probable or definite AIH. In 2008, the IAIHG proposed a simplified set of diagnostic criteria that included autoantibodies, immunoglobulin G, histology, and exclusion of viral hepatitis [29]. These parameters and the cumulative scores necessary for a diagnosis of probable or definite AIH are represented in

Table 2.

Table 1. International diagnostic criteria for the diagnosis of autoimmune hepatitis [9]

Parameter	Score
Gender	
Female	+2
Male	0
Serum biochemistry	
Ratio of elevations of serum alkaline phosphatase vs. aminotransferase	
>3.0	-2
1.5-3	0
<1.5	+2
Serum globulin or IgG above normal	
>2.0	+3
1.5-2.0	+2
1.0-1.5	+1
<1.0	0
ANA, SMA, or LKM-1	
>1:80	+3
1:80	+2
1:40	+1
<1:40	0
AMA positive	-4
Hepatitis viral markers	
Positive	-3
Negative	+3
Drug History	
Positive	-4
Negative	+1
Average alcohol intake	
<25g/day	+2
>60g/day	-2
Genetic factors: HLA DR3 or DR4	+1
Other autoimmune diseases	+2
Response to therapy	
Complete	+2
Relapse	+3
Liver histology	
Interface hepatitis	+3
Lymphoplasmacytic infiltrate	+1
Rosetting of liver cells	+1
None of the above	-5
Biliary changes	-3
Biliary changes	-3

Table 2. Simplified diagnostic criteria for the diagnosis of AIH [29]

Parameters	Score
Autoantibodies	
ANA or SMA	
>1:40	+1
>1:80	+2
LKM-1>1:40	+2
SLA positive	+2
IgG or total immunoglobulin level	
> upper limit of norma	+1
>1.156 upper limit of normal	+2
Liver histology	
Compatible with AIH	+1
Typical of AIH	+2
Absence of viral hepatitis	
No	0
Yes	+2
Pretreatment aggregate score	
Definite AIH	>7
Probable AIH	>6

Interpretation of Aggregate Scores

1. Definite AIH, a score >15 before treatment and >17 after treatment.
2. Probable AIH, a score 10-15 before treatment and 12-17 after treatment.

The simplified diagnostic criteria has high specificity (97% for probable and 99% for definite AIH) but a lower sensitivity 88% for probable and 81% for definite AIH as has been corroborated in a single center study from the United States [30].

Treatment

The diagnosis of AIH does not mandate therapy in every case and decision to treat a patient represents a careful balance between the risks and benefits of immuno-suppressive therapy. Table 3 lists accepted and relative indications for treatment [11].

The treatment of AIH has remained unchanged for nearly 5 decades. Most experts agree that combination therapy with prednisone and azathioprine is the preferred initial treatment of patients who have type 1 AIH [11-13]. Steroids alone should be used in patients who have severe cytopenia, have a concurrent neoplasm, are pregnant or wish to become pregnant, or have known intolerance for azathioprine. The common side effects of steroid therapy include facial rounding, acne, truncal obesity, osteopenia, diabetes, aseptic bone necrosis, psychiatric symptoms, hypertension, and cataracts. To avoid the typical side effects of prednisone, most

treating physicians use azathioprine as a steroid sparing agent. Side effects from azathioprine are relatively rare and occur in less than 10% of patients.

Treatment may be categorized into the phases of remission, relapse, treatment failure, and stabilization. Remission is a complete normalization of all inflammatory parameters including histology and is achieved by commencing prednisone 40mg per day. The steroid dose is then typically reduced, in the context of improving aminotransferase activity, by 10 mg every 2 weeks until the patient is stabilized on 10mg per day. Azathioprine is introduced at a dose of 1mg/kg/d, when AST falls to less than 2-3 times the upper limit of normal. Remission can be achieved in 65-75% of patients after 24 months of treatment and it can be sustained with azathioprine monotherapy of 2mg/kg [31]. However, side effects such as arthralgia, lymphopenia and myelosuppression have been observed. Complete remission is not achieved in ~20-30% of patients and these patients continue to carry a risk of progressive liver injury.

Relapse is characterized by an increase of aminotransferase levels and recurrence of clinical symptoms either under treatment, following tapering of steroid dose to determine the minimally required dose or after a complete withdrawal of therapy. Relapse is present in 50% of patients within 6 months of treatment withdrawal and in 80% after 3 years. Occurrence of a relapse requires reinitiation of standard therapy. In those relapsing on therapy, the strategy consists of augmentation of immunosuppression. This is achieved by an increase in the dose of prednisone to around twice the previous maintenance dose followed by institution of azathioprine. For those already receiving dual therapy, the azathioprine dose should be maximized until 2mg/kg/day is delivered. The duration of therapy following a single relapse is controversial. In general, a patient who has experienced more than one relapse should probably receive lifelong immunosuppression. This can consist of indefinite low dose steroids, indefinite azathioprine therapy or a combination of both.

Treatment failure is characterized by a progression of clinical, serological and histological parameters during standard therapy. This is seen in ~10% of patients. In these patients, the diagnosis of AIH has to be carefully reconsidered to exclude other etiologies of chronic hepatitis. In these patients, experimental regimens can be administered or liver transplantation should be considered.

Stabilization is the achievement of a partial remission. Because 90% of patients reach remission within 3 years, the benefit of standard therapy has to be reevaluated in this subgroup of patients. Ultimately, liver transplantation provides a more definitive treatment option.

Table 3. Accepted and relative indications for treatment of AIH

Accepted indications
1). Serum AST> 10-fold upper limit of normal
2). Serum AST >5-fold upper limit of normal and γ - globulin greater than twice normal
3). Bridging necrosis or multiacinar necrosis on histologic examination
Relative Indications
1). Symptoms (fatigue, arthralgia, jaundice)
2). Serum AST or γ -globulin less than accepted criteria
3). Interface hepatitis

Alternative Treatments

When standard treatment fails or drug intolerance occurs, alternative therapies such as budesonide, cyclophosphamide, cyclosporine, deflazacort, etanercept, methotrexate, mycophenolate mofetil, sirolimus, tacrolimus, rapamycin, rituximab, and ursodeoxycholic acid can be considered [32-43]. The efficacy of these drugs has not yet been definitively decided and results are only reported in small case series.

AIH and Pregnancy

AIH frequently affects women in their child bearing age. The outcome of pregnancy in AIH is generally positive, with a series reporting 31 live births from 35 pregnancies in 18 patients, 7 of whom were cirrhotic [44]. In total, 24 pregnancies were exposed to azathioprine but no fetal abnormalities were detected in this group. Current suggestions are that patients who have stable AIH and are on azathioprine could continue with azathioprine at low doses. In another study, a live birth rate of 80% was reported in 44 pregnancies in AIH patients [45]. A recent study evaluated 54 pregnancies, with 64% of patients having cirrhosis with a higher rate of fetal loss (29%). Despite this, maternal morbidity was only 8% and no maternal deaths were noted [46]. AIH typically improves in pregnancy and relapse is common in the post-partum period, with reports varying from 11-50% [44-46]. There is also a high likelihood of an AIH flare in the peripartum time period such that patients should have appropriate prophylaxis with increased immunosuppression following delivery [47].

Liver Transplantation

Liver transplantation remains the only lifesaving option in approximately 10% of AIH patients [48]. The indication for liver transplantation in AIH is similar to that in other chronic liver diseases and includes clinical deterioration, bleeding esophageal varices and coagulation abnormalities despite adequate immunosuppressive therapy [49-54]. Candidates for liver transplantation are usually patients who do not reach remission within 4 years of continuous therapy. Indicators of a high mortality rate associated with liver failure are histological evidence of multilobular necrosis and progressive hyperbilirubinemia. The long-term results of liver transplantation for AIH are excellent, with 5-year survival of up to 92% [51, 52, 55].

Recurrence of AIH after Liver Transplantation

The recurrence of AIH after liver transplantation has been extensively studied. The estimated risk for recurrence after liver transplantation is 8% for the first year and 25% after 2 years [51, 56]. The risk factors that might predict AIH recurrence after transplant include high-grade inflammation in the native liver, increased frequency of HLA-DR3, steroid withdrawal, and LKM-1 autoantibodies [57, 58]. There are not conclusive data to support the hypothesis that a specific immunosuppressive regimen represents a risk factor for the

development of recurrent AIH [59]. However, data indicate that patients transplanted for AIH require continued steroids in 64% versus 17% of patients receiving liver transplants for other conditions [60].

Primary Biliary Cirrhosis

Introduction

Primary biliary cirrhosis (PBC) is a chronic autoimmune cholestatic liver disease. As one of the first conditions in which specific autoantibodies were recognized, PBC is regarded as a “model autoimmune disease” [61, 62]. The disease tends to follow a progressive course and eventually causes fibrosis and cirrhosis of the liver without medical treatment. PBC is most common in middle-aged women and is characterized by biochemical markers of cholestasis, serum antimitochondrial autoantibodies (AMA) and lymphocytic infiltration of the portal tracts of the liver [63]. The annual incidence rate in the US is 27 cases per million and the prevalence rates range between 150 and 400 cases per million, thus leading to an estimate of 8100 new cases each year, with 45,000-120,000 prevalent cases among the US population [64, 65]. Both environmental factors and inherited genetic predisposition appear to contribute to its pathogenesis [66].

The most common symptoms in PBC are fatigue and pruritus [67, 68] and median survival in untreated patients has been reported to be 7.5 to 16 years [62, 69] but has improved since the introduction of ursodeoxycholic acid (UDCA) therapy and liver transplantation.

Clinical Presentation of PBC

At diagnosis, the majority of patients are asymptomatic and present for workup of elevated serum levels of alkaline phosphatase or cholesterol [70, 71]. In symptomatic patients, fatigue and pruritus are the most common complaints [72, 73].

Fatigue

Fatigue is the most common symptom in patients with PBC. It is found in up to 78% of patients and in severe cases it may impair the quality of life [67, 74]. Fatigue is not associated with histologic stage of the disease, degree of hepatocellular dysfunction, or autoantibody levels [67, 74-76]. The pathogenesis and effective treatment of fatigue in PBC is unknown. Recent progress in understanding the pathogenesis and impact of fatigue in PBC has been aided by the development of the fatigue impact scale (FIS) and the PBC-40 [77, 78]. It is generally considered that fatigue in PBC has a central origin [79] and to some extent there is also a link between the degree of fatigue and autonomic nervous system function [80]. There are other medical conditions in which fatigue is a very prominent symptom and these can coexist with PBC, such as hypothyroidism which occurs in about 20% of patients with PBC[81]. Severe fatigue may be associated with decreased overall survival and excessive daytime somnolence [82, 83].

Pruritus

Pruritus is a more specific symptom of PBC than fatigue and it occurs in 20% to 70% cases of PBC [70, 84]. Pruritus is not associated with histologic stage of the disease and the degree of cholestasis. Its pathogenesis remains poorly understood [85, 86]. It is proposed that pruritus in PBC is mediated at least in part by increased opioidergic neurotransmission [87] while other studies support a role for components of bile [88]. The pruritus can be local or diffuse, usually worse at night while lying in bed, and is often exacerbated by contact with wool, other fabrics, heat, or pregnancy.

Associated disorders

A number of mostly immune-mediated diseases are commonly observed in patients with PBC. Thyroid dysfunction, Sicca syndrome (dry eyes and/or mouth) and incomplete or complete CREST syndrome (calcinosis cutis, Raynaud syndrome, esophageal motility disorder, sclerodactyly and telangiectasia) are not uncommon [89-91].

Physical findings

In most cases physical examination is usually normal but occasionally, xanthelasma and xanthoma are recognized. Spider angiomata and splenomegaly are found in the setting of portal hypertension. Jaundice is a late finding in patients with advanced liver disease.

Portal hypertension

Portal hypertension is usually a late finding in the course of PBC, when patients have well-established cirrhosis but in some cases it may develop in early, precirrhotic PBC. Esophageal varices, gastric varices and portal gastropathy are the common presentations of portal hypertension. Patients with PBC can survive for many years after variceal hemorrhage without liver transplantation [92, 93]. Ascites and hepatic encephalopathy may develop in patients with histologically advanced PBC and cirrhosis.

Metabolic bone disease

Patients with PBC have increased risk of osteoporosis [94] and the cause is uncertain. The relative risk for osteoporosis in PBC compared to an age-matched and sex-matched healthy population is 4.4 [94]. Osteoporosis is usually asymptomatic and bone densitometry is an established technique for its diagnosis. The development of osteoporosis in PBC patients has been attributed to both decreased osteoblast activity and increased osteoclast activity [95-97]. Vitamin D metabolism is normal in PBC patients except for those with jaundice and clinically advanced disease [98-100]. Advanced stage PBC is associated with malabsorption of calcium and vitamin D. Pancreatic insufficiency and celiac disease which are associated with PBC may further aggravate malabsorption [101-103].

Vitamin deficiency

Patients with PBC may have decreased bile acid secretion resulting in malabsorption of fat and fat-soluble vitamins A, D, E, and K [98, 102, 104, 105]. The decreased levels of vitamin A, D, E, and K are associated with night blindness, osteopenia, neurologic impairment, and decreased prothrombin activity, respectively [106, 107].

Hyperlipidemia

Patients with PBC have elevated levels of serum lipids [108, 109] and the mechanism leading to hyperlipidemia is different from that in other conditions. Levels of high-density lipoprotein cholesterol are typically elevated and unusual lipoprotein particles, such as lipoprotein X, may accumulate [110]. High-density lipoprotein cholesterol is disproportionately elevated compared to low-density lipoprotein cholesterol and patients with PBC are not at increased risk of death from atherosclerosis [109, 111, 112].

Urinary tract infections

Recurrent urinary tract infections have been reported in up to 19% of women with PBC [113] and a pathophysiologic association with *Escherichia coli* strains has been suggested but not proven.

Pathogenesis

A florid bile duct lesion with damage to biliary epithelial cells and subsequent destruction of small bile ducts is the histopathologic hallmark of PBC. The exact pathogenetic mechanisms responsible for the damage of biliary epithelial cells in PBC remain unknown. Various experimental studies suggest that the florid bile duct lesion in PBC is initiated by environmental triggers acting on genetically susceptible individuals [114]. The primary event in the pathogenesis of PBC is the loss of tolerance to the E2 subunit of pyruvate dehydrogenase. It is also suggested that the destruction of biliary epithelium is based in part upon its unique apoptotic properties in which the mitochondrial autoantigens remain immunologically intact [115]. Genetic factors have an impact on PBC pathogenesis that is stronger than any other autoimmune disease [116-118]. A high concordance rate of about 60% among monozygotic twins has been reported and lymphocytes from women with PBC preferentially lose one chromosome [66, 119-121]. The relative risk of a first-degree relative of a PBC patient is 50-100-fold higher than for the general population [122].

The role of two main environmental factors such as xenobiotics and infectious agents has been suggested in the pathogenesis of PBC [123, 124]. Smoking, hormone replacement therapy and frequent use of nail polish are also linked to the risk of developing PBC, further support the potential impact of environmental factors in the pathogenesis of PBC [125, 126]. Xenobiotics usually trigger autoimmune reactions and also have direct toxic effect by apoptosis or oncosis, inducing the generation of immunogenic autoepitopes [117, 127]. The E-coli bacterium has been reported to be present in excess in the feces of patients with PBC. In addition, the incidence of urinary tract infections often induced by E-coli is high in PBC patients and history of urinary tract infections increases the risk of PBC [113, 125, 128].

Diagnostic Criteria

The diagnosis of PBC is currently based on the presence of AMA in serum, which is highly specific for the disease and elevation of biochemical indices of cholestasis based mainly on alkaline phosphatase. The diagnosis is supported by histological features in the

liver that are compatible with the diagnosis. The diagnosis of PBC can be established when two of the above three criteria are met.

AMA is found in nearly 95% of patients with PBC [62] and it has high specificity and sensitivity for the diagnosis of PBC. Half of the patients with PBC also have antinuclear antibodies and anti-smooth muscle antibodies [62]. Histology in PBC is characterized by chronic, nonsuppurative cholangitis that mainly affects interlobular and septal bile ducts. The infiltrate consists of plasma cells, macrophages, polymorphonuclear cells and in some cases epithelioid granulomas. Cross-sectional imaging of the liver and biliary tree is mandatory in all patients with biochemical evidence of cholestasis. If the diagnosis is uncertain, then cholangiography may be necessary, preferentially with noninvasive magnetic resonance imaging or endoscopically to exclude primary sclerosing cholangitis or other biliary tract diseases. Transient elastography is a new, simple and noninvasive imaging technique to evaluate the degree of liver fibrosis [129] in patients with PBC, but it is not yet approved by the United States Food and Drug Administration.

Treatment of PBC

Only those patients with PBC who have elevated liver biochemical markers are considered for treatment. A liver biopsy is not essential for diagnosis of PBC or initiation of treatment. Although therapy with UDCA is most effective in patients with stage I or II disease, patients at any stage are candidates for such therapy. The only drug approved for PBC is UDCA in a dose of 13-15 mg/kg/day. Several other drugs such as azathioprine, chlorambucil, colchicine, cyclosporine, malotilate, methotrexate, mycophenolate mofetil, penicillamine and thalidomide have been tested but none has been effective as a single agent [130-139]. UDCA is started gradually and generally given in divided doses. Starting the drug at full dose may precipitate pruritus and loose stools. Thus treatment should be started at a dose of 250-300mg per day with an increase in the dose every 3-4 days until the target dose of 13-15mg/kg/day is reached.

Effects of UDCA therapy

An initial response to therapy occurs in a few weeks and 80-90% of the maximum improvement is seen within 3 months. Normalization of biochemical values occurs within 2 years in 20% of patients and in 35% patients after 5 years [140]. The use of UDCA has been associated with the reduction of serum low-density lipoprotein cholesterol level [141]. Different studies have proved that UDCA therapy delays histologic progression, reduces the development of esophageal varices, and improves survival [72, 142-147].

Minor (5 lb) weight gain has been reported during the first year of therapy but is not progressive [148]. Loose stools and thinning of hair have also been reported infrequently.

Management of Symptoms

Fatigue may be multifactorial; causes other than PBC should be considered. These include anemia, hypothyroidism, depression and a sleep disorder. Different drugs such as

colchicine, fluoxetine, fluvoxamine, methotrexate, modafinil, ondansetron and UDCA have been used to treat fatigue in PBC [149-154]. Most of these drugs have no beneficial effects on fatigue except modafinil and methotrexate [152, 153]. Modafinil in uncontrolled pilot studies decreases somnolence, increases energy levels and also decreases total sleep time [153]. At this time, there is no recommended treatment for the fatigue resulting from PBC.

Box 1. Beneficial effects of UDCA in patients with PBC

- Improved liver biochemical functions
- Decreased total cholesterol
- Delayed histologic progression
- Reduced risk for the development of esophageal varices
- Improved survival in the treatment group

UDCA therapy usually does not relieve pruritus in PBC and bile acids sequestrants such as cholestyramine and colestipol are used as initial therapy [155]. The recommended dose of cholestyramine is 4g per dose to a maximum of 16g/day given 2-4 hours before or after UDCA. Treatment with rifampicin is also associated with relief of pruritus [156, 157] but the side effects such as hepatitis, hepatic failure, hemolysis, and renal impairment are concerns [84, 158, 159]. Opiate antagonist drugs such as naloxone, naltrexone and nalmefene have been found to reduce itch severity in some patients with PBC [156, 160-164]. The limiting factor in the use of opiate antagonists is the opioid withdrawal-like reaction which is characterized by abdominal pain, high blood pressure, tachycardia, goose bumps, nightmares, and depersonalization [160, 161, 163, 165]. It is not possible to predict who will develop an opiate withdrawal-like reaction. Clinical experience has suggested that patients who have severe pruritus may have a higher opioidergic tone and may be at risk for a more severe reaction. The selective serotonin reuptake inhibitor sertraline has been reported to improve pruritus in small clinical trials [166] and is considered a fourth-line treatment option [167]. Other experimental approaches include 5-hydroxytryptamine receptor type 3 antagonists, cannabinoids, subhypnotic doses of propofol, plasmapheresis, albumin dialysis, and nasobiliary drainage in desperate cases [167, 168]. Liver transplantation should be considered in serious cases in which all other strategies have failed, even if liver function is still conserved [167].

Treatment for dry eyes includes artificial tears and in refractory cases pilocarpine or cevimeline can be used. Cyclosporine ophthalmic emulsion is used when other agents are not effective, preferably under the supervision of an ophthalmologist. Management for xerostomia and dysphagia includes saliva substitutes and in refractory cases pilocarpine or cevimeline can be used. Vaginal dryness is treated with moisturizers but vaginal lubricants are not recommended for routine use.

Patients with late stage PBC are at increased risk for osteopenia and osteoporosis [169] and treatment with UDCA has no effect on bone loss. All perimenopausal and postmenopausal women should be provided 1000-1500mg of calcium and 1000 IU of vitamin D daily in the diet if there is no history of renal stones. Alendronate orally, 70mg weekly,

should be considered if patients are osteopenic in the absence of acid reflux or known varices.

Liver Transplantation

Liver transplantation is the treatment of choice in patients with late-stage PBC. Indications are decompensated cirrhosis with treatment-resistant ascites, recurrent spontaneous bacterial peritonitis, encephalopathy, recurrent variceal bleeding or hepatocellular carcinoma. The outcome of liver transplantation for patients with PBC is more favorable than for nearly all other disease categories. Osteopenia may worsen for the first 6 months after transplantation but bone mineral density returns to baseline after 12 months and improves thereafter [100]. Some 20-25% of patients with PBC who undergo transplantation develop recurrent disease over 10 years. Fortunately, recurrent PBC does not often affect long-term patient or graft survival [170]. Cyclosporin used for long-term immunosuppressive therapy reduces the incidence of recurrent PBC [171]. Liver transplantation improves fatigue and pruritus; sicca syndrome is unchanged; bone disease worsens initially and then improves; and AMA may persist or reappear but does not signal the recurrence of PBC.

Follow-up

Treatment with UDCA is continued indefinitely with periodic monitoring of liver biochemical markers. There are certain other recommendations during the follow-up period, such as annual check-up for thyroid status; bone mineral densitometry every 2-4 years; measurement of vitamins A, D, and K annually if bilirubin is more than 2mg/dl; upper endoscopy every 1-3 years if cirrhotic or Mayo risk score more than 4.1; and ultrasound and alpha fetoprotein in patients with known or suspected cirrhosis [172] or in men with PBC.

Primary Sclerosing Cholangitis

Introduction

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by inflammation and fibrotic obliteration of the biliary tree, resulting in bile stasis and hepatic fibrosis. Ultimately cirrhosis, end-stage liver disease and death ensue. PSC primarily affects young and middle-aged men and it is associated with inflammatory bowel disease (IBD)[173, 174]. Patients typically present with pruritus and fatigue at the early stage of the disease, although patients with incidental elevated liver enzymes may be diagnosed earlier. With the progression of the disease patients develop jaundice and signs of advanced liver disease. Rare presentations include variceal bleeding and cholangiocarcinoma [175, 176]. PSC is most commonly diagnosed with endoscopic retrograde cholangiopancreatography (ERCP), although magnetic resonance cholangiography (MRC) is rapidly emerging as a first-choice diagnostic test. A variety of therapeutic agents with different mechanisms of action have been evaluated in the treatment of this disease, none of which have shown convincing benefits. Among

eligible patients, liver transplantation is currently the only life extending therapy with end-stage PSC. Although PSC is a rare cholestatic disease, it is among the most common indications for liver transplantation in Europe and the United States [177, 178].

Clinical Presentation

PSC has a progressive course and patients are at increased risk for developing symptoms over time [174]. At presentation 15-55% patients with PSC are asymptomatic [176, 179-181]. Fatigue, pruritus, jaundice or abdominal pain develops in almost 60% of cases with PSC [174, 176, 179, 180, 182]. Symptoms such as pruritus and right upper abdominal pain are the most common intermittent symptoms, occurring with considerable individual variation and resolving spontaneously, in most cases. Patients also develop weight loss, fever, chills and hyperpigmentation.

Disease associated complications of PSC include steatorrhea, vitamin deficiencies, metabolic bone disease, bleeding peristomal varices, bacterial cholangitis, dominant biliary strictures, gallbladder stones and polyps, and cholangiocarcinoma.

Pathogenesis

The pathogenesis of PSC is not yet well understood. However, it is widely believed that immune dysregulation plays a key role in the development of the disease. Most studies have demonstrated association of human leukocyte antigen (HLA)-B8 and DR3 [183, 184]. A multicenter investigation found the HLA DR3, DQ2 heterozygous genotype to be associated with rapid disease progression in PSC, whereas a protective effect resulted from HLA-DQ6 [185]. Major histocompatibility class 1 chain A (MICA) and B (MICB) genes have also been studied in PSC. The prevalence of MICA5.1 (90% vs 74%) and MICB24 (58% vs 29%) alleles were considerably increased among patients with PSC compared with control subjects [186]. Among two independent PSC populations, MICA 008 allele was more common than in control subjects (66% vs 48%), whereas the MICA 002 allele had a protective effect from PSC [187]. The influence of a matrix metalloproteinase-3 functional polymorphism in PSC has also been investigated [188].

There is no specific autoantibody for PSC, although antineutrophil cytoplasmic antibody (ANCA) positivity has been known to occur in up to 88% of patients while antinuclear antibody (ANA) positivity has been observed in a substantial portion (53%) of PSC patients[189]. Anticardiolipin positivity has been reported in up to two-thirds of patients and is associated with prominent histologic changes and disease severity [190]. Some degree of association has also been linked with *H pylori* and PSC [191].

Diagnosis

A cholestatic picture of liver function with an elevation in serum alkaline phosphatase level is the biochemical hallmark of PSC, although some patients may have normal alkaline

phosphatase levels [173, 192]. Increase in serum aspartate and alanine aminotransferase levels are usually only mild to moderate. Patients with PSC often have fluctuations in bilirubin and alkaline phosphatase levels during the course of the disease. Periods of clinical and cholestatic relapses follow periods of clinical remission with less cholestasis [193].

Diagnostic features include diffuse multifocal strictures, usually involving both the intrahepatic and extrahepatic ducts. Strictures are typically short and annular, alternating with normal or minimally dilated segments to produce a characteristic “beaded” appearance [194]. Cholangiography is considered to be the gold standard for the diagnosis of PSC [195] and is still commonly used not only for diagnosis but also therapeutically to dilate or stent the dominant stricture and screen for cholangiocarcinoma by way of brush cytology and biopsy. ERCP in patients with PSC is associated with risk of complications such as cholangitis, pancreatitis, bile duct perforation and stent migration. Multiple studies have described highly variable rates of ERCP related complications in PSC patients, ranging from 3-18% [196-198]. MRC for detecting PSC has emerged as an accurate, rapid, noninvasive alternative for examination of the biliary tree and is commonly used in many centers. Other advantages of MRC over ERCP include cost savings and the lack of radiation exposure [198, 199]. The major disadvantage of MRC is that it is a purely diagnostic examination, although it can be used to identify patients who would benefit from subsequent ERCP [200].

The role of liver biopsy in the diagnosis of PSC appears to be limited [201]. Histology in PSC is characterized by damage, atrophy and ultimately loss of medium and large size bile ducts, within or outside the liver [202]. These are not typically captured in a percutaneous liver biopsy. The smaller ducts are affected by the resultant obstruction and gradually disappear (ductopenia). The characteristic pathological feature of PSC is concentric periductal fibrosis, which progresses to a narrowing and then obliteration of the small bile ducts, leaving a bile duct scar. Diagnosis is usually established by cholangiography and cholestatic liver profile.

Treatment

Different medical therapies have been tried for PSC, but no treatment has been proven to be effective in randomized controlled studies. Drugs evaluated to treat PSC include budesonide, colchicine, cladribine, cyclosporine, etanercept, infliximab, methotrexate, mycophenolate mofetil, nicotine, penicillamine, pentoxyfylline, pirfenidone, silymarin and tacrolimus [203-219]. Despite encouraging results from a few studies, none have demonstrated convincing evidence of benefits and some are associated with significant side effects [203, 210, 219].

Ursodeoxycholic acid has been aggressively studied at various doses for the treatment of PSC. Several pilot studies and subsequent controlled trials with UDCA have demonstrated some improvement in liver biochemistries in PSC, but no benefit in patient survival [220]. Recently, a large, placebo-controlled trial with UDCA at 28-30mg/kg/d has shown improvement in liver biochemistries but results do not correlate with clinical benefits and are associated with an increase in clinically important adverse events [221]. Therefore, high dose UDCA should not be used in PSC patients because it is associated with harmful effects.

Endoscopic therapy

Some patients present with clinical and biochemical deterioration and exhibit a dominant stricture that involves the larger extrahepatic biliary ducts. In such cases, endoscopic intervention is indicated with balloon dilatation, with or without stenting. This leads to symptomatic, biochemical and radiographic improvement. The use of endobiliary stents in PSC has been associated with greater frequency of intervention-related complications such as cholangitis; balloon dilation alone is preferred in this population [222, 223]. The incidence of dominant strictures in patients with PSC has been estimated to be as high as 45-58% [193].

Liver transplantation

Liver transplantation is the treatment of choice for patients with end-stage PSC. It should be considered before the disease becomes too advanced to increase the long term survival rates after liver transplantation [224]. Indications for liver transplantation include recurrent bacterial cholangitis despite intensive medical and endoscopic therapy, severe extrahepatic biliary obstruction that precludes operative repair, uncontrolled peristomal variceal bleeding, and intractable pruritus. PSC is among the indications for liver transplantation with the best patient survival [225]. Reports from single centers performing liver transplantation in PSC patients have demonstrated excellent survival rates of 90-97% at one year and 83-88% at five years [225, 226]. Recurrence of PSC in liver graft occurs in 2-40% of the transplanted grafts [59]. Proposed risk factors for recurrent PSC include recipient age, male sex, sex mismatch, co-existent IBD, presence of intact colon after liver transplantation, cytomegalovirus infection, recurrent and steroid resistant acute cellular rejection, use of murine monoclonal-CD3 antibody for acute cellular rejection and maintenance corticosteroids after liver transplantation [227-234]. The recurrence of PSC is associated with more severe course and graft loss.

Immunoglobulin G4-Associated Cholangitis

Introduction

Immunoglobulin G4 (IgG4) associated cholangitis (IAC) is a biliary disease characterized by formation of biliary strictures and response to steroid therapy. IAC frequently involves extrahepatic bile ducts and is associated with other fibrosing conditions, especially autoimmune pancreatitis (AIP). The serum level of IgG4 is usually elevated and there is infiltration of IgG4 positive plasma cells in bile ducts. Researchers have reported elevated levels of IgG4 in 9% of a large cohort of PSC patients[235]. IAC and PSC are more common in males [236-239] and this is somewhat paradoxical as autoimmune disorders are usually more common in females. IAC patients are older at diagnosis than patients with classic PSC and no cases of IAC have been diagnosed in children [240].

Clinical Presentation

Obstructive jaundice is a common clinical presentation and it occurs more abruptly in IAC [236-239, 241-248] which is seldom observed in PSC patients [240]. IgG4 is usually noncytotoxic [249] but has been shown to be a pathogenic autoantibody in some dermatologic disorders such as pemphigus vulgaris [250]. Elevated levels of IgG4 have been observed in some other allergic and parasitic disorders [251, 252]. IAC has no association with inflammatory bowel disease [253] or cholangiocarcinoma, whereas classic PSC has association with both IBD [240] and cholangiocarcinoma [240, 254, 255].

Diagnosis

The diagnostic criteria are usually based on histology, imaging of the bile tract, serology and other organ involvement. The histology is characterized by lymphoplasmacytic inflammation, fibrosis and obliterative phlebitis [237, 239, 247, 256, 257]. Despite the dense periluminal inflammation, the biliary epithelium is usually intact. This is in distinct contrast to PSC, which often produces mucosal erosion. In IAC, the inflammatory process is often more dense at the periphery of the bile duct [237, 256, 258]. The predominant cells in IAC are lymphocytes and plasma cells [237, 257]. Neutrophils, commonly seen in PSC, are not a feature of IAC. Imaging of the biliary tract shows one or more strictures involving intrahepatic, proximal extrahepatic or intrapancreatic bile ducts [259]. Although pancreatic involvement such as diffuse pancreatic enlargement or pancreatic mass is very common in patients with IAC, many cases have been described with isolated biliary tract strictures [237, 238, 260].

Patients with IAC generally have elevated levels of IgG4 [256]. It has been shown that some patients of IAC do not have increased levels of IgG4 initially but develop subsequently high levels during follow-up [247]. Recently, the IgG4-positive plasma cell/mononuclear cell ratio was found to be significantly higher in IAC in comparison with classic PSC patients and this ratio was suggested to be a useful index to help distinguish IAC from PSC [257]. Other associated conditions in IAC include, AIP, retroperitoneal fibrosis, renal lesions, and salivary/lacrimal gland enlargement.

Treatment

Corticosteroid therapy improves liver enzymes, causes resolution of strictures, improves associated pancreatitis and it distinguishes IAC from PSC [238, 239, 242, 244, 246, 257, 259, 261-272]. There is no specific recommendation on the dose and duration of steroid therapy; however, most researchers have used prednisone 40 mg daily for 4 weeks and then tapering of steroids 5 mg/week. Initial treatment is usually considered for 3-6 months but longer duration may be needed.

Overlap Syndrome

Introduction

The simultaneous or successive coexistence of PBC and AIH is called overlap syndrome [273]. This has also been named “hepatitic variant of PBC.” The diagnostic criteria, frequency, and appropriate treatment for overlap syndrome have not yet been standardized. The prevalence of PBC-AIH overlap syndrome has been reported in 5-19% of PBC patients [30, 274-278] and 5-8% of AIH patients. A scoring system for the diagnosis of AIH, proposed by the IAIHG, has also been used as a diagnostic tool for identifying PBC-AIH overlap among patients with PBC [9, 10, 275]. Treatments for both PBC and AIH when isolated are well established. PBC is treated with UDCA at a dose of 13-15mg/kg/day. This therapy is recommended by the American Association for the Study of Liver Diseases and is approved by the United States Federal Drug Administration [279]. Different studies have revealed that UDCA improves liver biochemical markers, decreases total cholesterol levels, delays histologic progression, reduces risk for the development of esophageal varices and improves survival [140, 141, 143, 145, 147]. Corticosteroid therapy, with or without azathioprine, markedly improves survival in patients with AIH, and UDCA is not helpful as treatment in this population [43, 280-282]. By contrast, despite its presumed autoimmune etiology, a clear benefit from immunosuppressive therapy in PBC has not been demonstrated to date [32, 134, 203]. Azathioprine monotherapy has no value in the treatment of PBC [135, 283, 284].

The overlap syndrome of PBC-AIH may represent an important and unrecognized cause of refractoriness to UDCA in patients with PBC and /or cause of refractoriness to corticosteroid therapy in patients with AIH [276, 285]. The combination of UDCA with immunosuppressive therapy could potentially provide benefits among patients with PBC-AIH overlap [278, 286]. Patients with PBC and features of AIH overlap are at higher risk of symptomatic portal hypertension and have worse outcome compared to those with PBC alone [287]. Therefore, distinguishing between patients with PBC-AIH overlap and patients with PBC or AIH alone is important because therapeutic options and long-term outcomes may be different between the groups.

Etiology and Pathogenesis

The etiology of overlap syndrome is unknown. In most of the autoimmune diseases, a triggering agent, genetic predisposition, autoantigen display, immunocyte activation and effector cell expansion are likely needed [288]. Similarly, the overlap syndrome is probably induced by one or more triggers or pathogens and is self perpetuating. In the context of overlap syndromes, it is also unknown how two autoimmune liver diseases act in the presence of another. Although the primary target cells and antigens are different, the common feature of autoimmune liver diseases is that they result from alterations of immune regulation [276]. Multiple triggering factors have been proposed including infectious agents (such as hepatitis A, hepatitis C, measles, and herpes simplex virus), drugs, toxins, environmental toxins and pregnancy [14, 16, 288, 289]. Minocycline is the drug that has been most commonly

implicated as a cause of AIH [18] but others have also been described including hydralazine, procainamide and statins [20, 290-292]. There can be a long lag time between exposure to the trigger and onset of the disease and the trigger factor is not needed for perpetuation of the disorder [288]. Most of the patients with overlap syndrome have the characteristic human leukocyte antigen (HLA) haplotypes of AIH, namely HLA-B8, DR3 and DR4 [293].

Diagnosis

There is no standardized diagnostic criteria and treatment for overlap syndrome. Most of the studies currently available are based on different criteria, none of which have been validated. Multiple authors have used criteria called “strictly defined criteria” for the diagnosis of overlap syndrome [276, 277, 293-295]. Those strictly defined criteria are based upon simplified criteria of PBC alone and AIH alone and overlap syndrome was defined by the association of PBC and AIH either simultaneously or consecutively. Several other authors have based the diagnosis of overlap syndrome on applying variations of the IAHG scoring system used for the diagnosis of AIH to patients with known diagnosis of PBC [9]. For diagnosis of each disease, the presence of at least two of the three accepted criteria was required. These criteria are mentioned in Table 4.

The determination of the most adequate set of criteria to use for diagnosis of overlap syndrome is challenging. AIH alone lacks pathognomonic criteria and its diagnosis is complex and is secured, especially in difficult cases, by the application of a scoring system that has been developed and refined over the years [9, 10]. The IAIHG scoring system is not only used for the diagnosis of AIH but also helps to separate autoimmune liver disease entities rather than looking for common features or the possible evolution of one disease into another [9, 296, 297]. There are a number of clinical and biochemical features common to AIH and PBC that are assigned positive scores despite their lack of discriminative ability, such as female gender, presence of other autoimmune disorders and lymphoplasmacytic infiltrate [298]. The scoring system provides points for the presence of SMA, ANA or LKM-1 and more recent developments in the area of autoimmune markers, such as soluble liver antigen/liver-pancreas (SLA/LP) which could be of significance in determining the presence of overlap syndrome, are not included in the scoring system.

Treatment

There is no standard treatment for the overlap syndrome. Corticosteroids have frequently been used in an empiric fashion to treat the overlap syndromes and results have been variable [285]. Remission has been reported with corticosteroids therapy in a group of patients with AIH and PBC, having serum alkaline phosphatase less than twofold the reference value [299]. Several different studies suggest that patients with PBC-AIH overlap syndrome benefit from a combination therapy with UDCA and corticosteroids [278, 293]. Studies assessing treatment response with immunosuppression in patients with PBC-AIH overlap, have demonstrated a beneficial effect of combination therapy of UDCA and immunosuppression, such as corticosteroids with or without azathioprine [276, 278, 293, 295, 300]. Combination therapy

not only improves biochemical markers but also delays fibrosis progression [278]. This opinion has been challenged by other authors who have reported that laboratory improvement could be achieved with UDCA alone and survival of overlap patients is similar to regular PBC patients. UDCA has been suggested by some authors as the first-line therapy, while the combination of UDCA and immunosuppression should be reserved to non responders to UDCA [300]. The heterogeneity in the criteria used to diagnose overlap syndromes in these studies might partially account for the variety of results obtained. It is clear that large and prospective trials are needed to evaluate the efficacy of UDCA and corticosteroids in patients with PBC-AIH overlap syndrome [276]. In addition, other important questions remain unanswered, such as the ideal drug, dosages, administration and duration of therapy.

Overlap of Primary Sclerosing Cholangitis and Autoimmune Hepatitis

In most of the reported cases of PSC-AIH overlap syndrome, PSC and AIH are believed to occur simultaneously [301-303]. In a few of these PSC-AIH patients, the diagnosis of AIH preceded that of PSC, often by several years [304-306]. In other cases, patients with PSC sequentially developed AIH [307, 308]. The overlap between PSC and AIH has been documented in both pediatric and adult populations [301, 302, 304, 309-311]. The study of HLA haplotype suggests a common genetic basis for both AIH and PSC [312]. The diagnosis of overlap syndrome of PSC with AIH is based on applying the IAIHG scoring system to a group of patients with PSC and in some cases conventional descriptive criteria are also used. Effective medical therapy is not yet available for the PSC. Most of the studies have used corticosteroids or a combination of corticosteroids and azathioprine for the treatment of PSC-AIH overlap syndrome [301, 304, 313-315]. The response to therapy depends on the predominance of PSC or AIH features.

Table 4. List of descriptive criteria for PBC, AIH and PBC-AIH overlap syndrome.

<i>PBC Criteria</i>
1) Serum ALP levels at least twice the upper limit of normal values or serum GGT levels at least five-times the upper limit of normal values
2) A positive test for AMA
3) A liver biopsy showing florid bile duct lesions
<i>AIH Criteria</i>
1) Serum ALT levels at least five-times the upper limit of normal values
2) Serum IgG levels at least twice the upper limit of normal values or a positive test for SMA
3) A liver biopsy showing moderate or severe periportal or periseptal lymphocytic piecemeal necrosis
<i>PBC-AIH overlap syndrome</i>
• Overlap syndrome is present when two out of three PBC criteria are met and two out of three AIH criteria are met either simultaneously or consecutively

Conclusion

Autoimmune liver diseases are characterized by immune mediated injury to hepatocytes or bile ducts. Autoimmune hepatitis typically involves hepatocytes while PBC, PSC and IgG4 associated cholangitis involve the biliary tract. The overlap syndromes of PBC-AIH and PSC-AIH involve both hepatocytes and cholangiocytes. It is generally accepted that pathogenesis of autoimmune liver diseases are multifactorial. Both environmental and genetic factors play an important role in disease onset and progression. The diagnosis of autoimmune liver diseases require the exclusion of other causes of liver damage such as viral, toxic, alcoholic, and metabolic or non-alcoholic fatty liver disease. These autoimmune liver diseases have specific clinical, biochemical, histopathological, and cholangiographic features. Only PBC, AIH and IgG4 associated cholangitis are responsive to standardized treatment regimens. There is no specific drug treatment for PSC. Patients with end-stage liver disease from these autoimmune disorders are treated with liver transplantation.

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Chapter 2

Immunopathological Events in an Experimental Autoimmune Kidney Disease and How Those Events Can be Terminated to Regain Tolerance to Self

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Abstract

For some time now Barabas and colleagues have been describing the term autoimmunity as encompassing two beneficial and two harmful processes. The beneficial processes aim (a) to maintain tolerance to self by eliminating cellular waste and (b) to discriminate properly against abnormal self resulting in the destruction of emerging abnormal cell lines. The harmful processes are the opposite of the beneficial ones, i.e. autoimmune disease and cancer.

In the case of autoimmune diseases, pathogenic immune responses directed against one or more target antigens in the system can cause cell mediated or pathogenic antibody initiated injuries.

In the case of cancer, groups of abnormal cells invade previously normal organs, tissues, etc., causing harm or even death because the immune system does not receive the information necessary to recognize them and produce lytic autoantibodies to kill them.

In this chapter we highlight the pathogenic IgG autoantibody mediated immune events observed in an experimental membranous glomerulonephritis called Heymann

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nephritis which cause the chronic progressive autoimmune kidney disease. We describe how the disease is initiated and maintained, and how it can be diagnosed and terminated by the application of a new vaccination method we have developed and call modified vaccination technique. We also touch on how the same technique may be used to stimulate or upregulate immune response against exogenous antigens and cancer.

Keywords: autoimmunity, cancer, autoimmune diseases, modified vaccination technique,

Introduction

In the medical literature the term autoimmunity almost always refers to autoimmune diseases, which are associated with morphological changes and functional deterioration of organs resulting from immune mediated injury. Our research group, on the other hand, as a result of extensive research into several aspects of autoimmune disorders, has for a number of years viewed the term autoimmunity as describing a much broader picture of immune functioning [4, 5, 9, 10]. As we describe in the present communication, we believe that autoimmune responses proceed in the living system mainly for the benefit of the organism, and in fact primarily to preserve immune tolerance to self [30, 32, 50, 51, 89, 134, 139]. In our view there are two beneficial aspects of autoimmunity, both of which aim throughout life to clear the internal environment of unwanted material, i.e. cell debris derived from cells at the end of their life span or damaged by physical injury or toxic agents (cigarette smoke, drugs, alcohol, burns, chemicals, etc.) and cells that acquire properties that would lead to cancer (Figure 1).

Unfortunately, there are two harmful aspects of autoimmunity as well, which must be kept at bay by the normally functioning immune system. One such harmful aspect of autoimmunity is the process typically classified as autoimmune disease. Disorders of this type emerge chiefly when normal self components (released from damaged cells) are not removed quickly enough from the internal environment [81, 111], and various modifying agents (chemicals, toxins, drugs, etc.) can alter their chemical composition and cause them to appear as non-self. Such altered self molecules, when presented to the cells of the immune system, can evoke pathogenic autoimmune responses and cause disease [14].

The other harmful aspect of autoimmunity manifests as cancer. It is characterized by groups of autologous but abnormal cells emerging and multiplying out of control, moving and establishing new colonies in sites other than their points of origin, and causing multiple morphological and functional disturbances which sooner or later become life threatening. These cancer cells are characterized by cancer specific antigens (ags) on their surfaces, which may be more or less easily discernable by the immune system.

To fully understand autoimmunity we must strive to comprehend the following:

- normal and abnormal immune events against endogenous ags;
- the etiology and pathogenesis of disorders initiated by endogenous ags;
- how to diagnose disorders initiated and maintained by endogenous ags (by histology, direct/indirect fluorescence antibody (ab) tests, electronmicroscopy, blood tests, etc.);
- how to prevent the occurrence of such disorders (if possible);
- how disorders are presently treated;

- how to utilize the modified vaccination technique (MVT) (a new vaccination method the Barabas research group has developed) in prophylactic and therapeutic regimens to specifically prevent or terminate disorders caused by endogenous ags.

The vast majority of scientists continue to use the term autoimmunity merely to denote autoimmune disease, and fail to note the critical relevance of the other three aspects of autoimmunity as processes involving or mediated by immune response against self. Likewise, there are those who continue to believe that once an autoimmune disease begins it continues *in perpetuum* [78, 101] and only drugs can slow down the harmful immune insults directed against target ags [63, 97]. Our research group, on the other hand, envisions a time in the near future when autoimmune disorders (i.e. both autoimmune disease conventionally so called and cancer) will be preventable, and when a disorder already present in the system will be treatable specifically and without side effects by the appropriate presentation of endogenous ags to the cells of the immune system [4, 9, 17, 18, 26].

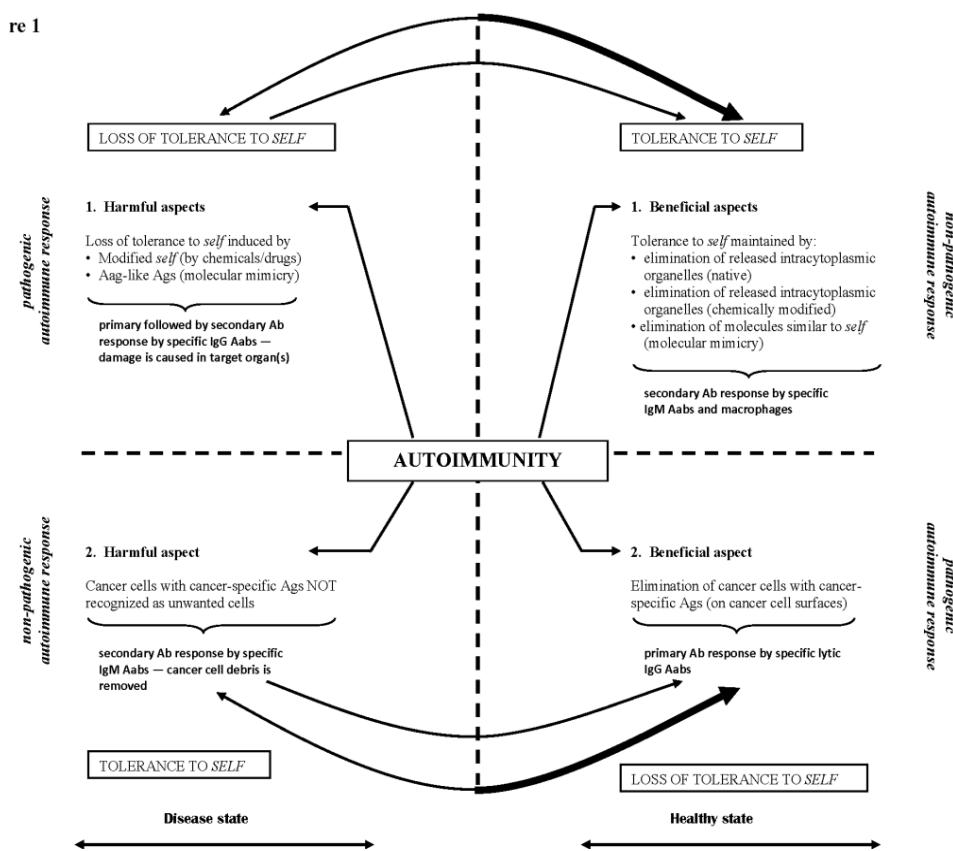


Figure 1. Two beneficial and two harmful aspects of autoimmunity are illustrated. The MVT by being able to correct immunopathological mishaps (autoimmune diseases and cancer) has the potential to evoke corrective immune responses in vaccinated hosts and reestablish normalcy/tolerance to self. [Figure reproduced by permission from BioProcessing Journal, 2007 Winter;6(4):12-18.]

Abbreviations: aab, autoantibody; aag, autoantigen; ab, antibody; ag, antigen; IgG, immunoglobulin G; IgM, immunoglobulin M; MVT, modified vaccination technique

In several of our experiments, we have demonstrated not only that an experimental autoimmune kidney disease called slowly progressive Heymann nephritis (SPHN) [12] can be prevented by the new vaccination technique that we have developed, but also that when the disease is in its progressive phase, the immunopathological processes can be terminated by the same technique [4, 5, 7, 9, 13, 15, 16, 17, 21, 26]. The technique is able to stimulate a specific corrective immune response. One reason why autoimmune diseases have not been preventable or effectively treatable so far is due to the lack of technical preparedness for the implementation of such a specifically targeted technique.

As we shall describe later in detail, during the autoimmune disease SPHN [12], production of both pathogenic immunoglobulin G (IgG) autoantibody (aab) (tissue damaging aab) and nonpathogenic immunoglobulin M (IgM) aab (cell debris removing aab) occurs [21]. In order to terminate the disease process, the level of circulating nonpathogenic IgM aabs capable of assisting in the removal of both modified and native autoantigens (aags) has to be sufficiently elevated. With the MVT this beneficial corrective immune response can be achieved specifically and without side effects [4, 26]. The vaccination technique is able to correct endogenous ag generated disorders (i.e. cancer and autoimmune diseases) by eliciting or enhancing immune responses that normally take place but at times are either insufficient or completely absent. The utilization of the immune system's natural ability to respond and correct autoimmune disorders and bring itself back to a normal healthy state could be the most welcome treatment solution for patients who benefit only marginally from presently available medicaments. Results achieved in the experimental autoimmune kidney disease provide encouraging possibilities for correcting a wide range of disorders involving, initiated by, or maintained by endogenous ags through the implementation of the MVT [7, 13, 15, 21].

We describe below the immune events that contribute to the development and termination respectively of the autoimmune disease called SPHN. In order to clarify physiologic versus disease causing processes and highlight possible means to downregulate the autoimmune disease causing pathological events, we repeat certain concepts as well as experimental evidence as appropriate throughout the text.

Background to Heymann Nephritis (HN)

HN was first described in 1959 by Heymann and colleagues. Many papers have subsequently been published on various aspects of this experimental autoimmune kidney disease. Classically, HN is produced in susceptible strains of rats by multiple IP injections of renal tubular ags incorporated most often into Freund's complete adjuvant. Proteinuria typically starts six to eight weeks after the first injection of the ag:adjuvant mixture, and progressively worsens [58, 59, 61, 100, 130]. During the progressive phase of the disease, morphological changes are observed in the kidneys [1, 22, 43, 49]. Silvermethenamine stained kidney sections show thickened glomerular capillary loops with silver positive spikes on their outer surfaces [12]. Electron microscope pictures reveal large osmiophilic deposits on the epithelial side of the glomerular basement membrane (GBM) partially or completely surrounded by basement membrane like material [2, 22]. Epithelial cell foot processes appear fused around the deposits. Osmiophilic densities are observed within the epithelial cells. The renal proximal tubules also show characteristic changes, especially at their brush border (BB)

regions where the cells appear swollen and fragmented into small pieces [80, 110]. Sloughing of the BB cells and cellular hyperplasia are also observed. Direct immunofluorescence ab tests show beaded depositions staining for rat IgG, rat C3 and rat C5b-9 around the glomerular capillary loops [12], and indirect fluorescence ab tests show serum samples of HN rats reacting with the BB regions of the proximal tubules of normal rat kidney sections with a diffuse staining pattern for rat IgG and with a linear staining pattern for rat IgM [12]. More detailed descriptions of the renal lesions and functional changes in the kidney are found in the various published material available [41, 42, 56, 60, 75, 80, 85, 86, 87, 90, 92, 103, 105, 109, 110, 114, 115, 128].

There are variants of HN. The best known variant is passive HN, which was first described by Barabas and colleagues in 1970 [23, 24, 47, 48, 96, 129]. Passive HN is induced in susceptible strains of rats [109] by a single IV injection of a heterologous (most often rabbit or sheep) anti-rat renal tubular ag IgG ab. Immediately after its administration the heterologous ab (e.g. rabbit IgG) reacts with the glomerular associated renal tubular ag [75, 76]. In a direct fluorescence ab test it appears as fine beaded depositions around the glomerular capillary loops (heterologous phase). Approximately one week after the injection of the heterologous ab, the rats produce abs (rat anti-rabbit IgG IgG ab) against the foreign serum protein (the rabbit anti-rat IgG), and these abs react with the heterologous ab localized in the glomeruli, intensifying and enlarging the glomerular deposits (autologous phase). Passive HN is not considered to be a true autoimmune disease as no actual autoimmune response, chronically progressive or otherwise, proceeds from the rat anti-heterologous IgG ab immune response. Nevertheless, passive HN has been and still is a very useful experimental model for investigating how immune complexes (ICs) develop and deposit in the glomeruli.

There are two other experimental model variants of HN that the Barabas group has developed. The first of these involves inducing the disease in rats by injections of chemically modified renal tubular ag incorporated into Alum and Distemper complex vaccine, followed by SC injections of an aqueous preparation of the same kidney ag [12]. The disease variant that develops – called SPHN – is associated with slowly progressive proteinuria and less severe morphological change in the kidney, allowing sufficient time to initiate immune intervention to prevent the occurrence of the disease, and during the progressive phase of the disease, to terminate it.

The second model variant of HN involves induction of the disease without incorporating adjuvant into the nephritogenic tubular ag injected into the rats. Instead, the renal tubular ag is chemically modified, and administered in repeated injections of a hapten-protein conjugate [14]. This method also produces SPHN. This is an important observation since in humans – especially those who are genetically predisposed to autoimmune disease – slowly progressing immune events could be initiated by inciting agents or toxins such as drugs, chemicals, smoking, alcohol, etc., which might couple with normal self ags and alter them into non-self. Altered self ags could subsequently start an autoimmune disease [36, 46, 94, 133].

There are differing opinions as to the role of self ags in the induction of autoimmune disease and in its maintenance during the chronic progressive phase. There are those who believe that once an autoimmune disease is initiated (for whatever reason) it can be perpetuated by an abnormal immune response against a target self ag, with the target ag stimulating continuous IgG aab production [8] and/or with pathogenic aabs continually produced by immortalized plasma cells [37, 78, 101].

Experimental evidence, however, strongly supports the view that a native self ag on its own can neither initiate nor maintain an autoimmune disease [8, 19]. Rather, the continuous presence of a *modified* self ag [12, 14, 58] – or of a self like ag (molecular mimicry) [38, 40, 52, 53, 142] – must be present in the system to start and continue pathogenic immune events.

So what is the role of a self ag in autoimmune disease development, e.g. of the nephritogenic ag in HN? First of all, it is a target, i.e. for pathogenic IgG aabs directed against the nephritogenic ag, which attack it where it resides in the BB regions of the renal proximal tubules, damaging the BB and releasing increased levels of the ag into the circulation and urine [12]. Secondly, the increased levels of nephritogenic ag liberated from the renal proximal tubules contribute to continuous deposition of ICs in the glomeruli. Thirdly, the ag stimulates the production of nonpathogenic IgM aabs [12], which aim to remove both modified and native nephritogenic ags from the circulation.

It should be noted that HN can only be induced in certain strains of rats [109, 117, 118]. In these susceptible animals the nephritogenic ag is localized in or on the epithelial cell foot processes and around the glomerular capillaries. Some descriptions have incorrectly assumed and reported that the nephritogenic ag, which appears to be similar to nephritogenic ags localized in the BB, is produced by the epithelial cells of the GBM and foot processes, and that they are redistributed on the outer surface of the foot processes and at their juncture with the GBM [70, 103]. According to these incorrect descriptions, when the nephritogenic ag incorporated in adjuvant is injected into the organism and pathogenic IgG aabs against it are produced, these abs react and localize to the GBM because the nephritogenic ag is essentially a part the membrane. Such descriptions further assert that the deposits (made up of the nephritogenic ag, pathogenic IgG aab against the nephritogenic ag, and complement components) increase in size as a result of continuous production of pathogenic IgG aabs by plasma cells, along with continuous production of nephritogenic ags by the epithelial cells of the GBM, contributing to layered depositions of ICs [12]. If the above were entirely correct, then it might be safe to conclude that HN and many other autoimmune diseases cannot be treated by any means other than by immunosuppressive agents, since once the disease process starts it necessarily continues into a chronic progressive condition, immortalized plasma cells continuously produce pathogenic IgG aabs against the target ag localized in the epithelial cells of the GBM [78, 101], and the epithelial cells continuously produce nephritogenic ags [70] to which pathogenic IgG aabs can join, causing the continuous increase in the size of IC depositions in the GBM.

The experimental findings of Barabas and colleagues have contradicted the assumptions built into the model described above. Our observations reveal that:

- the nephritogenic ag in the BB region of the renal proximal tubules is the primary source of circulating nephritogenic ag [41, 82, 115], and is the origin of the nephritogenic ag found both in the mesangium [8] and around the glomerular capillaries [31, 34, 66] of normal rat kidneys. This ag is actually trapped temporarily at glomerular and mesangial sites by IgM aabs [8], probably due to their charges.;
- the glomerular capillaries of neonatal rat kidneys – which are not open to the circulation – do not stain for the presence of nephritogenic ags [31], indicating that the nephritogenic ag is not produced locally by the epithelial cells of the glomerulus;

- the specific nonpathogenic IgM aabs directed against the nephritogenic ag assist in the removal (catabolism) of the nephritogenic ag entering into the mesangium [8] (designed to support the glomerular capillary loops and also to filter out large molecules that could interfere with glomerular filtration. The mesangium is filled with large macrophage like enzymes);
- the nonpathogenic IgM aabs react with and neutralize both native and modified nephritogenic ags [18, 19] (the latter could initiate continuous production of pathogenic IgG aabs against the target ag residing mainly in the renal proximal tubules' BB region);
- repeated injections of native nephritogenic ag into susceptible strains of rats only produce nonpathogenic, non-tissue damaging IgM aabs (even during the chronic phase of SPHN [14], though the increased ag supply might contribute to glomerular deposits);
- in order to maintain continuous production of pathogenic IgG aabs against the target nephritogenic aag, the inciting agent must be present in the system to chemically or otherwise modify the target ag [14, 104, 143];
- if the modified nephritogenic ag is removed from the system then no further production of pathogenic IgG aab is observed [92];
- the balance between normally occurring IgM production and induced IgG aab production can be tipped in favor of IgM aabs, *giving the IgM the chance to terminate the disease process by removing from circulation both the native nephritogenic aag (which is continually released from the renal tubules as a result of the pathogenic IgG aabs damaging the BB region by attacking the aag in situ, and which contributes to layered depositions of ICs in the glomeruli) and the modified native nephritogenic aag (which, while present in the system, stimulates the production of tissue damaging pathogenic IgG aabs that cause the injury to the renal tubules and contribute to the injury of the glomeruli)*;
- to bring this shift about, the animals must be subjected to a treatment protocol – which may be administered either (a) both pre- and post-kidney-disease-induction or (b) only post-induction – whereby ICs composed of the nephritogenic ag (i.e. rat kidney fraction 3 [rKF3]) and rat IgM ab directed against the nephritogenic ag at slight ag excess – e.g. [rKF3 X rat anti-rat kidney fraction 3 (rarKF3) IgM ab] rKF3 [21] – are injected into the animal at appropriate intervals;
- the IC so administered will produce elevated levels of the same ab with the same specificity against the target ag as resides in the inoculum, namely, rarKF3 IgM aab;
- specific IgM aab directed against the native nephritogenic ag, being cross reactive (just as the pathogenic IgG aab is), removes the native nephritogenic ag (that contributes to glomerular IC depositions) and the modified native nephritogenic ag (that contributes to continuous production of pathogenic IgG aabs), thereby preventing the development of SPHN or terminating it if instituted during the chronic progressive phase of the disease [7, 13, 18, 19].

This MVT has the potential not only of preventing most endogenous ag initiated and maintained autoimmune disorders (i.e. cancer and autoimmune diseases), but also of terminating the already present disorder [4, 5, 9, 10, 16, 17, 26]. It is the first vaccination

method to have been developed that is able to rectify endogenous ag related disorders specifically and without side effects utilizing the immune system's natural ability to respond to corrective information. The vaccination technique has proved in experiments carried out so far that tolerance to self can be regained [7, 13, 15].

Preparation of the inoculum used in the MVT requires the production by chemical or other means of components identical to the specific native target aags [9, 16, 74, 83, 102] and monoclonal/polyclonal abs directed against them. ICs made up of pure, specific components should evoke in the inoculated host the desired corrective immune response outcome.

Fate of rKF3 aag under Physiological Conditions

Figure 2 gives a detailed account what happens to rKF3 aag, a nephritogenic ag, after its release from the BB region of the renal proximal tubule. Many of the comments we provide below apply not only to rKF3 aag, but are generally applicable to all of the intracytoplasmic components of a living organism.

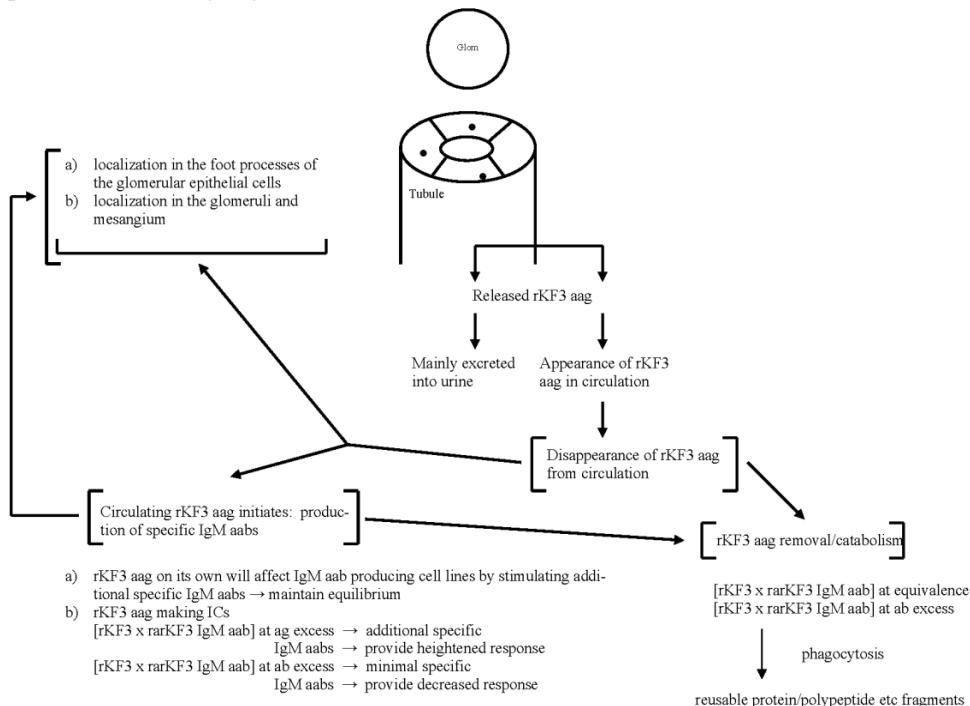


Figure 2. Fate of rKF3 aag under normal physiological conditions

At least three things can happen to the rKF3 aag continuously released from renal tubules into the circulation:

1. Stimulation of specific IgM aab production;
2. Removal/catabolism of rKF3 aags assisted by the IgM aabs;
3. Localization in glomerular/mesangial areas (minimal).

Abbreviations: aab, autoantibody; aag, autoantigen; ab, antibody; ag, antigen; Glom, glomerulus; IC, immune complex; IgM, immunoglobulin M; rarKF3, rat anti-rKF3; rKF3, rat kidney fraction 3

Like any other high demand absorption/filtration related structure, the BB zone of the renal proximal tubule undergoes rapid tissue component turnover in order to maintain its functional integrity. As a result, considerable amounts of BB related aags are shed and released from time to time into the urine [107, 108] and circulation [68, 82, 115, 116]. The released aags will be assisted in their removal by specific IgM aabs prior to being engulfed and digested by mononuclear cells and degraded into small molecular weight peptides, amino acids, etc [19, 77, 99, 140]. Some of the released nephritogenic ag will itself stimulate specific anti-nephritogenic IgM aab production, and some of the released nephritogenic ag will stimulate further production of such IgM aabs while in combination with the IgM aab in the form of IC. The circulating anti-nephritogenic IgM aabs will efficiently assist in the removal of the nephritogenic aag and will prevent its toxic accumulation or possible chemical alteration (which could lead to stimulation of lymphocytes and production of pathogenic tissue damaging IgG aabs). It is noteworthy that under normal conditions:

- native aags – including rKF3 aag – will not initiate the production of pathogenic IgG aabs, nor will they maintain their production during the chronic progressive phase of an autoimmune disease [14];
- glomerular localized rKF3 aag is present in a beaded pattern around the glomerular capillaries in ICs formed with IgM aabs directed against the aag [8]; and
- rKF3 aag associated in ICs with anti-rKF3 IgM aab is also present in the mesangium [8].

The nephritogenic ag (i.e. rKF3) found in both the glomerulus and the mesangium is derived from the renal tubular BB region (and not produced by the epithelial cells of the GBM as previously described in the medical literature) [70].

IgM aabs fulfill an important role in maintaining tolerance to self by clearing the system of released intracytoplasmic components. In a physiological sense we are not *per se* tolerant to the self components [137, 138] that would build up in our systems, and consequently we have specific IgM aabs against the intracytoplasmic aags naturally occurring there [134, 139]. As cells from the various parts of our bodies come to the end of their life spans or are damaged by toxic agents or physical injury (by drugs, chemicals, heat, cigarette smoke, alcohol, infectious agents, etc.), they release their intracytoplasmic contents into the circulation. The removal of these contents is assisted by the specific IgM aabs, which attach to them in preparation for their degradation by monocytes into reusable small molecular weight (MW) peptides [19, 77, 99, 140].

Fate of Native rKF3 and Altered rKF3 Aags During Pathological Response to Altered rKF3 aag

Most often autoimmune diseases start because modifying agents alter the chemical composition of self aags [136]. When an altered self or non-self component is recognized – as would be any foreign ag – by the cells of the immune system, then a pathogenic tissue damaging IgG aab response is initiated whose primary aim is to eliminate the altered self ag from the system. However, being cross reactive, the IgG ab not only reacts with the altered

self ag that initiated its production but also with the normal tissue that contains the native aag, targeting it for destruction and elimination [11]. If the inciting agent is able to maintain the presence of altered self and is present for a prolonged period of time in the system, then a chronic progressive autoimmune disease will ensue [14, 92], resulting in morphological and functional changes in the target tissue, organ, etc., as caused by the pathogenic activity of the IgG aabs [92].

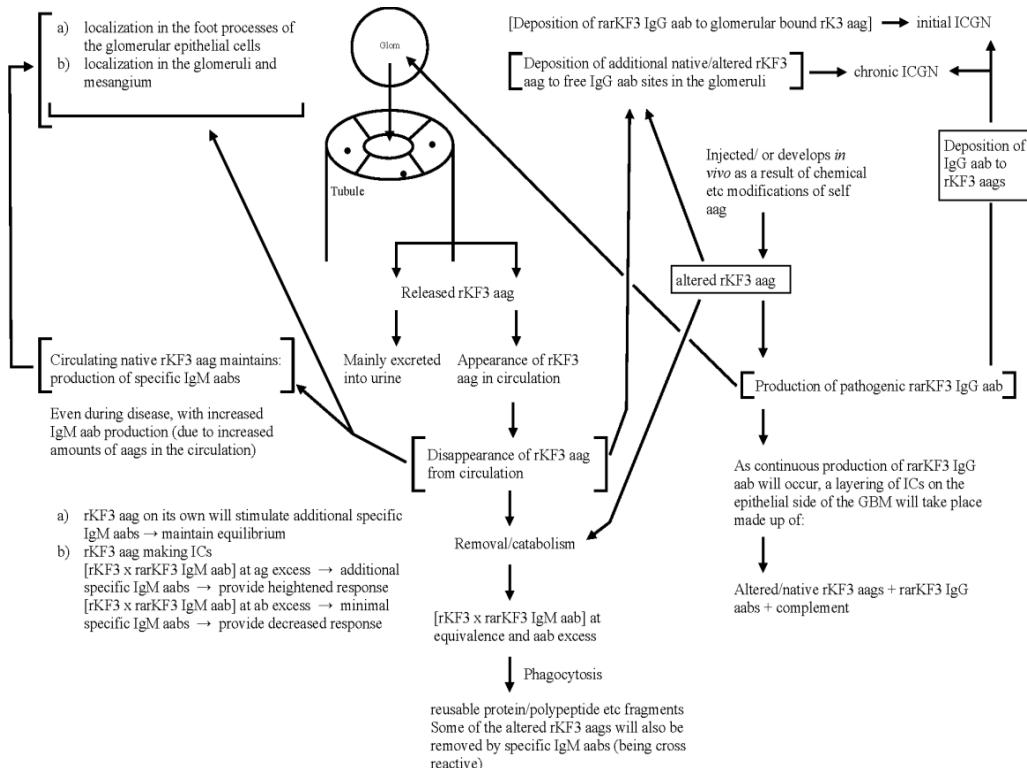


Figure 3. Fate of native rKF3 and altered rKF3 aags during pathological response to altered rKF3 aag With pathogenic aab response:

- Developing glomerular/tubular lesions will be mild, moderate, or severe, depending on the severity of pathogenic aab response;
- Intermittently or continuously deposited rarKF3 IgG aab + rKF3 aag + complement may affect the development/size and composition of IC deposits in the GBM;
- Pathogenic IgG aabs will liberate increased amounts of native rKF3 aag into the circulation from the BB regions of the renal proximal tubules adding to the complications of the disease e.g. by contributing to glomerular deposits;
- IgM aabs will attempt to halt the disease process by removing altered and native rKF3 aags from the circulation.

Abbreviations: aab, autoantibody; aag, autoantigen; ab, antibody; ag, antigen; BB, brush border; Glom, glomerulus; GBM, glomerular basement membrane; IC, immune complex; ICGN, immune complex glomerulonephritis; IgG, immunoglobulin G; IgM, immunoglobulin M; rarKF3, rat anti-rKF3; rKF3, rat kidney fraction 3

Modification of self ags by chemical or other means occurs in every living being continually during its life, but the efficiently functioning autoimmune system prevents an

abnormal response to self. Specific IgM aabs (being cross reactive as well) assist in the catabolism not only of released native intracytoplasmic components but also of aags that become chemically modified [18]. If modification of an aag (by an inciting agent) is short lived and an efficiently functioning immune system is able to remove/eliminate the modified self ag quickly, then the development of a genuine autoimmune disease will be averted.

Even during the course an autoimmune disease (such as HN or SPHN) there are two converse autoimmune reactions that occur. One is physiologic. Released native aags stimulate the production of nonpathogenic IgM aabs [12] (which assist in their eventual catabolism). These aabs are present in the system throughout life, but are more abundant during the chronic progressive phase of an autoimmune disease since more aags are liberated from target sites and stimulate their elevated production [12]. The other autoimmune reaction that occurs is pathogenic. Pathogenic IgG aabs continuously target the native ags situated in tissues and organs, which ags are essential for the morphological and functional integrity of those tissues and organs (Figure 3). Eventually the attack causes irreparable harm resulting in premature disability or even death.

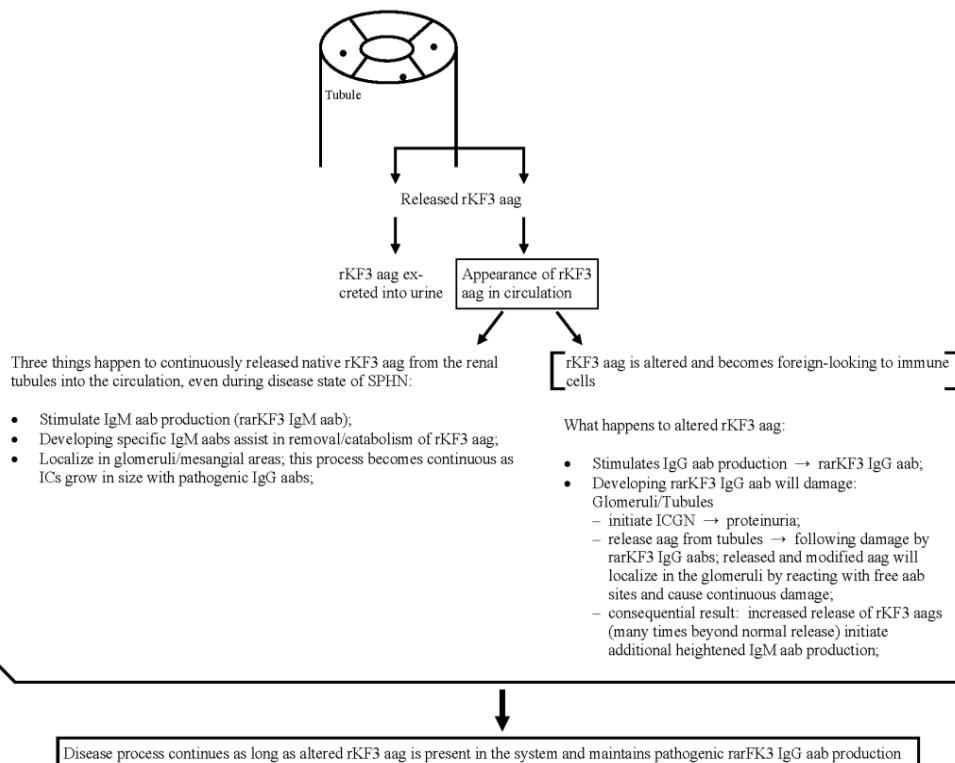


Figure 4. Fate of native and continuously present altered rKF3 aags during SPHN
If the disease process cannot be halted or does not slow down or terminate on its own (because more altered aag becomes available and continues to stimulate pathogenic aab production) then an irreversible autoimmune disease can ensue, culminating in single or multiple organ damage/failure.
Abbreviations: aab, autoantibody; aag, autoantigen; IC, immune complex; ICGN, immune complex glomerulonephritis; IgG, immunoglobulin G; IgM, immunoglobulin M; rarKF3, rat anti-rKF3; rKF3, rat kidney fraction 3; SPHN, slowly progressive Heymann nephritis

The level of pathogenic IgG aab and nonpathogenic IgM aab in the circulation provides a good indication of the progression of the disease, whether towards exacerbation or remission. Further, as long as pathogenic IgG aabs are present in the system, the symptoms of disease – in our case SPHN – will be observed (Figure 4). Damage to the BB area of the proximal convoluted tubules will persist, and aags will continue to be released. The released nephritogenic aags will contribute not only to continuous production of specific IgM aabs, but also to continuous deposition of ICs (together with rarKF3 IgG aab and complement components) in the glomeruli, causing chronic progressive IC glomerulonephritis characterized by nonselective proteinuria.

The fate of the modified self ag must be its complete elimination from the system in order for autoimmune disease maintaining processes to be terminated and tolerance to self to be regained. This can be achieved in two ways. The simplest way is to remove the inciting agent from the system. The other way is to specifically increase the level of IgM aabs against the target ag. Such cross reacting aabs are able to remove the modified aag from the system, thereby halting the further production of tissue damaging aabs [7, 13].

Fate of Altered and Native rKF3 Aags during Treatment with ICs Inducing Nonpathogenic Aab Response in Rats with SPHN

Throughout the life of the organism, the immune system does what it is instructed to do. For example, when it responds by producing pathogenic IgG aabs against a chemically altered self component, it carries out a normal response. However, this response, though normal, is not altogether beneficial to the operation of the targeted organ. Accordingly, there are inbuilt, immune mediated safety processes that work to avert, or at the very least, lessen tissue damage [139]. From birth, living organisms have specific IgM aabs directed against intracytoplasmic aags [138] which are present in various organs, tissues, etc. The function of these specific IgM aabs is to assist in the removal of the intracytoplasmic components that are released into the circulation following cell death. Being cross reactive, the IgM aabs can also remove aags that are chemically or otherwise modified [18], thereby preventing the development of an autoimmune disease by pathogenic aabs.

However, as long as the modifying agent remains present in the system and able to chemically modify a particular aag, and as long as the specific IgM aabs normally produced cannot neutralize and eliminate such chemically modified aags *in toto*, the pathogenic IgG aab response against the chemically modified aag will continue, causing damage to the target organ where the aag resides.

If the inciting or modifying agent is known, then its removal is of course exigent; but whether or not it is known, the logical approach for terminating the autoimmune disease process is to proceed with the removal from the circulation of both (a) the modified self ag that initiated and maintains the tissue damaging aab production, and (b) the native self ag that is targeted and liberated and contributes to lesion development (as in SPHN where glomerular IC depositions increase in size by continuous addition of pathogenic IgG aab, nephritogenic aag released from the renal proximal tubules, and complement components) [18].

Termination of pathogenic aab production should be possible to achieve by increasing the level of specific IgM aabs in the circulation against the native target aag. As stated above, these aabs, if present in sufficient amounts, can assist in the specific removal of both modified

and native aags from the circulation. In the absence of the aags from the circulation, immunopathological processes cease, a progressive autoimmune disease is halted, and tolerance to self is regained (Figure 5). The Barabas research group has shown that this outcome can be attained by the MVT [4, 5, 9, 13, 16, 26].

This third vaccination method, so called because it comes after the two previously developed techniques of active and passive immunization, has been shown to achieve prophylactic and therapeutic intervention in 100% of vaccinated animals, i.e. preventing SPHN if administered early, or when the disease is in its progressive phase, effecting its termination [7, 13, 15]. The MVT utilizes the immune system's natural ability to respond to ag information provided in the inoculum. The vaccine is made up of ICs composed of the target nephritogenic ag rKF3 and specific rarKF3 IgM ab in slight ag excess. Recipient rats injected with the IC produced heightened levels of IgM aabs against the target ag rKF3. The injected ICs accomplished ab information transfer. The recipient rats produced the same ab with the same specificity against the target ag as resided in the injected IC, namely rarKF3 IgM aabs [13, 15, 18]. The heightened levels of IgM aabs, being cross reactive, assisted in the removal of both modified and native rKF3 ag from the circulation, thereby terminating the autoimmune disease processes (production of pathogenic IgG aabs against the target ag, damage to the BB region of the renal proximal tubules and release of native aag therefrom, deposition of ICs in the glomeruli, etc.) and reestablished normalcy, i.e. tolerance to self.

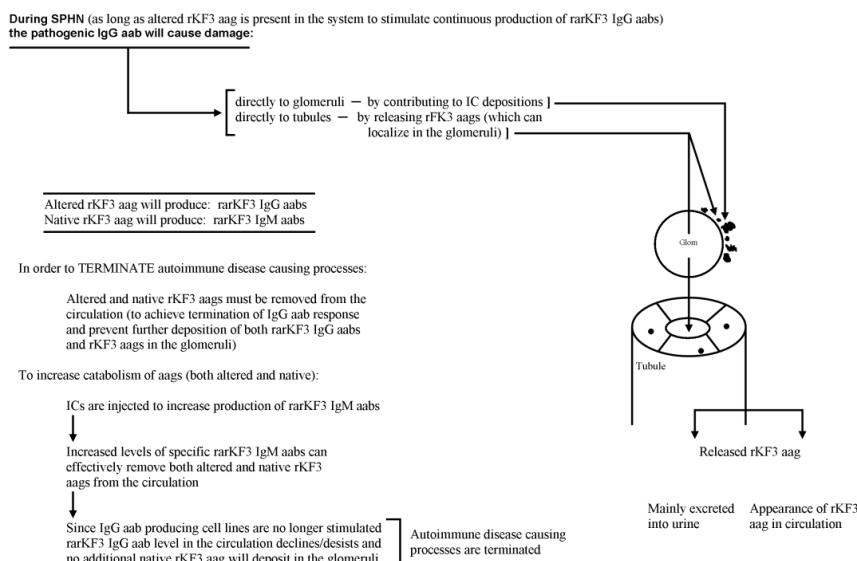


Figure 5. Fate of native and altered rKF3 aags during treatment with ICs of SPHN rats
Specific IgM aabs – produced by ICs [rKF3 x rarKF3 IgM] rKF3 – remove from the circulation and assist in the catabolism of both altered and native rKF3 aags. Their removal can bring about an ag specific downregulation of pathogenic rarKF3 IgG aab response as:

- No more altered aag will be available to stimulate the IgG aab producing cell lines;
- No more altered or native aags will be available to join free IgG aab sites in the glomeruli to increase the size of the deposits and further the progression of ICGN.

Abbreviations: aab, autoantibody; aag, autoantigen; ag, antigen; Glom, glomerulus; IC, immune complex; ICGN, immune complex glomerulonephritis; IgG, immunoglobulin G; IgM, immunoglobulin M; rarKF3, rat anti-rKF3; rKF3, rat kidney fraction 3; SPHN, slowly progressive Heymann nephritis

Renal ags Capable of Initiating and Maintaining HN (When Adjuvanted or Modified)

Several renal tubular preparations, when injected in adjuvants [12, 14, 42, 43, 58, 67, 69, 86, 88, 115, 128], will induce HN in susceptible strains of rats (Figure 6). However, the same renal tubular preparations injected on their own will not produce the autoimmune kidney disease [14]. So what is the difference? Native aags liberated from the intracytoplasmic environment into the circulation – from cells at the end of their life cycles or damaged by toxic agents [134, 135] – will stimulate the production of specific non-tissue damaging IgM aabs whose function is to assist in the clearance of the released aags. Native aags will not initiate the production of pathogenic IgG aabs, nor will they maintain their production during the chronic progressive stages of an autoimmune disease [6]. Nevertheless, they can contribute to lesion development, as in HN, when pathogenic IgG aabs damage the BB region of the renal proximal convoluted tubules and liberate associated nephritogenic aags therefrom into the circulation; and when these liberated nephritogenic aags join and enlarge ICs on the epithelial side of the GBM.

For pathogenic IgG aabs to be produced against a target ag, the aag (or homologous ag) must be presented to the cells of the immune system in adjuvants or in a chemically altered state [14, 58]. Such abnormal presentation of homologous or native aags, such as the tubular nephritogenic ag, will evoke pathogenic IgG aab production resulting in a chronic progressive autoimmune kidney disease, especially if the modified self ag is present for a prolonged period of time, for example as a result of repeated injection in the form of an ag:adjuvant mixture.

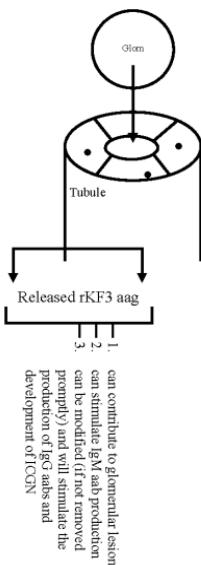
It should be noted that tissue damaging IgG aab production will only occur as long as the altered aag is present in the system to stimulate the pathogenic aab producing cell lines [92]. In the absence of a modified self ag, pathogenic aab production ceases. We do not believe that immortalized plasma cells (once pathogenic aab producing cell lines are established) are able to churn out tissue damaging aabs *ad infinitum* to maintain a chronic progressive autoimmune disease. Experimental findings do not support such a scenario [80].

Theoretically, one might expect HN to be establishable in every species of rat by repeated injection of nephritogenic ag in adjuvant. Yet it is not [109, 118]. HN can only be induced in species of rats in which nephritogenic aags detectably localize around their glomerular capillary loops. There is a controversy regarding the origin of the nephritogenic ag in the glomeruli. As noted above, one school of thought maintains that it is locally produced by the epithelial cells of the GBM and distributed around the glomeruli [70]. However, observations by the Barabas research group demonstrate that the glomerular localized nephritogenic aag originates in the BB regions of the renal proximal tubules [31], and localizes in and around the glomeruli in the form of small ICs formed in conjunction with specific IgM aabs directed against the nephritogenic ag [8]. These ICs are most likely temporarily trapped at these sites due to their charges, and do not produce any obvious morphological change in the kidney under normal circumstances. They do, however, serve as initial target antigenic sites (just like the *in situ* renal proximal tubular BB ag) for IgG aabs under pathogenic conditions. Subsequent cascading immune events result in layered deposition in the glomeruli of ICs made up of the nephritogenic aag, pathogenic IgG aab against the nephritogenic aag, complement components, etc. Nephritogenic ags capable of inducing HN in rats with appropriate inoculation schedules are shown in figure 6.

Nephritogenic aags: [all reside in renal tubules]

1. Heymann's ag
2. RTE - α_5
3. FX1A
4. rKF3
5. Pronase digested tubular ag
6. Sonicated u/c rKF3
7. gp 330
8. gp 660 + gp 70
9. gp 90 + gp 330
10. gp 108 & human FX1A
11. gp 120

Tissue localized nephritogenic aags are targeted by pathogenic IgG aabs, i.e., in tubular BB associated region and the glomerular localized nephritogenic aag



References: [to corresponding aags]

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Figure 6. Renal aags capable of initiating and maintaining HN (when adjuvanted or modified) Over the years, quite a number of renal tubular aags (homologous and heterologous) have been prepared and proven to be nephritogenic. The most nephritogenic aags (incorporated into adjuvants), capable of producing severe ICGN associated with proteinuria, are the high MW renal tubular aags (aags 1-6); while the more purified aags, though they do produce typical ICGN, do not produce proteinuria in most cases (aags 7-11).

These observations are very important in relation to other experimental autoimmune diseases as well as naturally occurring human autoimmune diseases, as they demonstrate that released large MW substances do not have to break down into smaller units or even to small polypeptide fragments to initiate and maintain an autoimmune disease process.

Following the release of large MW components from the intracytoplasmic environment into the circulation (if they are not removed swiftly by specific IgM aabs), these aags can be chemically altered making them appear foreign and thus making them responsible for the initiation and – if the altering agent persists – maintenance of an autoimmune disease.

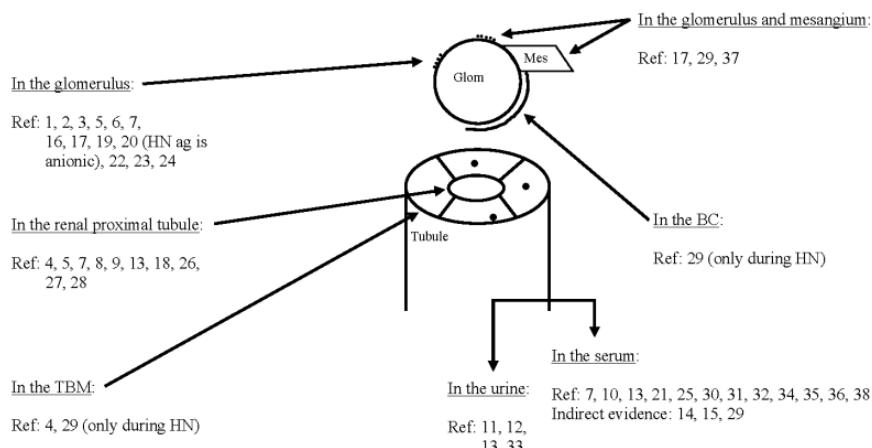
Abbreviations: aab, autoantibody; aag, autoantigen; ag, antigen; BB, brush border; Glom, glomerulus; HN, Heymann nephritis; ICGN, immune complex glomerulonephritis; IgG, immunoglobulin G; IgM, immunoglobulin M; rKF3, rat kidney fraction 3

Nephritogenic aags with the Potential to Induce HN – if Chemically or otherwise Modified – Are Present in the Following Locations

The nephritogenic aag is not only present in abundance in the BB regions of the renal proximal tubules (its principal location) but can also be found at various other sites, to which it travels after its release into the circulation. As stated previously, just like with any other tubular structure with a filtration/absorption function (such as the villi of the small intestine), the components of the BB regions of the renal proximal tubules are continually shed and released into the urine and circulation to keep the filtration/absorption/re-absorption function of the renal tubules operating at maximal efficiency. As a result, released native nephritogenic

ags can be detected in the circulation, in the urine, and at various other anatomical sites, i.e. the glomerulus, the mesangium, the Bowman's capsule, and the tubular basement membrane (the latter two locations during HN) where they are temporarily trapped because of their relative charges or because they are combined with nonpathogenic IgM aabs in the form of ICs [8, 12].

Under normal physiological conditions the nephritogenic aag is removed from the system by specific nonpathogenic IgM aabs [139] and degraded by mononuclear cells into reusable small MW peptides, amino acids, etc [19, 77, 99, 140]. However, as any intracytoplasmic ag released into the circulation can be modified chemically, by denaturing agents, etc., so can the nephritogenic ag [14]. Under such conditions, the production of pathogenic tissue damaging aabs will be initiated and maintained. Capable of cross-reacting with both native and modified aag, these abs attack the target ag *in situ* in the BB region of the renal proximal tubules [14]. We and others have shown that under experimental conditions native nephritogenic ags incorporated into various adjuvants or chemically modified can produce a chronic progressive autoimmune kidney disease, with primary site damage occurring in the BB regions as a result of attack directed against the ags located there, and secondary site injury to the glomeruli caused by the deposition of ICs and resulting in IC glomerulonephritis [12, 58, 100, 130] (Figure 7).



References: [to nephritogenic aag being present]

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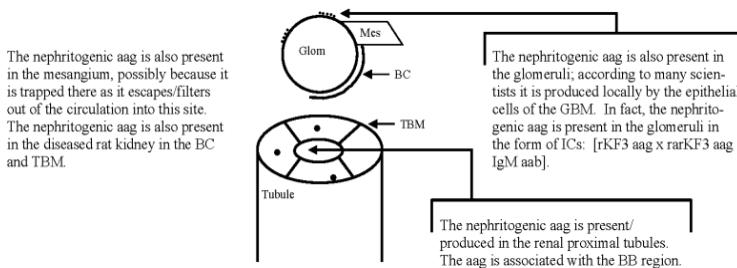
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Figure 7. Nephritogenic aags with the potential to induce HN (if chemically or otherwise modified) are present in the following locations.

Native nephritogenic aag released into the circulation from the BB region of the renal proximal tubules in the kidney is assisted in its catabolism by specific nonpathogenic IgM aabs. The nephritogenic ag is continuously present especially in the serum and urine and to a somewhat lesser extent in the mesangium and glomeruli. As long as the native ag is not altered in its chemical structure (i.e. not foreign in its appearance) the normally functioning immune system will prevent the development of an autoimmune disease.

Abbreviations: aab, autoantibody; aag, autoantigen; ag, antigen; BB, brush border; Glom, glomerulus; HN, Heymann nephritis; IgM, immunoglobulin M; Mes, mesangium; TBM, tubular basement membrane

The fact that chemically altered rKF3 ag without adjuvant can also cause SPHN [14] when injected repeatedly into rats suggests that similar events could occur upon the chemical modification of self aags in humans, also causing autoimmune disease. It is worth reiterating that native aags will not cause the production of pathogenic disease causing aabs, but they can be targets of pathogenic aabs either at the primary location where the aag resides (e.g. within an organ) or at secondary locations, e.g. in the glomeruli, incorporated into ICs and deposited there, causing IC glomerulonephritis.



References: [for the nephritogenic aag residing in the renal tubules]

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Figure 8. Where does the nephritogenic aag reside and where is it produced?

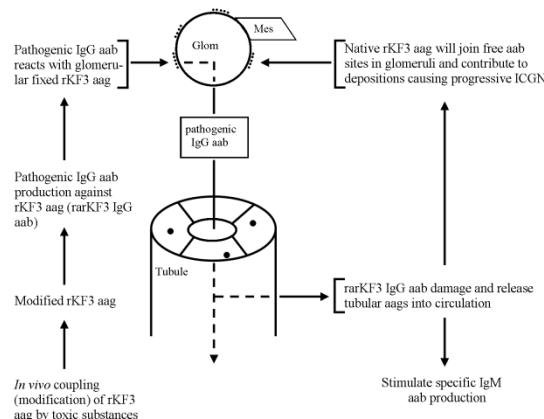
The nephritogenic aag is derived solely from the BB region of the proximal convoluted tubules. Once the BB related nephritogenic aag is released into the circulation it can be found in the serum, glomerulus, and mesangium; and during SPHN in the BC and TBM as well.

Abbreviations: aab, autoantibody; aag, autoantigen; BB, brush border; BC, Bowman's capsule; GBM, glomerular basement membrane; Glom, glomerulus; IgM, immunoglobulin M; Mes, mesangium; rarKF3, rat anti-rKF3; rKF3, rat kidney fraction 3; SPHN, slowly progressive Heymann nephritis; TBM, tubular basement membrane

Where Does the Nephritogenic aag Reside and Where Is It Produced?

The nephritogenic ag is produced only in the BB area of the renal proximal tubules [86, 128], which is virtually its sole residence under normal circumstances (Figure 8). There it fulfills an important role as an integral part of the BB in the latter's function of filtering out and retaining molecules that are no longer required in the system or that must be sequestered to keep fluid and electrolyte balances within physiological norms in the intravascular space. As noted, other researchers have asserted that the nephritogenic ag is also produced by the epithelial cells of the GBM [70] based on its observed distribution around the glomeruli in a small beaded pattern. While it is true the nephritogenic ag is also present around the glomeruli in the normal rat kidney, the Barabas research group has shown that (a) it is not present around the glomeruli of pre- and post-natal rat kidneys that are not open to the circulation [31], clearly indicating that the nephritogenic ag is not produced by the epithelial cells; (b) the nephritogenic ag is present around the glomerular capillary loops in the form of ICs made up of the nephritogenic ag and specific IgM aabs directed against it [8]; and (c) the nephritogenic

ag is present in the mesangium of normal rat kidneys in the form of ICs (nephritogenic ag X rat anti-nephritogenic ag IgM aab) and is also present in the mesangium of HN rat kidneys in the form of ICs (both nephritogenic ag X rat anti-nephritogenic ag IgM aab and nephritogenic ag X rat anti-nephritogenic ag IgG aab) [8]. These molecules are normally trapped by the mesangial cells (preventing the disturbance of glomerular filtration by such large MW complexes) and degraded into small MW components prior to being ejected back into the circulation [27].



Nature of nephritogenic aags:

1. Modified self aags: aags can be modified (*ex vivo* easily) *in vivo* by various toxic, bacterial, viral, drug etc products. Such modified aags are capable of evoking a pathogenic immune response against self. If such immune responses are prolonged (presumably because the modifying agent persists in the system) then a chronic progressive autoimmune disease can ensue such as HN or SPHN.
2. Native aags: as stated before, a native aag is unable to initiate a pathogenic aab response but can contribute to pathological events where developing pathogenic aabs are able to:
 - a) cross react with native aags (eg rKF3 aag) in the glomeruli (initial ICGN) and contribute to continuous enlargement/growth of ICs, causing chronic progressive ICGN;
 - b) reach the target ag site in the proximal convoluted tubules and damage and release aags into the circulation and urine;

Figure 9. What is a nephritogenic or pathogenic (kidney) aag?

A nephritogenic or pathogenic (kidney) aag can be defined as an aag (such as rKF3) which can under certain conditions initiate and/or contribute to an autoimmune kidney disease, e.g. by stimulating the development of pathogenic IgG aabs (which damage the tubular BB region and release increased amounts of BB associated aags), by depositing in the glomeruli in modified and native form, and by coupling with IgG aabs that join the glomerular localized aag, contributing to the enlargement of IC deposits.

Abbreviations: aab, autoantibody; aag, autoantigen; ag, antigen; BB, brush border; Glom, glomerulus; HN, Heymann nephritis; IC, immune complex; ICGN, immune complex glomerular nephritis; IgG, immunoglobulin G; IgM, immunoglobulin M; Mes, mesangium; rarKF3, rat anti-rKF3; rKF3, rat kidney fraction 3; SPHN, slowly progressive Heymann nephritis

What is a Nephritogenic Aag?

There is a lot of confusion as to what constitutes a self ag capable of initiating and maintaining autoimmune disease. Simply put, it is a self ag that is somewhat altered in its chemical composition or structure and therefore recognized by the cells of the immune system as foreign [14]. Molecules that are not derived from autologous ags but are similar in structure to self ags (molecular mimicry) can also be autoimmune disease causing ags [38, 39, 142]. Self like ags, whether modified ags or molecular mimics, are (under certain

circumstances) able to initiate and maintain pathogenic IgG aab responses against normal target ags present on the surfaces or in the inner compartments of cells because of the cross-reactivity of the pathogenic IgG abs (Figure 9). Normal self ags do not themselves initiate or maintain autoimmune disease causing immune events, i.e. production of pathogenic IgG aabs [12].

As described above and depicted in figure 9, pathogenic IgG aabs can damage the BB zone of the renal proximal convoluted tubules and cause the release of nephritogenic ags. These released normal self ags contribute to lesion development in the glomeruli by adding to layered depositions of pathogenic IgG aabs directed against the target nephritogenic ag and complement components, but will not produce pathogenic IgG aabs. However, they will produce nonpathogenic IgM aabs even during the progressive phase of the disease [6].

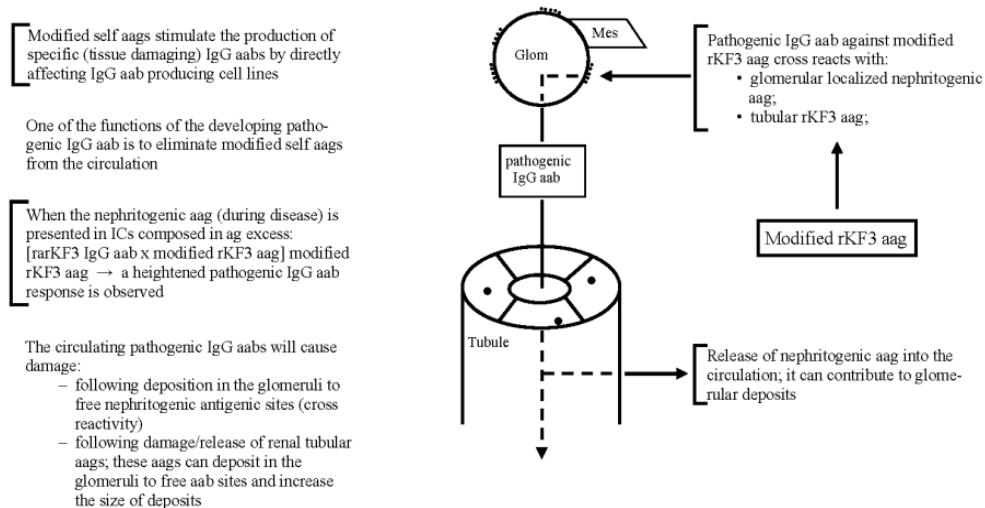


Figure 10. How can the autoimmune disease process be exacerbated?

As long as the modified aag is present in the system the chronic progressive phase of the autoimmune disease will persist by continued production of pathogenic IgG aabs.

Abbreviations: aab, autoantibody; aag, autoantigen; Glom, glomerulus; IC, immune complex; IgG, immunoglobulin G; Mes, mesangium; rarKF3, rat anti-rKF3; rKF3, rat kidney fraction 3

An Autoimmune Disease Process Can Be Exacerbated

As long as a modifying agent (i.e. chemical, drug, infectious agent, toxin, etc.) is present in the system and is able to modify the liberated aag (e.g. rKF3 aag released from the renal proximal tubules), a pathogenic aab response is initiated and maintained against the target aag wherever it is located, including in the tissue that produces the target ag, and in our case, in the glomeruli as well (Figure 10). Due to the damage of the target tissue, the aag is liberated into the circulation in unusually high amounts, and the specific IgM aabs available in the system cannot effect its rapid removal. Complement does not form in the system in amounts sufficient to expedite the removal of cell debris, and the aag is not efficiently cleared away [29], leaving it available for further modification.

A modifying agent can have a deleterious effect whether it is present in the system continuously or intermittently, as long as it can modify the aag in sufficient quantities to induce and maintain the production of pathogenic IgG aabs [14, 58]. An autoimmune disease is in a state of exacerbation when pathogenic tissue damaging IgG aabs are present in unusually high ab titres in the circulation [92]. During such a disease state, medical intervention with immunosuppressive agents would typically be required to alleviate the signs and symptoms of the condition [3, 63, 73, 95, 97].

An Autoimmune Disease Process Can be Downregulated

Experimental observation and experience in human medicine have heretofore demonstrated that once autoimmune diseases begin, they cannot be terminated by medical intervention, though their clinical signs and symptoms attenuated somewhat by the administration of immunosuppressive agents. In rare cases, however, even without medication the signs and symptoms of an autoimmune disease will subside, sometimes to a point where medication would be unnecessary.

Immunological approaches applied to downregulate autoimmune disease causing processes

Once an animal or human immune system is exposed to a modified self ag and begins to produce pathogenic IgG aabs against the target ag (primary immune response), it will respond to the same modified self ag via secondary immune response even months or years later [12, 15, 58, 92]. The removal of the original inciting agent, if its identity is known (it might be a drug, chemical, toxin, cigarette smoke, etc.), is one approach that is likely to prevent the autoimmune disease from recurring because of the lack of modification of the aag in its absence and the resulting lack of subsequent immune response. However, the removal of the inciting agent may be impossible, i.e. if it is hormonal, environmental, etc.

Another approach that has been tried to downregulate the events that cause autoimmune disease in experimental conditions is the administration of the target ag [28, 62, 84, 126] by various routes (most often orally in soluble form). Oral administration of a target ag has been effective in preventing or reducing autoimmune disease development in certain circumstances, especially when the ag has been administered prior to the induction of the disease. Unfortunately the same technique has been shown not to provide noticeable benefits in humans with established autoimmune diseases [127, 131, 132].

Heymann and colleagues carried out the administration of HN ag to neonatal rats for six weeks prior to the induction of HN [57]. Some of the animals that received the ag pre-disease induction did not develop the disease, and a few rats experienced milder forms of the condition. Heymann et al. concluded that the reason for the lack of disease or its attenuated form was that the rats had become tolerant to the injected ag.

Barabas and colleagues repeated and expanded the Heymann experiment. They injected aqueous rKF3 ag into some rats both before and after the induction of the disease, and in some rats only after the disease was already established. In both cases they observed downregulation of immunopathological processes. This downregulation was due to the production of cross reactive IgM aabs, which were able to neutralize both modified and unmodified nephritogenic ags, removing them from the circulation and terminating the

production of pathogenic IgG ab production [6]. No pathogenic IgG aabs in the system meant no kidney disease development or progression.

Barabas and colleagues also experimented with administering high titred IgM abs directed against the nephritogenic ag [6]. The effect on disease development and progression was minimal, indicating that passive immunization is not the most effective way to downregulate pathogenic immune events.

Monoclonal abs, especially Rituximab [35, 37, 55], are also used in humans to treat autoimmune diseases with limited success [25, 37, 44, 45, 64, 79, 98]. While the monoclonal ab is effective in controlling some aspects of the disease through the elimination of B cells, it is nonspecific in its action, has side effects [37], requires repeated administration, and will not terminate immunopathological processes.

The vaccination method the Barabas group has developed – the MVT – is the best immunological approach to date for preventing pathogenic IgG aab mediated autoimmune disease (prophylactic vaccination), and terminating disease when already present (therapeutic vaccination) [4, 5, 9, 10, 16, 17, 26] Figure 1. The vaccination technique (provided that pure and specific components are present in the inoculum) induces corrective immune responses, without side effects, capable of removing from the system the modified self ag that stimulates pathogenic IgG aab and the native aag that contributes to lesion development. The vaccine is essentially made up of two components, the native target ag and specific IgM ab against the native ag, combined into an IC mixture at slight ag excess [7, 13]. When the IC is injected into animals, with or without an autoimmune disease, it produces a heightened immune response, stimulating production of the same class of ab with the same specificity against the target ag as is present in the inoculum [14]. E.g. when the IC is composed of [rKF3 X rarKF3 IgM ab] rKF3, and injected into rats it produces elevated levels of rarKF3 IgM aabs. These aabs, being cross reactive, will clear the circulation of modified and native aags, thereby terminating the immunological events that cause the disease.

The use of ICs to study ab responses against various exogenous ags is not new [20, 71, 72, 91, 119, 120, 121, 122, 123, 124], nor is their use in increasing the production of abs against exogenous ags in vaccinations [54, 65, 141]. However, the application of the MVT – utilizing specific endogenous ags and specific abs against them in the form of ICs to induce a corrective immune response and reestablish tolerance to self in vaccinated recipients – is a novel approach, exploiting the immune system's natural ability to regain normal health. The immune system is capable of responding to and correcting mishaps (which cause autoimmune disease and cancer) in a very short time, provided the necessary information (presented in the form of ICs) is transmitted to its effector cells.

Conclusion

Autoimmune disease in humans are mainly incidental to genetic [33, 112] and gender predispositions (females are generally more susceptible), but are also influenced by environmental factors [93, 113, 125]. There are many agents (smoke, alcohol, toxins, infectious agents, chemicals, cold, heat, etc.) that can modify aags released into the circulation, e.g. from the intracytoplasmic space. If specific IgM aabs are not sufficiently available to assist in their efficient and quick removal and elimination, these aags can become

modified (to hapten-self ag conjugates) and the cells of the immune system will subsequently handle them as foreign molecules and produce pathogenic IgG aabs against them [106]. The developing pathogenic aabs aim primarily to react with and neutralize/eliminate the modified native aag which initiated their production (just as any pathogenic IgG ab produced against a foreign ag, e.g. bacterial or viral, would do). However, being cross reactive the pathogenic IgG aab is also able to react with the normal target ag that resides in the tissue or organ of origin, and mounts a tissue damaging attack on that tissue or organ [80]. In SPHN, for example, pathogenic IgG aabs directed against modified nephritogenic ags can damage and release nephritogenic ags from the BB region of the renal tubules [80] and contribute to layered depositions of ICs in the glomeruli composed of the nephritogenic ag, pathogenic IgG aab directed against the modified nephritogenic ag, and complement components.

The Barabas research group has demonstrated over the past few years that the term autoimmunity covers four aspects of immune response against self [4, 9, 19], two beneficial and two harmful ones (Figure 1). The two beneficial aspects of autoimmunity are dedicated to removing from the internal environment both cell debris and (cancerous or pre-cancerous) intact cells with altered surface ags. The cellular waste is degraded into small molecular weight substances by mononuclear cells and into components reusable as functional and structural proteins etc. The two harmful aspects of autoimmunity are the obverse of the beneficial ones, the disease conditions, i.e. cancer and autoimmune disease, that manifest when the beneficial functions are attenuated or go awry. To date the prevention and treatment of these conditions has been undertaken by nonspecific means, i.e. drugs, and not by inducing a specific response from the immune system that would be free of side effects.

The reason why proper immune mediated preventative and therapeutic options have not been available so far is that technical challenges have provided obstacles to our ability to present the offending endogenous ags to the cells of the immune system in such a way as to specifically compel it to mount a corrective immune response. The MVT described by Barabas and colleagues [4, 5, 16, 17] is a novel immunization method that utilizes ICs with the ability to produce the same ab response against an endogenous target ag in the vaccinated host as is present in the inoculum. In SPHN, for example, IC made up of the native nephritogenic ag (rKF3) and homologous IgM ab against it at slight ag excess ([rKF3 X rarKF3 IgM ab] rKF3) stimulated the elevated production of rarKF3 IgM aab production in the animal. These aabs, being cross reactive, removed both modified and native nephritogenic ags from the circulation. In the absence of circulating nephritogenic aags the disease process came to a halt and tolerance to self was regained [7, 13].

In preliminary experiments we have further shown that the MVT can also be applied to upregulate pathogenic IgG ab responses against an exogenous ag [20] as well as an endogenous cancer specific ag on cancer cells (unpublished observation). If this treatment modality proves generally effective in preventing and/or treating different types of cancers (with the appropriate specific IC components produced in each instance), then specific vaccinations could be instituted at the very least for people to whom currently available treatments pose an increased risk.

The aim of the present chapter is to explain how mishaps caused by or involving endogenous ags (i.e. autoimmune disease and cancer) can be terminated by appropriate presentation of endogenous ags to the cells of the immune system utilizing the MVT. The MVT can be used to stimulate a specific response in the immune system, resulting in the elimination of the specific disease causing endogenous ag from the internal environment.

Providing this treatment to patients with autoimmune disorders would undoubtedly assist them to regain normal immune functioning by directing the immune system to eliminate altered self and reestablish tolerance to normal self, leading the patient back to normal health.

List of Abbreviations

aab, autoantibody; aag, autoantigen; ab, antibody; ag, antigen; BB, brush border; GBM, glomerular basement membrane; HN, Heymann nephritis; IC, immune complex; IgG, immunoglobulin G; IgM, immunoglobulin M; MVT, modified vaccination technique; MW, molecular weight; rarKF3, rat anti-rKF3; rKF3, rat kidney fraction 3; SPHN, slowly progressive Heymann nephritis

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Chapter 3

Hematopoietic Stem Cell Transplantation for Severe Autoimmune Diseases: Time for a Reappraisal

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Summary

Two different sets of investigations are at the origin of hematopoietic stem cell transplantation (HSCT) for severe autoimmune diseases (SADs). The experimental evidence consisted in the transfer/cure of animal SADs as murine lupus by means of HSCT, allogeneic but also, almost paradoxically, autologous. The clinical one came from serendipitous reports of patients allotransplanted for coincidental diseases, and finally cured of both conditions. The encouraging results of autologous HSCT (ASCT) in experimental ADs were enthusiastically translated into human therapy by clinicians hoping to achieve great results without incurring into the rigors associated with the allogeneic procedure.

Allogeneic STC has elicited great expectations, but the burden of higher mortality and morbidity, with GVHD in the first place, that it may elicitely, must be considered, even when making recourse to reduced conditioning regimens (RIC). Paradoxical relapses notwithstanding complete donor chimerism have been reported. Further experience is clearly needed, but the early enthusiasm for an attractive one shot therapy must be tempered with a realistic evaluation, at least until new significant breakthroughs will be made.

Well over 1000 ASCT for SADs have been performed worldwide at this time, with multiple sclerosis (MS) and connective tissue diseases in the foreground. Transplant-related mortality (TRM) and morbidity have decreased to well under 5%. A dramatic disease-arresting effect is a constant benefit, but the whole course of the disease appears to be

influenced favorably. Profound changes of the autoimmune circuitry have been demonstrated, but no authentical eradication of disease (cure?) should realistically be expected. Important multicentric prospective trials are ongoing to compare ASCT to the best available non-transplant therapies, but it may be argued that in the end both approaches will be integrated for single patients, and that new agents will possibly alter present strategies.

Introduction

Stem cell therapy for severe autoimmune diseases (ADs), here discussed as hematopoietic stem cell transplantation (HSCT), both allogeneic and autologous, but also more recently as gene therapy-assisted autologous HSCT [1, 2], has become one of the hottest areas of clinical immunology. It has been developing progressively in the last decades, and has generated “excitement and promise as well as confusion and at times contradictory results in the lay and scientific literature” [3]. It is important to realize, however, that the utilization of the almighty [4] stem cells for regenerative medicine must be distinguished from the purpose of suppressing autoimmune cellular and humoral aggression (Figure 1). This does not mean that both areas aren’t tightly connected, since supplying new pancreatic beta cells to patients with type I diabetes cannot resolve the disease, if the causative autoimmune process is not cancelled [5]. Only in a few ADs both effects coincide, as in aplastic anemia [6] and its subtypes.

Two streams of research, experimental and clinical, are at the origin of the increasing utilization of HSCT, autologous and allogeneic, for SADs [7,8]. The first animal experiments on New Zealand mice showed that murine lupus could be transferred or cured by means of HSCT [ref. in 7]. These results have been elegantly confirmed recently [9]. In addition, a Graft-versus-Autoimmunity (GVA) effect was postulated [10-11], and confirmed experimentally [12]. The second stream in favor of allo-SCT came from the clinical observation of patients affected by coincidental diseases, that is patients with ADs having developed a hematologic malignancy for which they received an allo-SCT, and were ultimately cured of both diseases [13].

About at the same time it was found that autologous HSCT (ASCT) was curative in determined experimental ADs, and also efficacious in human disease. ASCT has become an effective and widely utilized procedure, and most of this commentary will address its present status worldwide. Nonhematopoietic mesenchymal stem cells (MSC) are a third cellular line derived from bone marrow (BM) and from cord blood, and approximately 80 clinical trials are here currently exploring their applications (14). The consensus is that they act mainly by means of immunomodulation and paracrine processes, and they will not be discussed further.

Allogeneic Transplantation

The idea of suppressing an autoaggressive immune system, supplying a new healthy one (even if from siblings), and finally harnessing a GVA effect is appealing. In an International Workshop held in 2005 it was stated that “the potential for a 1-time delivery of a curative therapy is outstanding” [15]. But will it really be so? Clinical trials are being pursued

worldwide, but I shall briefly discuss only published material and our own personal experience.

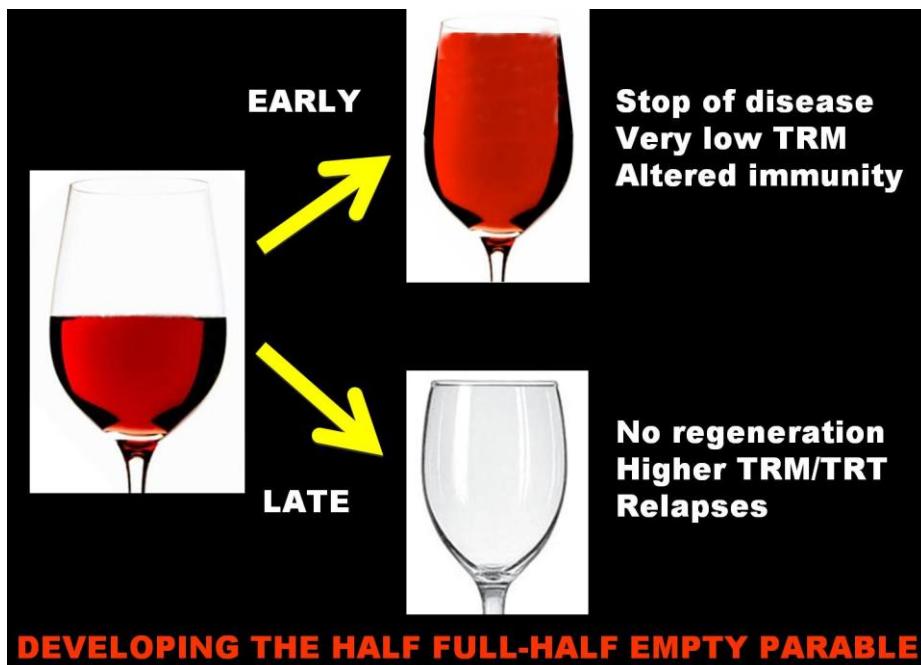


Figure 1. The different ways by which hematopoietic stem cell transplantation (HSCT) may affect autoimmune diseases.

A retrospective EBMT study [16] has collected 35 patients having received 38 allogeneic transplants for various ADs, hematological and non-hematological. The donors were identical siblings for 24 patients, matched unrelated donors (MUD) for 3, mismatched related for 2 and syngeneic for 3 patients. Treatment related mortality (TRM) was 22.1% at 2 years and 30.7 at 5, while death due to progression of disease was 3.2% at 2 years and 8.7% at 5. Of the 29 surviving patients 55% achieved complete clinical and laboratory remission, and 24% partial remission. A cross-sectional study was done in America on MS patients transplanted for coincidental haematological disease. Eleven patients were evaluated in Seattle. Early neurological events occurred, but long-term survivors appeared to be neurologically stable, and all patients beyond 5 years after transplant were negative for oligoclonal bands in the csp (17). A prospective study of Allo-HSCT for MS (ALLSTAR) has been proposed. Nonmyeloablative, reduced intensity conditioning regimens (RIC) have been recommended (18). A clinical retrospective study showed, in analogy with an established pattern in oncohematologic diseases, that there were more relapses of coincidental ADs in patients transplanted for hematologic malignancies with no GVHD, than in those who developed it [10]. However this effect could not be detected in the EBMT study [16], and a much greater clinical material would be necessary to obtain significant evidence [19, 20].

Efforts have been made, as already attempted in oncohematologic diseases, to separate GVHD from GVA. As already mentioned, a potent GVA effect was demonstrated in rat models of experimental autoimmune encephalomyelitis (EAE) [12]. Clinically there is a group of patients who have been allotransplanted for SADs, in whom donor lymphocyte

infusions (DLI) were necessary to achieve full donor chimerism, which ultimately ensured complete remissions of the SADs [lit in 19; 20]. These results are counterbalanced with others, which favor the hypothesis that mixed chimerism might be capable of inducing long-term remissions [21]. However full chimerism was present in two patients with rheumatoid arthritis (RA) with long-term post-transplant remissions [22], and in a 7-year old boy with Evans syndrome, in whom two autologous transplants had previously failed [23].

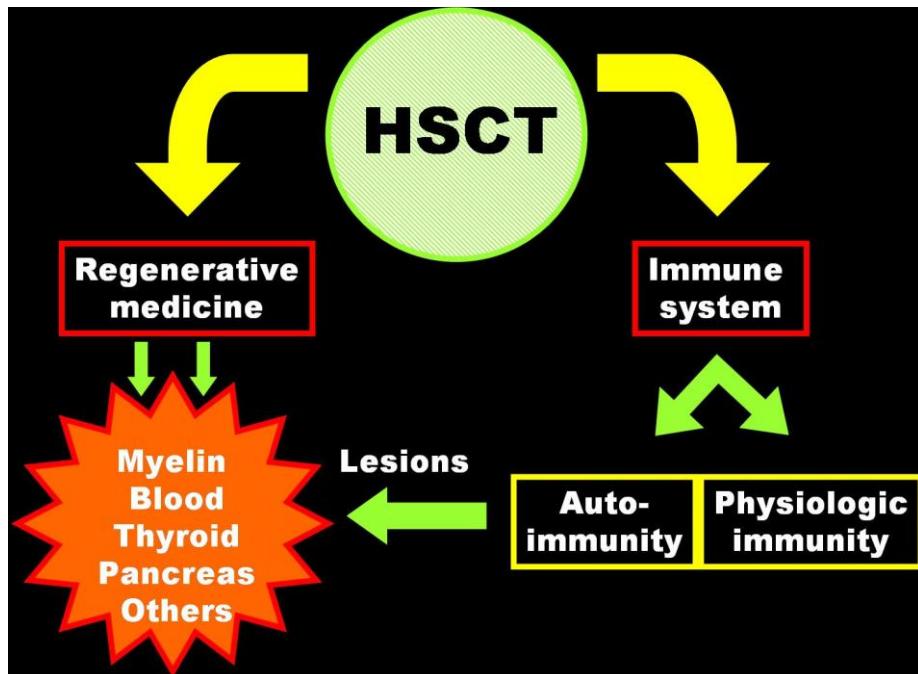


Figure 2. A more detailed view of the half-full empty parable of autologous HSCT in early and late autoimmune diseases.

Controversial evidence, however, comes from the study of non-responsive patients. There appear to be two types. An example of the first one is the report of a failure of Allo-SCT to arrest disease activity in a patient with multiple sclerosis (MS), having been successfully transplanted because of coincidental chronic myeloid leukemia [24]. Even more disquieting are the already mentioned reports of patients with SADs having received Allo-SCT, but having subsequently relapsed notwithstanding full donor chimerism. The first and widely acknowledged case was a female patient with rheumatoid arthritis (RA), who received an HLA-identical transplant because of gold-induced aplastic anemia [25], and the second another patient with RA and multiple myeloma (MM), in whom the myeloma was cured but the RA relapsed [26]. The most demonstrative case of this type of paradoxical relapse is the one of a patient with severe Evans syndrome, who was transplanted from his HLA-identical sister but needed a series of DLI in order to achieve full donor chimerism and complete hematologic remission. This patient unfortunately relapsed and died with a terminal hemolytic-uremic syndrome 5 years later [27]. The patient was male and had received the bone marrow of his HLA-identical sister. The immunoglobulins (IgG, IgM) eluted from his

100% XX expanded B cells were not the ones eluted from his Coombs-positive cells. It was hypothesized that the autoantibodies might have been secreted by long-lived host plasmacytes surviving in postulated marrow niches [28]. However, even allowing for the hypothesis that relapses in donor cells in patients transplanted for leukemia might be less uncommon than generally thought to be, it is still an extremely rare event, having been identified in 14 out of 10,489 transplants in a recent survey [29]. In contrast, 3 relapses in the much smaller group of autoimmune allografted patients inevitably cause some perplexity. Only further careful investigations will hopefully elucidate this unexpected problem. For the time being, allografting should be reserved for individual patients with coincidental diseases [13], for patients severely relapsed notwithstanding former autologous transplants [23], and potentially for controlled clinical trials [15, 30, 31].

Syngeneic transplants (a niche event) have been utilized for few patients. Three patients with RA received syngeneic transplants following high-dose immunosuppression. The first was a patient with severe seronegative RA, who enjoyed a long-term remission [32]. However a second patient with progressively erosive, rheumatoid factor positive RA, who was treated with high-dose CY and received an unmanipulated peripheral blood graft (PBSCT) from her identical twin sister, had a poor clinical response, associated with serological persistence [33]. A still unpublished case is the one of 45 year old lady with severe seropositive RA who was transplanted in Genoa from her identical twin sister on July 29, 2005. The conditioning regimen consisted in CY, 160 mg/Kg. Both rheumatoid factor and anti-cyclic citrulline peptide (CCP) titres decreased significantly (CCP from 234 to 2 IU/ml), but later there was a clinical relapse with fever, polyarthritis and elevation of ESR, requiring further treatment.

Autologous Transplantation: Progress and Questions

The rationale for an apparently paradoxical procedure such as autologous HSCT, in which the patients' immune cells, despite varying degrees of T-cell depletion *in vitro* and/or *in vivo*, are administered back to them, came from the pioneering studies by van Bekkum and his group, who were able to cure (EAE) and adjuvant arthritis (AA), considered as models of human MS and rheumatoid arthritis (RA), by means of autologous ("pseudoautologous") HSCT [34]. These results considerably strengthened the philosophy of autologous HSCT (ASCT) for human ADs, even if it was pointed out later that, in animal models, the abnormality of the antigen-induced type seems to reside in immunocompetent T/B cells but not in the HSC, and therefore ASCT may be curative, while in spontaneous ADs new, unaffected HSC were necessary to achieve a cure [35]. In contrast to the long interval having taken place between the first allogeneic transplants for animal ADs and translational clinical trials, clinical ASCT quickly followed the experimental investigations. It was proposed by Slavin for SADs in general [36] and by myself for severe SLE [37] in 1993. The first transplants were performed for a connective tissue disease [38] (1996) and for severe SLE [39] (1997). The following utilization of ASCT for SADs is growing almost exponentially. In a landmark EBMT retrospective study by Farge et al [40] 900 patients with various ADs were analyzed, and the procedure's impact has been recently discussed by Sullivan et al [41].

Excellent reviews of specific diseases have been published recently, and a monographic issue of Autoimmunity has just been devoted to this theme [42]. Here I shall discuss the most significant general questions.

1. *Autologous HSCT for ADs has been considered a relatively safe procedure from its inception, but is it becoming safer?* Autoimmune diseases represent an extremely heterogeneous spectrum of diseases, and in most of them the severe-refractory forms have a poor prognosis and a greatly impaired quality of life. However, one cannot disagree with Burt's statement that "Treatment-related mortality needs to be very low for non-malignant diseases" [3]. Treatment-Related Mortality (TRM) reached 12% in the initial EBMT Registry, decreased to 7 + 3% in 2005 [43], and finally did not exceed 5% in the most recent EBMT study [43]. In this last one evidence was also found of a clear center effect, indicating that experienced teams, well acquainted with the multi-organ involvement of SADs, produce superior results. In the case of a single disease such as SLE, a collection of 153 patients transplanted in 30 Centers showed a TRM of 11% [44]. However, of 200 patients transplanted at Northwestern University, Chicago, TRM using non-myeloablative conditioning regimens in 200 patients was 1.5% [45]. This does not mean, of course, that TRM cannot grow much higher in very severe conditions such as advanced scleroderma. Scleroderma-related organ dysfunction contributed to treatment-related deaths [46]. In conclusion, the answer to this first question is that ASCT may be considered reasonably safe when performed by experienced teams, appropriate conditioning regimens, and not for end-stage disease patients. Although the inclusion of patients in approved or investigational protocols is the best policy, in selected patients with advanced, refractory SADs, the decision to perform ASCT may ultimately rely on a combination of clinical acumen, experienced teams and good patient-doctor relationship. It is important, however, that lupus specialists be aware of the procedure rather than tend to ignore it. An excellent review of all aspects of contemporary global therapy for SLE including ABMT, has been published recently [47].

2. *Which are the most appropriate mobilization and conditioning regimens?*

The source of HSCs was initially the bone marrow (BM), but has now changed to the peripheral blood (PB) following mobilization procedures. In the EBMT study of 900 patients, the source was PB in 827 cases [40]. The most popular mobilizing regimens generally consist of combinations of cyclophosphamide (CY) and G-CSF [48]. Mobilizing regimens incorporating CY (from 2 to 4g/m²) have the additional, significant advantage of acting as an important therapeutic procedure *per se* (therapeutic mobilization). In our own experience of 9 SLE patients, the achievement of a complete remission (CR) following mobilization with CY 4g/m² enabled, in 2 of them, to dispense from performing the initially programmed ASCT [49].

A variety of conditioning regimens has been utilized, but it could be shown that high-intensity protocols were followed by a lower probability of disease progression, albeit with a higher risk of TRM [43]. The strategy of performing intense immunosuppression without affecting the whole of the hematopoietic system [50] is most generally accepted. A combination of both strategies, in which Rituximab 500 mg is given before and after the regular 200 mg/kg CY protocol ("sandwich technique"), is being currently utilized at Northwestern University, Chicago [51].

Anti-CD20 immunotherapy for the control of relapse following ASCT in patients with rheumatoid arthritis (RA) had been utilized with success [52], so that the strategy using an additional immunotherapy in this area is attractive. Caution is required because of the possibility, even if remote, of a devastating complication, progressive multifocal leukoencephalopathy (PML), due to the activation of the John Cunningham virus (JCV). A recent review has reported 52 patients having developed PML, 7 of which had received HSCT (3 allogeneic, 4 autologous) for lymphoproliferative diseases [53]. It is clear that maximal immunosuppression produces greater benefits, but, at the same time, may be associated with unforeseen iatrogenic complications.

3. *What significant changes in the immune system take place following ASCT ? Are we really curing autoimmunity ?*

No other aspect of the ASCT-based procedures has been the object of so much research, enthusiasm and controversy. Powerful immunosuppression reduces the autoimmune cells to minimal residual autoimmune disease (MRAD). While the cure of oncohematological disease requires the eradication of cancer stem cells [54, 55], different views have been proposed for ADs. The expression “not just immune suppression” has been used by Muraro [39] to indicate the immune resetting-reeducation of the faulty immune system. However this concept may be countered by the alternate one “not only immune resetting”. Summarizing an important group of investigations, two closely related mechanisms have been identified. It is clear that they interact substantially.

The first pathway has been defined as a “re-education” of the faulty immune system [56], which is obtained by restoring a diverse antigen-specific repertoire also through the reactivation of the thymic output (“thymic rebound”), that has been shown to persist, albeit in lesser measure, also in adults [57]. In a recent study of ASCT in 7 SLE patients the Berlin group has found evidence for an overwhelming regeneration of the adoptive immune system and of the B-cell lineage, which became apparently tolerant to self-antigens [58]. The second mechanism is closely related, and consists in the reconstitution of the regulatory T-cell pool following ASCT. T-reg (CD4+ CD25+) expressing the transcription factor Foxp3 are crucial in preventing autoreactivity and restraining autoimmunity throughout life. Experimental and clinical studies have demonstrated the impact of the T regulatory network for inducing post-transplant immune tolerance in SLE [59, 60]. However, in a recent study of 2 SLE patients having been infused with autologous, expanded MSC there was a marged increase in T-reg, but no association with clinical benefit [61].

Are these changes sufficient and stable enough to guarantee a rebuilding of the immune system, configured in a way that is less likely to redevelop autoimmunity? These sophisticated investigations have also met with some controversies. In a first study in autotransplanted MS patients, the T cells recognizing myelin basic protein were indeed initially depleted by immunoablation, but then rapidly expanded from the reconstituted T cell repertoire in 12 months [62]. More recently, an early recovery of CD4 T-cell receptor diversity was found after “lymphoablative” conditioning and autologous CD34 cell transplantation in systemic sclerosis (SSc) patients, suggesting that the treatment is not completely T-cell ablative, and thus not ultimately curative [63]. In a comprehensive recent study analysing original and

pooled data from autotransplanted MS patients, Mondria et al [64] confirmed not only the already known persistence of oligoclonal bands in the CSF of 88% of the reported cases, but also the persistence of the soluble lymphocyte activator CD27, thus concluding that complete eradication of activated lymphocytes from the CNS had not been established, in spite of an intensive immunosuppressive regimen including ATG, CY and total body irradiation (TBI), in two fractions of 5 Gy a day at days -2 and -1 [65]. Active demyelination and axonal damage have been found to continue after ASCT [66]. Our own clinical experience in SLE has included late (and very late) relapses, in a way that suggested a recapitulation of the natural history of lupus. So, whether pressing the reset button will turn out to be ultimately curative is still uncertain. In 5 lupus patients having developed highly malignant B lymphomas (DLCBL), intensive chemotherapy was followed by cure of the lymphomas, but not of SLE [67].

4. *What Type of Benefit Does ASCT Confer to Severe, Progressive, Relapsing-Refractory Ads?*

It is important to realize that the effects of ASCT must be divided in two phases: the early suppression of ongoing, immuno-inflammatory events, and the later resetting of the autoimmune clock, which is closely related to the length and grade of remission. The first effect is clearly due to the immunosuppressive conditioning regimens, and is proportional to the dose intensity, also independently from HSC rescue [68]. No sophisticated biodynamics occur here, besides the combination of immunosuppression and abrogation of its attending inflammation. This first effect is responsible for its dramatic disease-arresting (“nosostatic”) properties, which have been observed in the aggressive phases of disease, where ASCT may well be the most potent salvage therapy available. A clear distinction of the diverse sensitivity to ASCT according to the phases of disease has been recently made by Shevchenko et al [69], who have divided the transplant strategies for MS in “early”, “conventional” and “salvage-late” procedures. Among the many examples of this early, dramatic therapeutic effect are, besides the cancellation of systemic symptoms, the almost immediate clearance of inflammatory urinary sediments in lupus nephritis [70], the prompt improvement of nailfold capillaroscopy in SSc [71], and the early abrogation of Gadolinium-enhancing lesions in MS [72], most dramatically in its so-called malignant form [73, 74]. Intermediate changes may be considered the striking disappearance of diffuse calcinosis in a child with overlap connective disease [75], and the regression of dermal fibrosis in patients with severe scleroderma [44].

The impact of ASCT on SADs in the long run has been discussed in several contributions. In the most important study, Progression Free Survival (PFS), which may be considered as the most accurate estimated outcome of a therapeutic procedure, was 43% at 3 years [40], but further follow-up is needed. Three apparently contrasting aspects emerge: first, that in the overwhelming majority of patients no authentical immunological cure may be realistically expected; second, that dramatic remissions occur, may be life-saving, and even long term. Third, that in most relapses the subsequent utilization of conventional therapies, to which the patients were formerly refractory, is generally possible. Thus, the question, whether the glass is half-empty or half full [76], can be answered in a positive way when the disease is still in its early, inflammatory phase and no irreversible lesions have

occurred (Figure 2). Randomized prospective trials are being pursued; however the pace of medical progress is such, that the control arm of the prospective trials may often turn out to be obsolete.

Conclusion

Is there, at the time of this writing, sufficient evidence to answer the question, whether HSCT, in its various forms, is and will be the best available therapy for SADs? There has been a tendency to place the cause of autoimmunity on a faulty immune system, thus assimilating ADs to the neoplastic lymphoproliferative diseases. However most ADs result from a combination of faulty immune systems and antigen (target organ) dysfunctions. The distinction between primary and secondary ADs, the first being sustained by primary immune defaults and the latter by a predominant antigenic trigger [77], might be considered as helpful for the evaluation of SCT interventions [78]. However the interaction between immune system and target organ antigenicity is extremely tight [79].

The autologous procedure is being performed worldwide because of its combination of safety and efficacy. It is capable of arresting progressive, otherwise refractory ADs. In addition, if utilized early in appropriate patients, it favorably changes the course of disease, even ensuring varying degrees of regeneration. Whether the autoaggressive immune system is being re-educated or, more simply, resetted is still not fully clarified. With this background, I believe that the best and more cogent indications are when ASCT is performed in an early stage of disease, and not after too many lines of therapy. Anyway, independently from the results of the randomized prospective trials, my belief is that there will ultimately be an integration between the two approaches, based on the careful selection of individual patients.

A word of caution must be said concerning the potential development not only of PML, as already discussed, but also of therapy-related myelodysplasia and leukemia (t-MDS, t-AML), which must be closely watched for when utilizing alkylating drugs and others. Fortunately, there haven't been such reports in this area, and recourse to ASCT in patients with SADs should not be hindered by the fear of late malignant complications, although careful long-term surveillance is mandatory.

Great expectations have been associated with allogeneic SCT, but its position is still uncertain. Ongoing trials will hopefully offer some answers to the question, or hope, whether the total eradication of a faulty immune system will be sufficient, and whether there is solid evidence of a clinically exploitable GVA effect. However the unexpected relapses notwithstanding full donor chimerism are still a problem, and further experience is needed.

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Chapter 4

Thyroid Arterial Embolization for the Treatment of Graves' Hyperthyroidism

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1. History of Thyroid Arterial Embolization

The first successful thyroidectomy on record using endovascular interventional techniques to embolize the thyroid arteris was reported by Russian authors, EV Galkin et al, in 1994 in the journal of Vestn Rentgenol Radiol. In order to suppress thyroid pathologic activity in diffuse toxic goitre, these authors have for the first time resorted to roentgenoendovascular functional thyroiedctomy in 32 patients with stages III-IV diffuse toxic goitre. Following superselective catheterization of the left and right thyroid arteris, they embolized these arteries with embolic materials which consisted of nonlyzed synthetic, organic and inorganic substances. Followed up for over 1.5 years after endovascular thyroid arterial embolization, a stable clincial and hormonal remission and reduction of thyroid size to the first degree were observed in all the patients. However, in their report, they did not describe which thyroid arteries were occluded with those embolic materials. Have all the superior and inferior thyroid arteries been embolized? Have any arteries been spared embolization? Have they performed animal experiments of thyroid arterial embolization before they tried this embolization technique in human subjects? No records regarding the embolization details can be found in the English literature.

In the Chinese literature, the earliest thyroid arterial embolization on animals could be dated back to 1992 while the human trial of thyroid arterial embolizaiton was also performed in this year. In 1992, Jin-Ming Li et al were the first to report thyroid arterial embolization for the treatment of experimentally-induced hyperthyroidism in adult canines. They chose 44 dogs (male:female=1:1) with body weight ranging 12-15 kg. These dogs were randomly

divided into 5 groups, with 8 dogs in each of the first three groups, 9 in the fourth group, and 10 in the fifth group. The first group served as the normal control group while the other four were hyperthyroidism groups. Hyperthyroidism was induced by feeding the dogs with levothyroxine (10mg once, 3 times daily) for 30 days until the dogs had hyperthyroid symptoms like weight loss and irritability with increased serum T3 and T4. The operation was performed under general anesthesia using 3% armobarbital sodium 30mg/kg. Bilateral thyroid arteries and veins were exposed, and the arteries were embolized with embolic agents which could be observed to enter into the thyroid glands. In the first and third groups, the bilateral superior thyroid arteries were embolized while the thyroid middle vein was ligated. Only the bilateral superior thyroid arteries were embolized in the second group. In the fourth and fifth groups, the bilateral superior thyroid arteries were embolized. One inferior thyroid artery was embolized in the fourth group but ligated in the fifth group. The embolic agent was octyl-alpha-cyanoacrylate (TH glue) and every gram thyroid tissue was injected 0.5ml TH glue. In the control group, the serum T3 and T4 were decreased at different time points after embolization (7, 14, 21 and 28 days) with a significant difference ($P<0.01$) compared with before embolization. In all the other groups, the serum T3 and T4 all dropped significantly at different time points after embolization compared with before embolization ($P<0.01$). The serum rT3 level had no significant difference before and after embolization ($P>0.05$) in all the groups at all time points. The TH glue is a permanent embolic agent and can permanently occlude the blood supply and collateral circulation of the thyroid gland. Consequently, the embolized thyroid tissue will become ischemic and necrotic, leaving a small amount of thyroid tissue alive to secret reduced thyroid hormones for the maintenance of normal function. This was the first animal experiment on thyroid arterial embolization in the world and had provided sound theoretical and experimental basis for clinical application of thyroid arterial embolization to treat Graves' hyperthyroidism.

However, Jin-Ming Li et al did not perform any clinical application of thyroid arterial embolization in Graves' hyperthyroid patients until 1994 when a group of researchers headed by Xin-Guo Zhang reported both animal experiments and clinical application of thyroid arterial embolization in Graves' hyperthyroid patients. They tried thyroid arterial embolization on 12 male canines weighing 15-17 kg before clinical application in treating 11 patients with Graves' hyperthyroidism. Their clinical trial was actually between June 1992 and March 1993, but the animal experiment was even earlier. The canine experiments began by using open surgery (6 dogs) for direct catheterization of the superior thyroid artery or by using Seldinger technique to puncture the femoral artery (6 dogs) for catheter navigation to the superior thyroid artery. The 12 dogs were divided into 6 groups for test of the embolization effect of the embolic agents of lipiodol, myodil, gelfoam, algin granules and sodium morrhuate. Among the 6 groups, one was the control group treated with physiological saline. After thyroid arterial embolization, no severe side effects occurred in the animals except dysphonia in the two dogs in the sodium morrhuate group. The dysphonia lasted 1 month before disappearing gradually. Ten days after thyroid arterial embolization, the serum level of T3 and T4 significantly decreased in all the groups except for the control group treated with physiological saline (Table 1.1). Focal necrosis was observed in the groups of lipiodol, gelfoam and algin granules. In the myodil group, only inflammation filtration was observed with no necrosis. However, diffuse necrosis was present in the sodium morrhuate group in which the parathyroid glands were also apparently affected by the embolic agent leading to serum calcium decrease. No serum calcium decrease was observed in the other

groups. Lipiodol and algin granules had a good tracing effect. Myodil did not have a good embolization effect, neither did lipiodol and gelfoam. Their results suggested that the algin granules not only were safe and convenient but also had a good embolization effect.

Table 1.1. T3, T4 and serum calcium changes before and 10 days after thyroid embolization in dogs

Variables	Preembolization	postembolization	P value
T3(ng/ml)	0.91±0.07	0.66±0.05	P<0.01
T4(ng/ml)	8.52±1.04	5.04±0.83	P<0.01
Ca(mg/l)	8.46±0.95	8.51±1.10	P>0.05

In the report by Xin-Guo Zhang et al, clinical trial of thyroid arterial embolization was performed in 11 Graves's disease patients with stages III-IV diffuse toxic goitre (Table 1.2), with 3 males and 8 females (age range 26-57 years). The disease course of these patients was all over one year and all the patients had had medications before thyroid arterial embolization. All these patients had side effects of the medication, could not adhere to the medication or were recurrent after surgery or radioiodine therapy. Six patients had severe side effects resulted from medications, two patients had thyroid crisis during the medication treatment, and the other three patients had recurrence of hyperthyroidism after treatment with medications, radioiodine or surgery.

Table 1.2. Results of thyroid arterial embolization in 11 Graves' patients

	Basal metabolism (%)	T3(ng/ml)	T4 (ug/dl)	Neck circumference (cm)
Preembolization	+63.83±8.72	7.64±1.34	28.34±5.21	37.33±3.26
1 w post	+23.31±6.57	2.32±0.48	11.85±1.82	35.51±3.10
2 w post	+19.03±4.64	2.14±0.36	10.75±1.41	34.52±2.84
4 w post	+10.32±2.57	1.63±0.20	9.49±0.83	33.27±2.41
6 w post#	+10.86±2.68	1.82±0.27	10.73±1.42	34.02±2.78
4 m post#	+7.82±2.04	2.03±0.32	9.90±0.94	33.87±2.47
8 m post#	+8.06±2.10	1.77±0.22	10.07±1.25	33.50±2.45

#n=10; w, week; m, month.

After thyroid arterial embolization, no significant changes were observed in the blood phosphate or calcium. One patient experienced a fever up to 39.2°C following embolization but returned to normal within 4 days. All the other patients experienced a fever less than 38°C within three days. Front neck pain was reported in all patients with no special treatment. After embolization, the thyromegaly decreased rapidly within one week in all the patients, and the thyroid gland remained the same 4 weeks later. The vascular murmur disappeared immediately after embolization, and exophthalmos was relieved in five out of seven patients. No aggravation was observed in exophthalmos in later follow-up. Follow-up was performed for 2-12 months after embolization, and only one patient had recurrence of hyperthyroidism 1 month later. No recurrence was reported in all the other patients. This patient with recurrence had strong positive autoantibodies (TGAb and TMAb) and was finally referred to surgery for resection of the thyroid glands.

After animal experiments and clinical application of thyroid arterial embolization in Graves' hyperthyroidism patients, these researchers reached some conclusions. Thyroid arterial embolization is easy to use and the blockage of both superior thyroid arteries will be sufficient for most hyperthyroidism patients (7/10). For a few patients whose thyroid blood supply comes primarily from the inferior arteries, two superior and one inferior arteries should be embolized. The embolization principle should be first angiography and then embolization. No reverse flow of contrast agent should be allowed. Otherwise, the embolic material contained in the contrast agent could get into other branches and result in misembolization. Thyroid arterial embolization can also be used as the preparation for thyroid resection in hyperthyroidism patients who has strong positive TGAAb and TMAb. In these patients, thyroid arterial embolization can relieve the hyperthyroidism symptoms, reduce the size of the thyroid gland, and decrease surgical hemorrhage and complications.

From then on, thyroid arterial embolization for the treatment of Graves' hyperthyroidism has been performed both in the animal experiments and in clinical application in China for almost twenty years, with more than 700 hyperthyroidism patients treated.

2. Thyroid Anatomy, Hyperthyroidism and Tests of Thyroid Function

2.1. Anatomy

2.1.1. Thyroid Gland

The normal butterfly-shaped thyroid gland weighs about 20g in adults and is attached to the anterior and lateral aspects of the trachea by loose connective tissue. It is composed of right and left lateral lobes, one on each side of the trachea, which are connected by an isthmus anterior to the trachea. A small, pyramidal-shaped lobe sometimes extends upward from the isthmus. The gland upper margin lies just below the cricoid cartilage. The two thyroid lobes are found along the lower half of the lateral margins of the thyroid cartilage. The thyroid is surrounded by a thin, fibrous capsule that penetrates the gland to form pseudolobules.

The thyroid gland has an abundant blood supply and the main blood supply of the thyroid is from the superior thyroid artery, a branch of the external carotid artery, and the inferior thyroid artery, a branch of the thyrocervical trunk from the subclavian artery. After arising from the external carotid artery on each side, the superior thyroid artery descends several centimeters in the neck to reach the upper pole of each thyroid lobe, where it branches. The inferior thyroid arteries, after arising from the thyrocervical trunk of the subclavian artery, cross beneath the carotid sheath and enter the lower or midpart of the thyroid lobe. The thyroidea ima artery may also be present, varying in size from a minute vessel to one the size of the inferior thyroid and arising from either the innominate artery or the aortic arch and coursing upward anterior to the trachea to the inferior border of the thyroid. The branching of the large arteries takes place on the surface of the gland, where they form a network. After much branching, small arteries are sent deep into the gland. These penetrating vessels arborize among the follicles, finally sending a follicular artery to each follicle. This in turn breaks up into the rich capillary basket-like network surrounding the follicle.

The excellent blood flow of the thyroid, presumably related to its endocrine function, is in the range of 4-6 ml per gram per minute, or approximately 50 times as much blood per gram as in the body as a whole. A venous plexus forms under the thyroid capsule. Each lobe is drained by the superior thyroid vein at the upper pole, which flows into the internal jugular vein, and by the middle thyroid vein at the middle part of the lobe, which enters either the internal jugular vein or the innominate vein. Arising from each lower pole is the inferior thyroid vein, which drains directly into the innominate vein.

The microscopic appearance of the thyroid shows numerous follicles (acini) of spherical sacs filled with proteinaceous colloid. The wall of the acinus is made up of a single layer of cuboidal cells resting on a basement membrane that is richly supplied with capillaries. The acini are arranged in subunits of 20-40, which are demarcated by connective tissue to form lobules, each supplied by an individual artery. The height of the epithelial cells lining the follicles varies with the state of functional activity but normally is about 15μ . The size of the follicles also varies but approximates 200μ in diameter. The wall of each follicle consists of two types of cells. Those cells that reach the surface of the lumen of follicle are called follicular cells, and those that do not reach the lumen are called parafollicular cells. When the follicular cells are inactive, their shape is low cuboidal to squamous, but under the influence of TSH, they become cuboidal or low columnar and actively secretory. The follicular cells produce two hormones: thyroxine which is also called tetraiodothyronine or T4 because it contains four atoms of iodine, and triiodothyronine or T3, which contains three atoms of iodine. Thyroxine is normally secreted in greater quantity than triiodothyronine, but triiodothyronine is three to four times more potent. Moreover, in peripheral tissues, especially the liver and lungs, much of the thyroxine is converted into triiodothyronine. These two hormones have a similar function, controlling metabolism, regulating growth and development, and increasing reactivity of the nervous system. The parafollicular cells produce calcitonin or CT. This hormone decreases blood levels of calcium and phosphate by inhibiting bone breakdown and accelerating calcium absorption by bones.

Thyroid tissue may migrate frequently into the anterior mediastinum. Substernal goiter may develop in this location and often is continuous with the cervical thyroid. Rarely, posterior mediastinal thyroid tissue is found, and from it may arise large goiters of the posterior mediastinum that are usually not continuous with the cervical thyroid gland.

2.1.2. Parathyroid Glands

The parathyroid glands are small, round masses of tissue partially embedded in the posterior surface of the lateral lobes of the thyroid gland. Each parathyroid gland has a mass of about 40mg. Usually, one superior and one inferior parathyroid gland are attached to each lateral thyroid lobe. Microscopically, the parathyroid glands contain two kind of epithelial cells. The more numerous cells, called chief cells, produce parathyroid hormone or parathormone. The function of the other kind of cell called an oxyphil is not known. Parathyroid hormone is the major regulator of the levels of calcium, magnesium and phosphate ions in the blood. The blood calcium level directly controls the secretion of both calcitonin and parathyroid hormone via negative feedback loops that do not involve the pituitary gland. The parathyroid glands are abundantly supplied with blood from branches of

the superior and inferior thyroid arteries. Blood is drained by the superior, middle and inferior thyroid veins. The nerve supply of the parathyroid glands comes from the thyroid branches of cervical sympathetic ganglia and appears to be vasomotor in function.

A normal amount of calcium in the extracellular fluid is necessary to maintain the resting state of neurons. A deficiency of calcium caused by hypoparathyroidism makes neurons depolarize without the usual stimulus. As a result, nerve impulses increase and result in muscle twitches, spasms, and convulsions. This hypoparathyroidism may result from surgical removal of the parathyroids or from damage caused by parathyroid diseases, infection, hemorrhage or mechanical injury. Hyperparathyroidism causes demineralization of bone. If uncorrected, this condition may lead to osteitis fibrosa cystica, in which the areas of destroyed bone tissue are replaced by cavities which fill with fibrous tissue. The bones consequently become deformed and are highly susceptible to fracture. Usually, hyperparathyroidism is caused by a tumor in the parathyroid glands.

2.2. Dysfunction of the Thyroid Gland

2.2.1. *Hyperthyroidism*

Hyperthyroidism is caused by overproduction of thyroid hormone. Clinically, it is manifested by a state of hypermetabolism associated with cardiovascular and neuromuscular alterations. It is most commonly caused by diffuse primary hyperplasia of the thyroid glands (Graves' disease). Infrequently, hyperthyroidism may be caused by a hyperfunctioning focus within a colloid adenomatous goiter, a benign or malignant tumor possessing the capacity to elaborate thyroid hormone, and Hashimoto's thyroiditis.

The clinical manifestations of hyperthyroidism are varied, including warm and moist skin, sweating, increased sensitivity to heat, nervousness, a fine tremor of the hands particularly when outstretched, weight loss, increased appetite, fatigability, muscular weakness, tachycardia, elevation of systolic blood pressure and sometimes cardiac arrhythmias in older patients. Eye changes, particularly exophthalmos, are encountered in Graves' disease but only rarely with other forms of hyperthyroidism.

2.2.2. *Hypothyroidism*

Hypothyroidism is caused by a deficiency of thyroid hormone. It presents in a wide range of clinical syndromes, depending upon the severity of the hormonal lack and on the age when the deficiency first appears. If the hormone deficiency is present from birth and is severe, it results in cretinism. If the hormone lack appears later or is less severe, the hypothyroidism may cause little or no clinical manifestations or may induce the syndrome called myxedema. Severe hypothyroidism can be diagnosed clinically by myxedema, as well as by slowness of affect, speech, and reflexes. Circulating thyroxine and triiodothyronine values are low. The serum thyroid-stimulating hormone (TSH) level is high in all cases of hypothyroidism that are not caused by pituitary insufficiency, and it is the best test of thyroid function. In severe hypothyroidism, both the morbidity and the mortality of surgery are increased as a result of the effects of both the anesthesia and the operation. Such patients have a higher incidence of perioperative hypotension, cardiovascular problems, gastrointestinal hypomotility, prolonged anesthetic recovery, and neuropsychiatric disturbances. They metabolize drugs slowly and are very sensitive to all medications.

2.3. Tests of Thyroid Function

A variety of clinical laboratory tests are available both to measure the extent of thyroid activity and to help determine the cause of disease. Measurements of thyroid function include the basal metabolic rate, the levels of protein-bound iodine in the serum, the uptake of radioiodine, and both total (TT4 and TT3) and free (FT4 and FT3) thyroid hormone concentrations in the serum. The causes of abnormal thyroid function can be investigated by tests in which thyroid function is stimulated by TSH or suppressed by T3 and by the search for autoantibodies. The recognition that autoimmunity is a major cause of thyroid dysfunction has led to the development of tests for thyroid autoantibodies such as thyroid peroxidase antibodies (TPOAb), thyroglobulin antibodies (TGAb) and TSH receptor antibodies (TRAb). Currently, thyroid testing is performed on serum specimens using either manual or automated methods employing specific antibodies. Methodology is still evolving as performance standards are set up by the professional organizations and newer technology and instruments are developed by manufacturers.

2.3.1. Basal Metabolic Rate

It has long been recognized that hyperfunction of the thyroid gland is associated with an increase in oxygen consumption. With this increase in basal metabolism, a corresponding increase in energy expenditure occurs, with resultant heat production. This is reflected clinically by weight loss and is associated with an increased intake of calories to provide the additional energy. Since the direct measurement of heat loss from the body is difficult, it is usually assessed indirectly through measuring the oxygen consumption. Under truly basal conditions, the amount of oxygen utilized is relatively constant. The energy equivalent of a liter of oxygen is 4.83 Calories, or equal to a respiratory quotient of 0.82. Basal oxygen consumption is slightly higher in males than in females and declines rapidly from infancy to the third decade, with a slower decline thereafter. The basal metabolic rate is related to body surface area. All these factors must be considered in determination of the final value. The results are expressed as a percentage difference from predicted normal values, and the basal metabolic rate is generally ranged between -10% and +10% in healthy subjects. Low values are suggestive of hypothyroidism and high values reflect thyrotoxicosis. Emphasis should be placed upon the fact that a series of disorders can alter the basal metabolic rate, including fever, neoplastic diseases, hypertension, diabetes mellitus, heart failure and pulmonary insufficiency. Drugs can also affect the basal metabolic rate. Body heating of large amounts of ingested or intravenously administered fluid, as in diabetes insipidus, will also raise the basal metabolic rate. Although this test is no longer a part of the routine diagnostic armamentarium, it is still useful in research.

2.3.2. Thyroidal Radioiodide Uptake

This test is the most commonly used thyroid test requiring the administration of a radioisotope. The radioisotope is usually given orally in a capsule or in liquid form, and the quantity accumulated by the thyroid gland at various periods of time is measured. Correction for the amount of isotope circulating in the blood of the neck region, by subtracting counts obtained over the thigh, is of particular importance during the early periods following its administration. The percentage of thyroidal radioactive iodide uptake is calculated from the counts cumulated per constant time unit.

The percentage of thyroidal radioactive iodide uptake 24 hours after the administration of radioiodide is most useful, since in most cases the thyroid gland has reached the plateau of isotope accumulation, and because at this time the best separation between high, normal, and low uptake is obtained. Normal values for 24-hour radioiodide uptake are 5%-30% in most parts of North America and 1%-50% in many other parts of the world. The increase in dietary iodine intake following the enrichment of foods may affect the normal values. The intake of large amounts of iodide (>5 mg/day), primarily from the use of iodine-containing radiologic contrast media, antiseptics, vitamins, and drugs like amiodarone, suppresses the radioiodide uptake values to a level hardly detectable using the usual equipment and doses of the isotope. The need to inquire about individual dietary habits and sources of excessive iodide intake is obvious.

The test does not measure hormone production and release but merely the avidity of the thyroid gland for iodide and its rate of clearance relative to the kidney. Disease states resulting in excessive production and release of thyroid hormone are most often associated with increased thyroidal radioiodide uptake and those causing hormone underproduction with decreased thyroidal radioiodide uptake.

2.3.3. Thyroid Releasing Factor Stimulation Test

Administration of thyroid releasing factor will cause a release of thyrotropin (TSH) from the pituitary with an associated increase in circulating TSH levels. This is an excellent test of pituitary thyrotropin reserve and is especially valuable in defining pituitary failure as a cause of hypothyroidism.

2.3.4. Circulating Thyroxine and Triiodothyronine

Measurements of thyroxine (T4) and triiodothyronine (T3) in serum and the estimation of their free concentration have become the most useful tests for the evaluation of the thyroid hormone-dependent metabolic status. This approach comes from the development of simple, sensitive and specific methods for measuring these iodothyronines and because of lacking specific tests for direct measurement of the metabolic effect of these hormones. One advantage is the requirement of only a small blood sample and the large number of determinations that can be completed by a laboratory during a regular workday. T4 and T3 can both be measured most specifically and easily by radioimmunoassay, but in many centers the use of T3 resin binding test is still used as an indirect measurement of circulating thyroid hormone.

2.3.5. Resin Triiodothyronine Uptake (rT3)

This test measures the competitive binding for radioactive T3 between serum thyroxin-binding globulin and a resin. The radioactive T3 added to the system will be bound preferentially by the resin if the thyroid hormone binding sites on thyroxin-binding globulin are saturated with endogenous T3 and T4, and T3 uptake by the resin will be high. The resin uptake of T3 is directly proportional to the fraction of free T4 in the serum and inversely related to the binding sites of the thyroxin-binding globulin. The uptake is high in thyrotoxicosis and low in hypothyroid states. The test serves as an indirect measurement of the unbound fraction of T4 and is valuable since it is simpler to perform than other measurements of T4.

2.3.6. Thyroid Suppression Test

This test evaluates the integrity of the pituitary-thyroid axis. It is based upon the principle that the administration of thyroid hormone will not suppress the patient's thyroid function when normal homeostatic mechanisms are disrupted. After an initial radioactive iodine uptake test, triiodothyronine is administered in a dose of 100 µg daily for 7 days. The radioactive iodine uptake test is then repeated, and if the radioactive iodine uptake is less than 20% in 24 hours, it indicates a disturbance of homeostatic control, which might be present in hyperthyroidism or occur in the presence of thyroid neoplasms. Care should be taken not to administer this test to patients in whom excessive metabolic activity induced by the test would lead to complications, including patients with cardiac conditions or vascular diseases.

2.3.7. Thyrotropin Stimulation Test

This test is employed to differentiate primary thyroidal failure from thyroid hypofunction as a result of inadequate TSH stimulation. If an increase in radioactive iodine uptake of $\geq 10\%$, or a rise in thyroxine of $\geq 2 \mu\text{g}$ per 100 ml, can be demonstrated, then it is likely that the thyroid can respond to exogenous TSH stimulation. Thyrotropin is given in a dose of 5-10 units intramuscularly to assess primary thyroid insufficiency or diminished thyroid reserve. Increased amounts of TSH may be necessary in the presence of pituitary failure. Using TSH determinations at rest and after the stimulation of thyrotropin-releasing factor, coupled with a dependable index of thyroid hormone release following TSH administration, it is possible to define the site of the lesion in hypothyroidism.

2.3.8. Thyroid Autoantibodies

The humoral antibodies most commonly measured in clinical practice are directed against thyroglobulin (Tg) or thyroid cell microsomal (MC) proteins. The MC protein is principally represented by the thyroid peroxidase (TPO). More recently, immunoassays have been developed using purified and recombinant TPO. Other circulating immunoglobulins, which are less frequently used as diagnostic markers, are those directed against a colloid antigen, T4 and T3. Antibodies against nuclear components are not tissue specific.

Some techniques have been developed for the measurement of Tg and MC antibodies. These procedures include a competitive binding radioassay, complement fixation reaction, tanned red cell agglutination assay, the Coon's immunofluorescent technique, and enzyme-linked immunosorbent assay. Although the competitive binding radioassay is a sensitive test, agglutination methods combine sensitivity and simplicity and have now largely superceded other methods. Current commercial kits utilize synthetic gelatin beads rather than red cells.

A large number of names have been given to tests that measure abnormal immunoglobulins present in the serum of some patients with autoimmune thyroid disease, in particular Graves' disease. The interaction of these unfractionated immunoglobulins with thyroid follicular cells usually results in a global stimulation of thyroid gland activity and only rarely causes inhibition. It has been recommended that these assays all be called TSH receptor antibodies (TRAb).

2.3.9. Thyroid Biopsy

Frequently, biopsy of the thyroid gland is indicated in order to establish a firm pathologic diagnosis. The biopsy procedure depends on the intended type of microscopic examination. Core biopsy for histologic examination of tissue with preservation of architecture is obtained by closed needle or open surgical procedure; aspiration biopsy is performed to obtain material for cytologic examination.

Open biopsy is the superior biopsy and needle biopsy is the simpler. Needle biopsy has been criticized as a technique when a neoplastic disease is suspected. Some have found needle biopsy useful in the diagnosis of Hashimoto's disease. Biopsy can establish a diagnosis of benign and malignant tumors, chronic thyroiditis, nodular goiter, and hyperthyroidism.

In closed core biopsy which is an office procedure, a large (about 15-gauge) cutting needle of the Vim-Silverman type is most commonly used. The needle is introduced under local anesthesia through a small skin nick and firm pressure is applied over the puncture site for 5-10 minutes after withdrawal of the needle. In experienced hands, complications are rare, but may include transient damage to the laryngeal nerve, puncture of the trachea, laryngospasm, jugular vein phlebitis, and bleeding. Because of the fear of disseminating malignant cells, biopsy was restricted for many years to the differential diagnosis of diffuse benign diseases. With the improvement of cytology and biopsy techniques, open biopsy carried out under local or general anesthesia has been virtually abandoned.

Percutaneous Fine Needle Aspiration has been promoted by the development of more sophisticated staining techniques for cytologic examination, the realization that fear of tumor dissemination along the needle tract was not well founded, and especially the high diagnostic accuracy of the technique. Moreover, the procedure is exceedingly simple and safe. The patient lays supine, and the neck is hyperextended by placing a small pillow under the shoulders. Local anesthesia is usually not required. The skin is prepared with an antiseptic solution. After the lesion is fixed between two gloved fingers, it is penetrated with a fine (22-27 gauge) needle attached to a syringe. Suction is then applied while the needle is moved within the nodule. A non-suction technique using capillary action has also been developed. The small amount of aspirated material, usually contained within the needle or its hub, is applied to glass slides and spread. As biopsy of small nodules may be technically more difficult, the use of imaging tools like ultrasound and computed tomography to guide the needle is preferred. It is important that the slides be properly prepared, stained and read by a cytologist experienced in the interpretation of material from thyroid gland aspirates. The rates of false-positive and false-negative results are acceptably low, and the accuracy of this technique in distinguishing benign from malignant lesions may be as high as 95%.

3. Thyroid Arterial Embolization for the Treatment of Graves' Hyperthyroidism

3.1. Introduction

The most common cause of hyperthyroidism is Graves' disease, whose etiology and pathogenesis have not been defined clearly even though considerable progress has been made. Currently, three modalities are established in the treatment of this disease, including surgery, radioactive iodine and antithyroid medication. Medication is widely accepted as the first choice in Europe, Japan, China and New Zealand whereas radioactive iodine remains the mainstay of therapy in the United States. However, these modalities of therapy all have their own intrinsic limitations and disadvantages. Medical management consists of antithyroid medication for a duration of 12 to 18 months. The main disadvantage of medical treatment is high relapse rate between 20% and 75%. Moreover, some patients are noncompliant with or have serious side effects to antithyroid medications. The use of radioactive iodine is associated with delayed onset, a high cumulative hypothyroid incidence of more than 70% over 10 years and a theoretic mutagenic risk in young patients. Surgery as the initial modality of treatment is infrequently recommended. Although surgery offers the advantage of quick control of hyperthyroidism and in experienced hands carries extremely low morbidity, it may be complicated by recurrent laryngeal nerve injury, or permanent hypoparathyroidism after near-total thyroidectomy.

In recent years, considerable progress has been made in the endovascular technology, and with this progress, there emerged a therapeutic approach for Graves' disease through thyroid arterial embolization. This approach involves embolization of most of the thyroid tissue to greatly reduce its thyroid hormone secretion, hence restoring the patient to euthyroidism. Up to now, the clinical experience in using this endovascular technique for hyperthyroidism is still minimal.

3.2. Preembolization Preparation

In the practice of thyroid arterial embolization, patients were usually selected for thyroid arterial embolization because they were noncompliant with or had serious side effects to antithyroid drugs, and refused surgical and radioactive iodine therapy. The study protocol has to be approved by the Institutional Review Board and the Ethics Committee of the hospital. The patients should be informed of the method and potential risk and side effects of endovascular embolization before providing signed informed consent.

Before embolization, a β -blocker (propranolol, 10mg, 3 times daily) is administered to control the patients' heart rate under 100 per minute and methimazole (20~40mg, 3 times daily) or propylthiouracil (PTU, 50~100mg, 3 times daily) is also administered to control hyperthyroidism. Sedation (diazepam, 5-10 mg) is given to the patients the night before the embolization procedure.

All patients have thyroid function tests including total T4 (TT4), total T3 (TT3), free T4 (FT4), free T3 (FT3), reverse T3 (rT3), TSH, thyroglobulin antibody (TGA_b) and thyroid

microsome antibody (TMAb) 3 days before embolization and at follow-up. Ultrasonography is performed on all patients three days before embolization and at follow-up to assess the thyroid tissue, volume and blood supply. Follow-up thyroid angiography is not performed as a rule unless the patients have symptoms and signs of recurrence and need further intervention.

3.3. Blood Supply of the Thyroid Gland and the Parathyroid Gland

The major blood supply to the thyroid glands is from two pairs of arteries: bilateral superior thyroid arteries arising from the external carotid artery and bilateral inferior thyroid arteries originating from the subclavian artery. The superior thyroid artery divides into an anterior and a posterior branch, with the former branch supplying principally the anterior part and sending a branch across the upper border of isthmus to form anastomoses with the artery from the oposite side. The latter branch descends along the posterior border of the gland, supplying the medial and lateral parts of the thyroid gland in addition to anastomosing with the inferior artery. Not infrequently, a lateral branch supplies blood to the lateral surface of the thyroid. The inferior thyroid artery divides into a medial and a lateral branches to anastomose freely with branches from the superior thyroid artery as well as those from the trachea and esopagus. It is common to find that blood flows between each lobe of the thyroid via branches across the isthmus. The high vascularization nature of the thyroid glands not only facilitates endovascular embolization of the gland tissue, but also dictates the use of extra measures to ensure safety of this procedure by performing selective angiography before and after embolization for confirmation of catheter position and success of the procedure.

The parathyroid glands usually derive most of their blood supply from the inferior thyroid artery, although branches from the superior thyroid artery supply ar least 20% of upper glands. This is the reason why in thyroid surgery ligation of the main trunk of the inferior thyroid artery is not a standard procedure and also is the reason why in thyroid arterial embolization both inferior thyroid arteries will not be embolized at the same time. Vascularization of other important adjacent structures (the oesophagus, trachea and recurrent laryngeal nerves) does not depend on thyroid arteries in an important manner, so their blood supply is not likely to be affected by thyroid arterial embolization.

3.4. Embolization Protocol

Thyroid arterial embolization should be performed by experienced interventional radiologists. Percutaneous access is gained through right or left femoral artery with the use of Seldinger technique. A typical procedure requires about 1-2 hours. In brief, the patient is placed in a supine position, and the inguinal pulsation point of either the left or right femoral artery is chosen as the the puncture site. A small skin incision is made under local anesthesia (1% procaine, 2-3 ml). Along with a cannula, the puncture needle is inserted into the femoral artery through the incision. Then, the needle is removed while the cannula remains in the arterial lumen as an entry portal for an angiographic catheter. The catheter is advanced from the femoral vessel via the abdominal aorta and sequentially to both superior and inferior

thyroid arteries. Navigation of the catheter is visualized by use of a digital subtraction x-ray system. Before embolization, contrast media is injected into the arteries for visualization of the arteries and regions of the thyroid glands they supply blood to. This angiography helps doctors determine how much blood supply each artery provides and to which part of the thyroid gland. With this information in hand, the doctors can decide which and how many arteries are to be embolized. Usually, the embolization of three arteries including two superior and one inferior thyroid arteries will be sufficient to block the majority of blood supply to the gland.

After superselective catheterization of the thyroid bilateral superior and inferior arteries, dexamethasone (DXM) of 5mg is slowly injected through the catheter and then, the mixed product of polyvinyl alcohol (PVA, 150~500 μ m, Boston Scientific), papaverine and contrast agent is slowly injected through the catheter under fluoroscope into the arteries of the thyroid until blood flow is static. During embolization procedure, Pingyangmycin (12mg, bleomycin A5) is administered through the catheter to each patient, papaverine is repeatedly employed through the catheter to prevent and relieve spasm of the thyroid arteries for complete embolization while at the same time, special attention is paid to preventing regurgitation of embolization agent for avoiding mis-embolization of other arteries.

PVA granules ranging 150~500 μ m indiameter are predicted to be sufficient for blocking the lumen of the thyroid arteries, because histologic analysis of surgically-removed hyroid tissue shows that the diameter of the small arteries vary from 0.04-0.11 mm. The injection of these particles should be slowly performed for the prevention of reflux. The procedure begins with infusion of 150-200 μ m particles because this size particle exceeds the diameter of the smallest arteries with no chance of passing through into the systemic circulation. Then, slightly larger particles, of 200-300 mm, are used to completely block the vessel, a standard technique in embolization. Some researchers would prefer a third step, that is to embolize the trunk of the superior or inferior thyroid arteries by using a stainless wire coil of appropriate size based on the diameter of the arterial lumen. Selective angiography is performed postembolization to ensure that the targeted arteries are completely occluded with disappearance of the parenchymal phase.

3.5. Postembolization Management

After the embolization procedure, dexamethasone (DXM) is administered (10mg, 3 times daily for 3-7 days following embolization) to inhibit the body's immune system and to relieve symptoms of chemical inflammation reaction of the thyroid gland caused by arterial embolization. Propranolol may be continued for tachycardia, but gradually decreased. All patients receive an antibiotic, IV ampicillin for 3-7 days postembolization for preventing possible bacteria infection. Antithyroid medications are used within 2 weeks as before embolization. If thyroid function returns to normal or lower than the normal range 2 weeks later, antithyroid medications will be reduced by half. The drugs will be withdrawn completely if thyroid function remains normal or lower than the normal value range at 1, 2, 6 and 12 months, and continued at small dosage (1/3 to 1/2 of the dosage used before embolization) if thyroid function is slightly higher with FT3>10PMol/l or TT3>5nmol/l.

3.6. Efficacy Assessment

The clinical effect of thyroid embolization is categorized as follows: 1) euthyroid, if the patient's hyperthyroid symptoms and signs disappear, thyroid function is restored to normal and antithyroid drugs have been withdrawn for 1 year without recurrence; 2) improved, if the patient's symptoms and signs have changed greatly for the better (including pulse slowing down and smaller thyroid size and thyroid function has decreased without being restored to euthyroidism) but with maintenance dose of antithyroid drug equal to or less than half that before embolization;

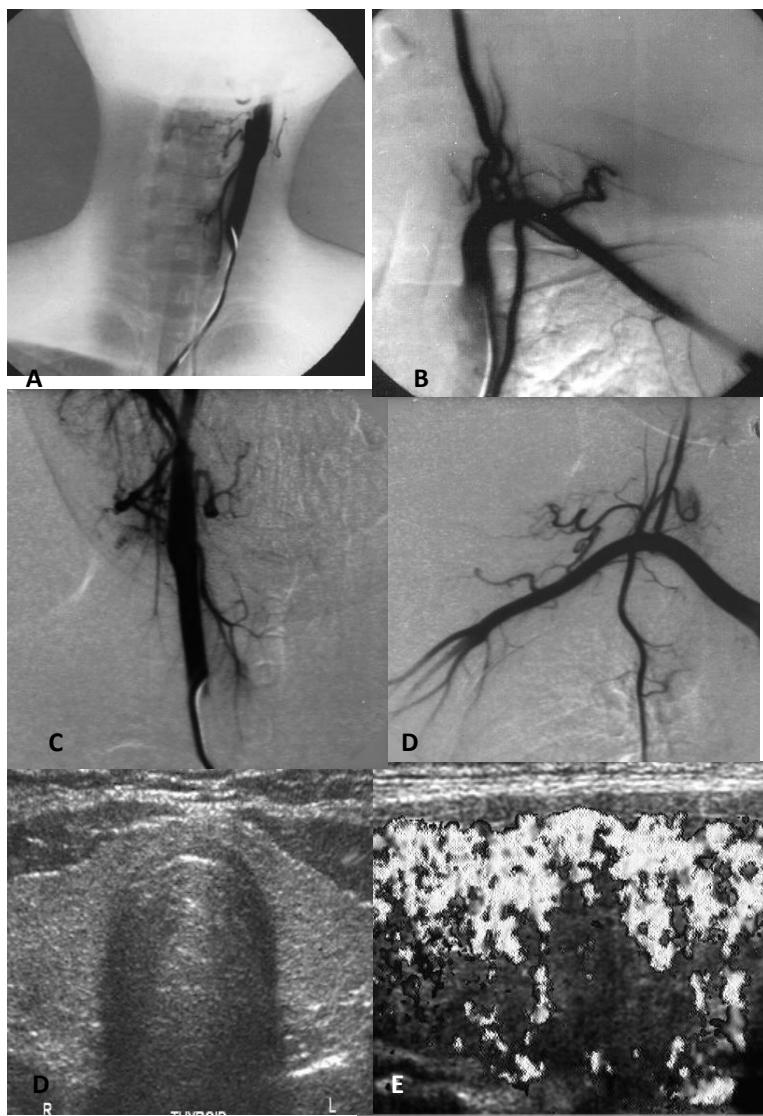


Figure 3.6.1. Normal and abnormal thyroid glands. A, B, C&D. Normal angiography of the thyroid glands through left carotid (A), left subclaval (B), right carotid (C) and right subclavian arteries demonstrates no abnormal stain of the thyroid gland and no winding of the thyroid artery. D. Normal thyroid gland in sonography. E. Hyperthyroidism caused by Graves' disease in sonography.

3) ineffective, if there are no great changes in hyperthyroidism compared with pre-embolization and the required antithyroid drug dose exceeds half that before embolization; 4) recurrent, if a cured or improved patient has hyperthyroid symptoms once more, or the symptoms become worse necessitating an antithyroid drug dosage exceeding half that required before embolization.

After the embolization procedure, vascular murmurs in the anterior neck region disappears immediately, and the enlarged thyroid gland decreases apparently by 1/3 to 1/2 of the original volume before embolization (Fig.3.6.1-3). No patients are classified as ineffective. In the euthyroid patients, the hyperthyroidism symptoms disappears, thyroid function is restored to normal, and antithyroid drugs have been withdrawn for over 12 months. Recurrent patients have thyroid vascular murmurs and hyperthyroid ultrasonogram once more. When examined with digital subtraction angiography, recurrent patients' thyroids regain blood supply through collateral circulation with obvious thyroid dyeing of contrast agent.

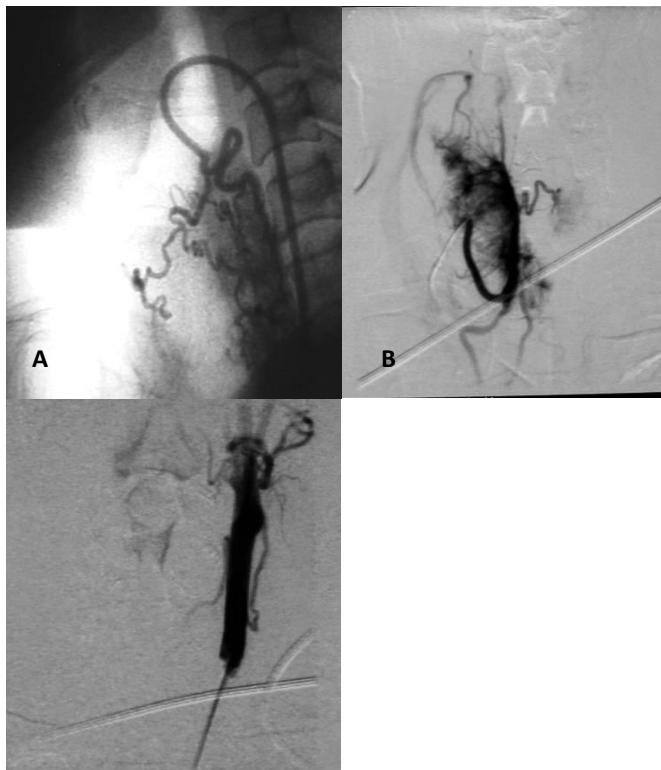


Figure 3.6.2. Super-selective thyroid angiography of a 30-year-old woman who presented with hyperthyroid Graves' disease. A. Super-selective catheterization of left superior thyroid artery (lateral view) revealed extreme winding and thickening of the gland branches of the left superior artery. B. Super-selective catheterization of right inferior thyroid artery (anteroposterior view) demonstrated obvious stain of the thyroid gland, and winding and thickening of right superior, middle and inferior thyroid veins. C. Postembolization angiography of the thyroid gland through left carotid artery showed occlusion of the left superior thyroid artery and no abnormal stain of the thyroid gland.

Followed up for two years, the mid-term effect rate is 96.4% (euthyroid plus improved) with the euthyroid rate of 78.6%, improved 17.8% and recurrent 3.6%. The euthyroid patients have no hyperthyroid symptoms, the thyroid function is restored to normal and the antithyroid

drugs have been withdrawn for 12 months or more. A few patients who have embolization of only bilateral superior thyroid arteries experience recurrence of hyperthyroidism several months later and are re-embolized. After the second embolization where three thyroid arteries (bilateral and one inferior thyroid arteries) are blocked, the hyperthyroid symptoms and signs disappear completely, the thyroid function become normal, and they are considered to be cured. 17.8% patients are considered improved with symptoms, signs and thyroid function all changed for the better, but still need 25mg to 50mg PTU a day for maintenance.

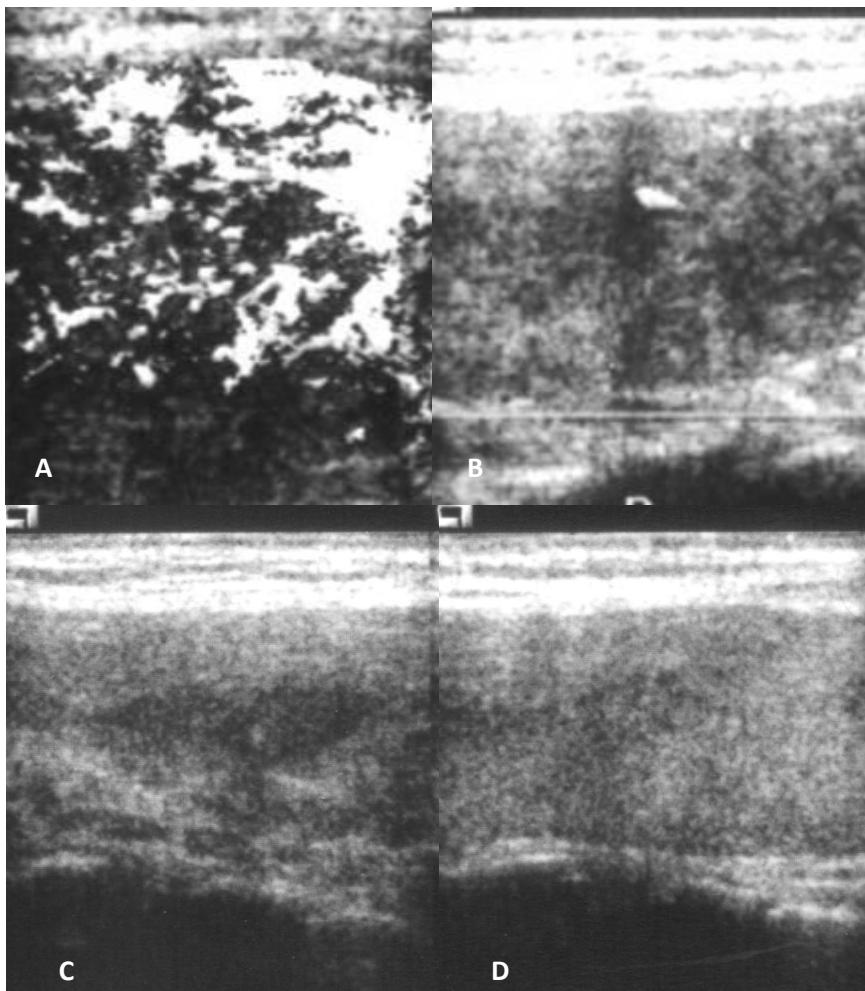


Figure 3.6.3. A 31-year-old female with ultrasound examination before and after thyroid arterial embolization. A. Pre-embolization Doppler scan shows abundant blood supply within the thyroid gland consistent with hyperthyroidism. B. Seven days after embolization, ultrasound scan demonstrates that the blood supply within the thyroid has greatly decreased, with some low-level echo areas implying tissue necrosis. C. Six months later, grayscale ultrasound reveals greatly reduced size and blood supply of the gland. D. Ultrasound at 3 years demonstrates decreased thyroid volume with some echo areas, indicating tissue fibrosis of the gland.

3.7. Undesirable or Side Effects

Adverse effects mainly include anterior neck pain (sore throat), toothache, hoarse voice and fever caused by occlusion of relevant arterial branches (Table 3.7). These effects usually relieve within 2-4 days with or without medications. No adverse effects have been reported at long-term follow-up after thyroid arterial embolization. But temporary hypothyroidism and hypoparathyroidism have been reported shortly after embolization. In the study by Zhao et al, one patient experienced a temporary severe episode of hypocalcemia with tetanus (total serum calcium, 1.4-1.9 mM) on day 2 after procedure. Besides embolization of unilateral superior artery, this patient was also embolised two inferior arteries which mainly supply blood to the parathyroid glands. Serum calcium returns to normal within one week after oral supplementation with a calcium preparation (calcium carbonate, 500mg, 3 times daily). At 12-month follow-up, this patient has no hypoparathyroidism or other adverse effects left. Temporary hypothyroidism was also reported by Zhao et al in one patient at 3-month follow-up, but the hypothyroidism was restored to euthyroidism after complete withdrawal of antithyroid drugs at 12-month follow-up. Another patient with III^o goiter had a fever up to 39.8 degree which lasted for 3 weeks. After examination of chest x-ray, ultrasonography, blood culture, laboratory tests of blood and urine, no infection was confirmed and the patient was considered to have comparatively severe embolization syndrome.

Table 3.7. Untoward effects of thyroid arterial embolization

Effects	Incidence (%)	Severity	Duration
Fever	22(73.33)	Mild-moderate	2-4d
Neck pain	30(100.00)	Moderate	2-4d
Hoarse voice	10 (33.33)	Mild	2-4d
Increased pulse	6 (20.00)	Mild	2-4d
Toothache	5 (16.67)	Mild	2-4d
Periodic paralysis	2 (6.67)	Mild	2-4d
Temporary hypocalcemia	1 (3.33)	Severe	7d
Temporary hypothyroidism	1 (3.33)	Mild	3m

d=day, w=week, m=month

The bilateral superior arteries of the thyroid originate from near the bifurcation of the common carotid artery and are closely related to the internal carotid artery. In addition, the thyroid arteries have a lot of “dangerous” anastomoses with those of spinal medulla, trachea and esophagus, and the external carotid arteries also have some anastomoses with intracranial arteries and ophthalmic arteries (Fig.3.7). Therefore, special attention should be paid in thyroid embolization to avoiding both mis-embolization of dangerous and important anastomoses and regurgitation of embolic agents which may cause ischemia and even infarction of the brain, spinal medulla, trachea, esophagus and eyes, possibly leading to severe complications. Familiarity with the applicant anatomy of the thyroid arteries and skilled endovascular techniques are crucial to avoiding and decreasing undesirable effects and complications.

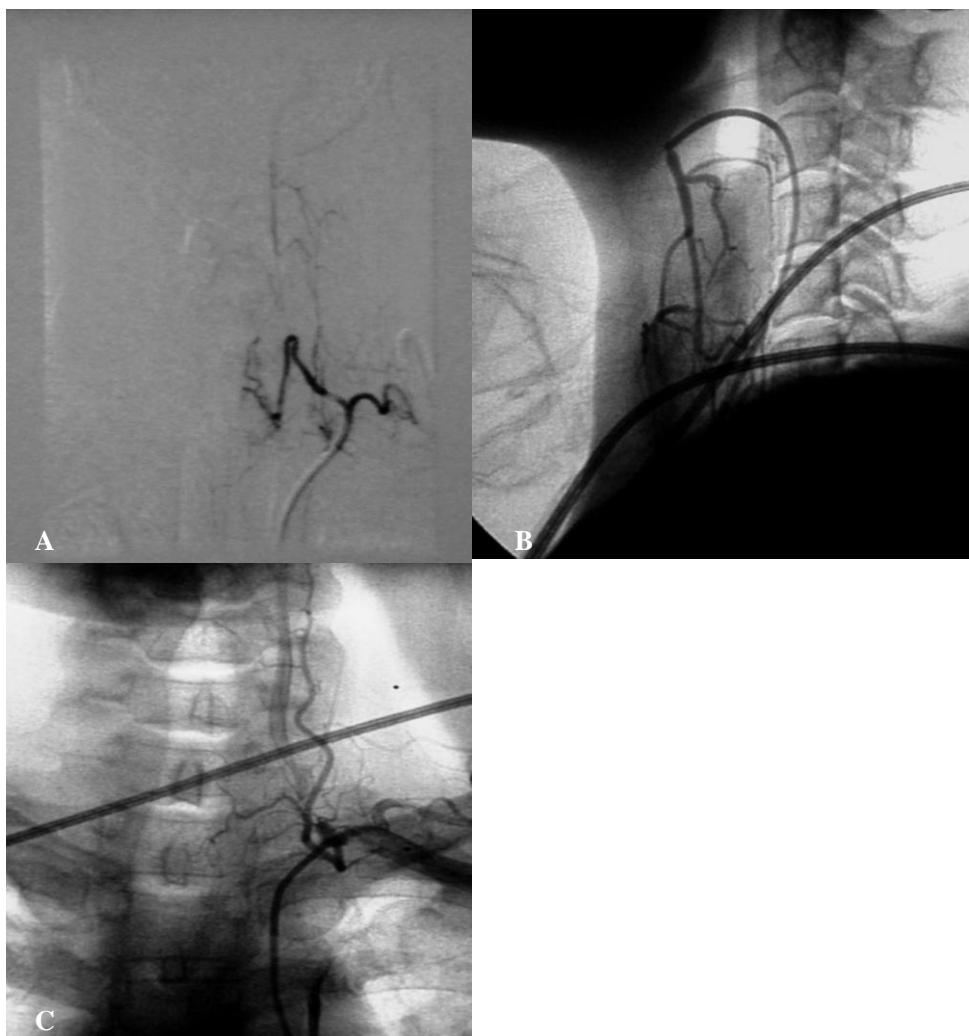


Fig.3.7. A. Angiography of left inferior thyroid artery demonstrates anastomoses of the ascending cervical artery with the ipsilateral vertebral artery at two sites (arrows). B. Angiography of left superior thyroid artery reveals one arterial branch supplying the esophagus and another branch supplying the sternomastoid muscle. C. Angiography of left subclavian artery shows the left thick ascending cervical artery has several branches extending to the spinal cord and the left inferior thyroid artery is smaller.

In the English literature, Feng Wen et al reported a case of branch retinal artery occlusion after thyroid artery interventional embolization for the treatment of Graves' disease hyperthyroidism. A 33-year-old man with hyperthyroidism complained of visual loss and scotoma in the left eye after thyroid artery embolization. Full ophthalmologic examination revealed that visual acuity was 20/25, with inferior and superior scotomas present in the left eye. Fluorescein angiography of the left eye demonstrated delayed filling of a superotemporal branch retinal artery and nonfilling of an inferotemporal branch retinal artery. This case indicates that a small risk of retinal artery occlusion after thyroid artery interventional embolization should be considered.

3.8. Case Report

A 35-year-old female patient was admitted to the hospital on 11 November, 2004 because of progressive weakness, dyspnea, diarrhea, weight loss, increased appetite and anxiety for 2 months. Since 2000, the patient had suffered from weight loss, increased appetite, restlessness, palpitations, heat intolerance, increased perspiration and fine tremor of the hands with no special treatment. In August 2002, she had sudden onset of dizziness, headache, nausea and vomiting. The vomiting was like ejection without coffee-like substances. Then, hyperpyrexia and coma ensued, and the patient was emergently admitted to our hospital. The patient was diagnosed as "hyperthyroidism and thyrotoxic crisis" which was managed properly including the use of propylthiouracil (PTU). The patient was discharged from hospital after the symptoms were improved. However, she discontinued the use of PTU later on without instruction from a doctor. Then, the above symptoms re-appeared on 3 December 2002 with concurrent cardiac arrest and the patient was referred to another hospital. After tracheotomy, cardiopulmonary resuscitation and other medical measures in the hospital, the patient was discharged once again on improvement of the symptoms. The patient took PTU regularly and had regular follow-up examination of thyroid function thereafter. In October 2004, the patient had shortness of breath with concurrent loss of consciousness and aconuresis. The patient was emergently referred to our hospital. Thyrotoxic crisis was diagnosed and 30 drops of sodium iodine, 500 mg of hydrocortisone acetate and 4 mg of propranolol were immediately infused. The methimazole therapy was administered at a dosage of 30 mg/6h and other measures for cardiac failure were also started. On admission, the temperature was 40 °C, pulse rate 170 beat/min, and BP was 150/80mmHg. She was pale and comatose, and her skin was flush and moist. Bilateral exophthalmos and a diffusely enlarged thyroid gland with an audible bruit were noted. The thyroid gland was III°. Thyroid function tests revealed that the patient had aggravated hyperthyroid state as shown in the table (Table 3.8): TT3 7.1nmol/L (normal 1.08-3.1), TT4 251nmol/L (normal 77.2-154.4), FT3 20.3pmol/L (normal 2.50-9.82), FT4 43pmol/L (normal 10.0-25.0) and TSH <0.01μIU/ml (normal 0.25-4). Chest film and CT scan of her neck showed tracheal stenosis caused by thyromegaly (Fig.4). The CT scan demonstrated the right thyroid lobe being 5mm×13 layers with the largest layer area being 5.9 cm×3.8cm and the left thyroid lobe occupying 5mm×8 layers with the largest layer area being 4.8cm×1.8cm. The narrowest place of the trachea was only 0.4 cm.

The patient responded well to the medical management. Since the thyrotoxic crisis occurred during the process of medication, her hyperthyroidism might be refractory to medication and other therapies should be applied. Therefore, other medical measures for the treatment of hyperthyroidism caused by Graves's disease were introduced to the patient including radioactive iodine and surgical ablation of the thyroid gland when clinical conditions of the patient got stable. At that time, a clinical trial of thyroid arterial embolization was performed in our hospital, and together with other therapies, the patient was informed of the benefits and risks of these measures of management. After careful consideration, the patient chose to undergo thyroid arterial embolization as the therapy for her hyperthyroidism.

Table 3.8. Thyroid function before and after embolization

	TT3(nmol/L)	TT4(nmol/L)	FT3(pmol/L)	FT4(pmol/L)	TSH(uIU/ml)
NR	1.08-3.1	77.2-154.4	2.50-9.82	10.0-25.0	0.25-4
TC	7.1	251	20.3	43	<0.01
PrE	2.29	90.52	3.19	20.22	<0.01
3d PoE	6.7	245	21.3	39	<0.01
2w PoE	4.5	209	15.7	30.6	0.08
1m PoE	0.56	36.07	2.13	17.48	2.34
2m PoE	2.1	112	7	16.8	0.31
1y PoE	2.0	106	6.8	16.2	0.5
3y PoE	1.06	66.31	3.83	15.87	3.94

TT4: total T4; TT3: total T3; FT4: free T4; FT3: free T3; TSH: thyroid-stimulating hormone; NR: normal range; TC: thyrotoxic crisis; PrE: pre-embolization; PoE: post-embolization; d: day; w: week; m: month; y: year.

After the treatment plan had been approved by the Institutional Review Board and the Ethics Committee of the hospital, thyroid arterial embolization was performed following control of hyperthyroidism. Before arterial embolization of the thyroid gland, a tracheal stent was placed within the trachea to expand the stenosis caused by enlarged thyroid compression (Fig.3.8.1). The stent was 5.0 cm in length and 2.0 cm in width, with the upper end of the stent about 1.0 cm above the upper edge of the stricture and the lower end 3.5 cm above the tracheal carina. One week later, thyroid arterial embolization was performed under local anesthesia. The angiography suite used was German SIEMENS BICOR PLUS/T.O.P model (1250mA). An experienced interventional radiologist performed thyroid arterial embolization. Following percutaneous approach through the right femoral artery using the Seldinger technique, selective angiography and embolization of the thyroid gland was performed. The thyroid major blood supply comes from two pairs of vessels: the left and right superior thyroid arteries that arise from the external carotid arteries, and the left and right inferior thyroid arteries originating from the subclavian arteries. After selective thyroid angiography, a 3F SP microcatheter (Boston Scientific, Fremont, CA, USA) was employed to catheterize bilateral superior and left inferior thyroid arteries (Fig.3.8.2). On catheterization of each of these arteries, 5 mg dexamethasone (DXM) was injected and then, the mixed product of polyvinyl alcohol, papaverine and a nonionic contrast agent (Omnipaque 300, Amershan

Health, Shanghai, China) was slowly injected through the catheter under fluoroscopic control into the thyroid arteries until blood flow ceased. During embolization, 12 mg pingyangmycin (Tianjian, China) was administered through the catheter, and papaverine was repeatedly injected to prevent and relieve spasm of the thyroid arteries for complete embolization. Special attention was paid during the procedure to preventing regurgitation of the embolic agent to avoid mis-embolization of other tissues.

Because the embolized thyroid tissue may become necrotic and release more hormones into the blood, thereby causing severe immune reactions, DXM was administered (10mg, 3 times daily for 3-7 days) following embolization to inhibit the body's immune system and to relieve symptoms of chemical inflammation of the thyroid gland caused by embolization. The patient received IV ampicillin for 3-7 days post-embolization to avoid bacterial infection. Antithyroid drugs were continued for 2 weeks after embolization in unchanged dosage and gradually reduced or withdrawn based on the thyroid function test.

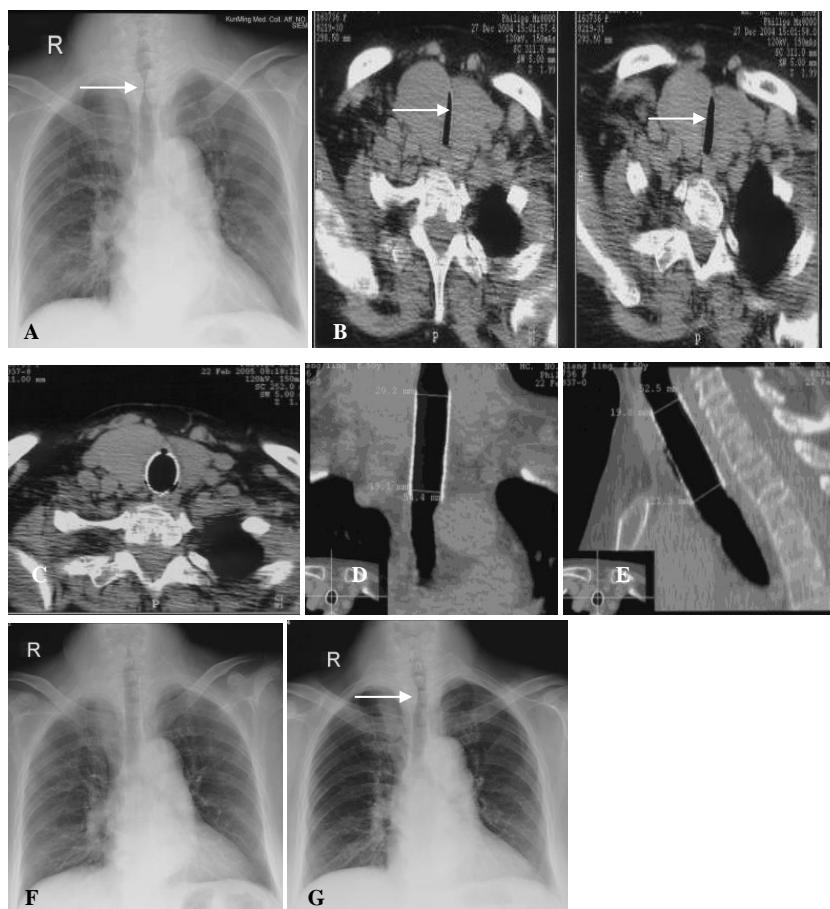


Figure 3.8.1. Chest film (A) and CT neck scan (B) before a stent was put within the trachea. The thyroid gland was greatly enlarged and compressed the trachea with significant stenosis. After a stent was deployed within the stenotic trachea (C-F), CT scan demonstrated the stenosis had disappeared and the tracheal diameter had returned to normal. The stent had been withdrawn after thyroid arterial embolization (G), and the right thyroid lob was still enlarged to compress the trachea (arrow).

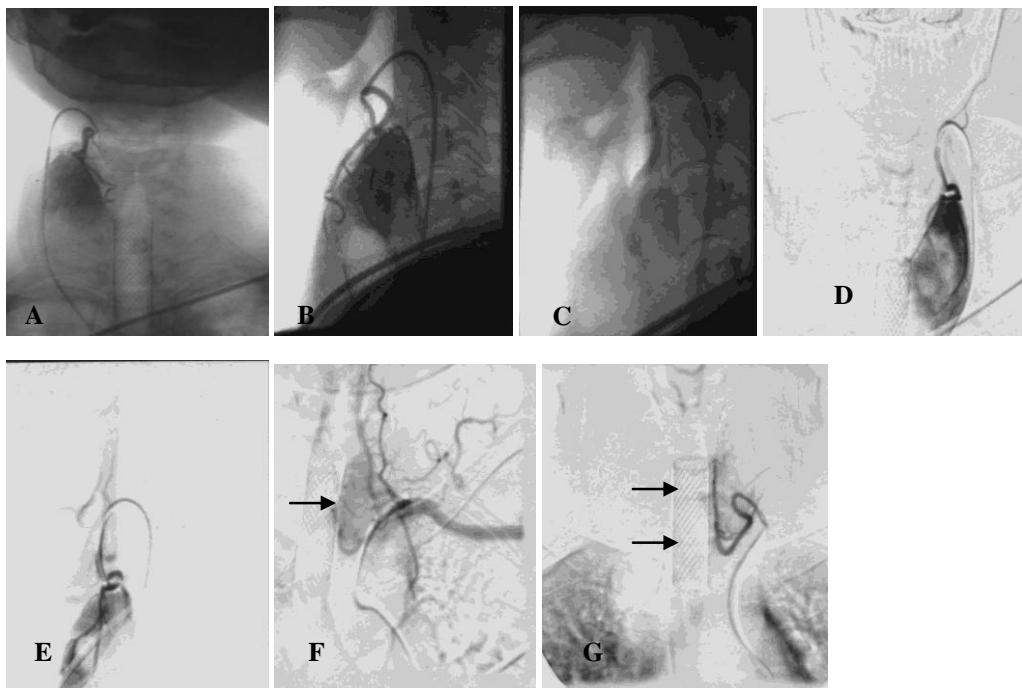


Figure 3.8.2. Thyroid arterial embolization. A-C. Right superior thyroid arterial embolization. Right superior thyroid artery angiography demonstrates significant staining of the right thyroid lob (A&B). After embolization with polyvinyl alcohol, the gland abnormal staining has disappeared (C). D&E. Left superior thyroid arterial embolization. Angiography of left superior thyroid artery shows abnormal staining of left thyroid lobe (D). Following arterial embolization, the abnormal staining has significantly reduced in size and concentration (E). F&G. Left inferior thyroid artery embolization. Angiography of left inferior thyroid artery reveals abnormal staining of the thyroid (F, arrow). After arterial embolization, the staining has greatly decreased (G). Double arrows show the tracheal stent.

4. Embolization Techniques

4.1. Mechanism of Thyroid Arterial Embolization

The purpose of thyroid arterial embolization for the treatment of hyperthyroid Graves' disease is to block most of the blood supply to the thyroid gland, hence resulting in necrosis and later, fibrosis of most of the thyroid tissue. The thyroid gland has two bilateral superior and two bilateral inferior arteries. More than 70% of blood supply to the thyroid gland is supplied by the bilateral superior arteries. If two bilateral superior arteries and /or one inferior artery were embolized, most of the thyroid tissue would lose blood supply and become necrotic and the secretion of thyroid hormone would greatly decrease, thus leading to the same effect as subtotal surgical thyroidectomy. This is thought to be the mechanism of arterial embolization to treat hyperthyroid Graves' disease.

4.2. Scope of Embolization

In the earlier stages of thyroid arterial embolization, most authors believe that embolization of two major supplying arteries (usually bilateral superior thyroid arteries) of the thyroid glands will be sufficient and that only if the thyroid gland is very large (larger than four times normal), additional embolization of one unilateral artery will be considered.

Although bilateral superior arteries supply more than 70% of the thyroid gland, embolizing these two major thyroid arteries does not actually mean that more than 70% thyroid tissue will become necrotic and later, fibrotic. Thyroid arterial embolization is quite different from surgical thyroidectomy where the tissue is excised and eliminated from the body and only a small amount of thyroid tissue is left for maintenance of its function. With thyroid arterial embolization, the embolized thyroid tissue is not eliminated from the body and may have complicated relations with surrounding tissues and organs as far as blood supply is concerned. If the embolic agents do not reach and completely occlude the peripheral branches of the thyroid arteries because of improper sizes of embolic agents, the embolized tissue can establish collateral circulation from surrounding tissues and re-gain its blood supply. If so, over 30% thyroid tissue can be viable, leading to possible recurrence of hyperthyroidism. Furthermore, as is typical with hyperthyroid Graves' disease, the four arteries of the thyroid gland enlarge and connect with one another forming an abundant vessel network. Once two major arteries and their branches are occluded, the other two minor arteries can enlarge to supply blood to the thyroid tissue, with a possible result of over 30% thyroid tissue to be alive.

In the initial period of the study by Wei Zhao et al, six patients had only the two bilateral superior arteries embolized, and two of these patients had recurrence within 6 months after the first embolization procedure. These two patients initially had only grad I goiter. Angiograms of the right thyroid superior artery showed formation of collateral circulation which was responsible for the recurrence (Fig.4.2). After a second procedure where 3 arteries were embolized (two superior and one inferior arteries), the two patients were restored to euthyroidism and were considered to be cured at 12-month follow-up. The embolization of only two superior thyroid arteries may be insufficient to reduce secretion of the thyroid hormone to normal, with a possible result of hyperthyroid recurrence. Therefore, after experiencing the first two recurrent patients where only the two superior arteries were embolized, the authors tried to occlude three arteries for each patient, primarily two superior and one inferior, in one procedure in order to achieve the desired result.

Some clinical researchers in China have studied the blood supply to the thyroid gland. They performed thyroid angiography on 55 patients with Graves' disease hyperthyroidism and measured the diameter of every thyroid artery and the ratio of blood supply every thyroid artery provide to the thyroid gland. If the blood supply to the thyroid gland provided by one of the superior or inferior thyroid arteries on one side is greater than 60%, the artery is defined as the major supplier on this side. If the superior or the inferior arteries provide blood supply in the range of 40-60% to one side, these two arteries are not superior to each other in blood supply on this side. In comparison of the diameter, no significant difference is detected between the right and left superior thyroid arteries ($P>0.05$). However, there is a significant difference in the diameter between the right and left inferior thyroid arteries, with the mean diameter of the right inferior artery greater than that of the left ($P<0.02$) (Table 4.2).

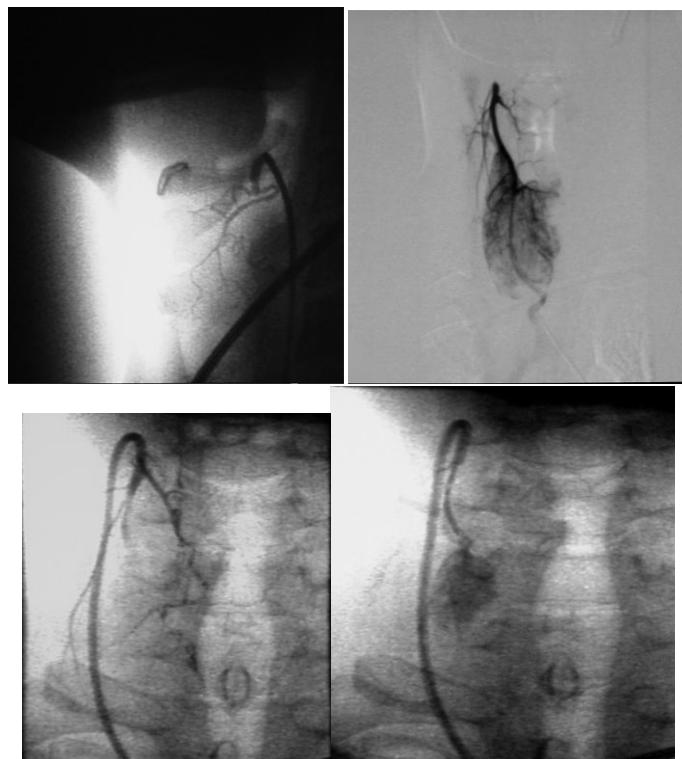


Fig.4.2. A and B are angiograms of the first embolization of a 32-year-old man, C and D are angiograms of the second embolization of the same patient. A. Angiograph of right superior thyroid artery showed obvious thyroid dyeing of contrast agent. The cricothyroid artery and stenocleidomastoid artery were patent. B. PVA particles of $150\text{-}250\mu\text{m}$ diameter were used to embolize the right superior artery. The thyroid dyeing disappears and the cricothyroid and stenocleidomastoid arteries remain patent. C. Three months after the first embolization, repeated angiograph shows the right upper pole of the thyroid gland regains its blood supply, resulting in dyeing of part of the thyroid. D. A microcatheter was inserted into the right superior artery and PVA was employed for the second embolization. The dyeing of thyroid disappears and non-thyroid arteries remain patent after the embolization.

On the right side, no significant difference exists in the diameter between the superior and inferior thyroid arteries ($P>0.05$), whereas on the left side, a highly significant difference in the diameter exists between the superior and inferior thyroid arteries with the superior artery larger ($P<0.01$). As for the blood supply to the thyroid gland on the right side, the superior artery is the dominant in 56.4% (31/55), the inferior artery is the dominant in only 3.6% (2/55), and in 40% (22/55), the superior and inferior thyroid arteries provide similar amount of blood supply. On the left side, the superior thyroid artery is the dominant in 49.1% (27/55), the inferior one is the dominant in 14.55 (8/55), and in 36.4% (20/55), the superior and inferior thyroid arteries provide similar blood supply to the gland (Table 4). These results suggest that only half of the blood supply to the thyroid gland is provided by the bilateral superior thyroid arteries in patients with Graves' disease because thyroid arteries of these patients have become pathologically enlarged and connected with one another to form an affluent arterial network, and embolization of only the bilateral superior thyroid arteries will not be sufficient to block more than 70% blood supply to the thyroid gland. Thus, it will be impossible to reach the goal of euthyroidism by embolizing only the bilateral superior thyroid arteries.

Table 4.2. Thyroid artery diameter and blood supply to thyroid gland

Thyroid artery	Diameter range	Mean diameter	Major supplier
Right superior	2.2-6.0 mm	3.8±0.9 mm	31/55(56.4%)
Right inferior	1.7-5.6 mm	3.5±1.3 mm	2/55(3.6%)
Left superior	2.4-6.0 mm	3.7±0.9 mm	27/55(49.1%)
Left inferior	1.0-5.2 mm	2.9±1.0 mm	8/55(14.5%)

4.3. Thyroid Arteries to be Embolized

Thyroid arterial embolization is a procedure without established guidance or protocols. Before embolization, it is important to define the arteries to be targeted. Selective angiography of bilateral superior and inferior arteries must be performed to demonstrate the scope of blood supply of each of these arteries. This essential information allows the performers the option of embolizing three greatest blood supplying arteries. Generally speaking, the arteries to be embolized should be both superior thyroid arteries and one larger inferior thyroid artery which supplies the most blood supply to the gland, with the minor inferior thyroid artery left untouched. On the one hand, the inferior arteries are usually more tenuous and tortuous than the superior ones. On the other, the parathyroid glands are mainly supplied by the inferior arteries. If bilateral inferior arteries are occluded, there may appear hypoparathyroidism either temporarily or permanently, as demonstrated by one female patient in the study by Wei Zhao et al. This patient was embolized one superior and two bilateral inferior arteries. After the procedure, she developed serious temporary hypoparathyroidism and recovered a week later after appropriate treatment. Once the parathyroid glands' main blood-supplying arteries, the bilateral inferior arteries, were totally embolized, collateral circulation of the glands could not be established in a short time, leading to decreased secretion of the parathyroid hormone and hypocalcemia. A week later, the establishment of collateral circulation of the parathyroid glands will restore the glands to normal secretion owing to the fact that the parathyroid glands have abundant capillary network with surrounding tissues and organs. Even so, the thyroid bilateral superior arteries and one larger inferior artery should be occluded, leaving one smaller inferior artery intact to avoid possible hypoparathyroid risk.

The trunk of the bilateral superior and unilateral inferior thyroid arteries should not be occluded with embolic agents, because occlusion of the large arterial trunk contributes little to either the obliteration of the thyroid peripheral branches or the necrosis of the thyroid tissue. Collateral circulation forms quickly from surrounding tissues and organs once the large arterial trunk is occluded. Only when occlusion of tiny peripheral vessels is achieved, do the tissues become necrotic. Moreover, a second embolization procedure may be carried out through the patent trunk of the previously-targeted thyroid arteries in case of insufficient embolization or collateral circulation formation leading to recurrence of hyperthyroidism.

4.4 Embolizing Techniques

In catheterizing the thyroid arteries, soft-tip catheters should be used and the catheter tip should be re-shaped according to the originating angle and course of the thyroid artery. A microcatheter can be used to facilitate super-selective catheterization of the gland branches of the thyroid arteries. On the one hand, super-selective catheterization using the microcatheter to navigate deeply into the tenuous branches of the thyroid gland can not only promote complete embolization but also prevent regurgitation of the embolic agents which might cause serious complications. On the other hand, super-selective catheterization can avoid mis-embolization of non-gland branches of the thyroid arteries. Besides branches supplying the thyroid gland, the thyroid arteries also have some other branches such as superior laryngeal, sternocleidomastoid, ascending cervical, cricothyroid, esophageal and inferior laryngeal arteries that do not supply blood to the thyroid gland. Mis-embolization of these branches may result in some possible complications. With super-selective catheterization, mis-embolization may be avoided, and post-embolization reactions and complications may be greatly reduced.

4.5. Use of Pingyangmycin

In the practice of thyroid arterial embolization, some authors used pingyangmycin to achieve a higher degree of arterial occlusion. Pingyangmycin is derived from a kind of soil rich in fungi. Molecular biochemistry analysis has shown that pingyangmycin is a subbranch of bleomycin A5 and is used for chemotherapy of neoplastic diseases as a glycopeptide antibiotic. Its antineoplastic function seems to inhibit DNA synthesis and radical formation, resulting in scission and fragmentation of DNA molecules. The side effects of Pingyangmycin depend on its first-pass effect on target organs. The most common and serious side effects are lung damage and pulmonary fibrosis. With high concentrations in target tissues, it can destroy the vascular endothelial structure and induce proliferation of endothelial and smooth muscle cells, leading to thrombosis and vascular cavity disappearance. However, no one has performed a controlled study on its effectiveness, and no clear results have been revealed as to whether or not this drug really plays an important role in thyroid arterial embolization. The actual role of this drug still needs to be addressed with a rigid control clinical study.

4.6. Embolic Materials and Recurrence

At follow-up, a few patients were recurrent. Several factors may contribute to hyperthyroid recurrence. Firstly, the embolizing scope with only two thyroid arteries being embolized may not be sufficient for definite efficacy. Embolization of three thyroid arteries is suggested. Secondly, the embolic agent PVA may not be the best material for embolization. The choice of appropriate-sized particles of PVA may not be so easy for complete occlusion of the thyroid artery branches and for prevention of possible collateral circulation. If the particle is too small, it may pass through the arteriovenous anastomoses into the systemic circulation, causing mis-embolization. If the particle is too large, it may not occlude small

artery branches and achieve complete blockade of the targeted thyroid tissue, consequently resulting in the formation of collateral circulation which may affect treatment efficacy. Better embolic materials like purified ethanol, cyanoacrylate and morrhuate sodium should be investigated to achieve better embolic effects.

5. Body Reaction to Thyroid Arterial Embolization

5.1. Pathologic and Histologic Features of the Embolized Thyroid Tissues

Embolization of the thyroid arteries occludes most of the vessels and no bruit can be heard immediately following the procedure. On the very first day post embolization, the thyroid gland begins to decrease in size and continues to decrease long after embolization (Table 5.1).

Table 5.1. Changes of thyroid gland size before and after arterial embolization

	cases	normal	I ° goiter	II ° goiter	III ° goiter	Blood supply
PrE	37	0	5	12	20	Abundant with typical sign of goiter
7 d PoE*	37	5	15	10	7	Scarce with large ischemic regions
6 m PoE*#	19	9	6	3	0	Scarce with small ischemic regions
3 y PoE*#	14	7	5	2	0	Relatively scarce

Note: *P < 0.05 compared with pre-embolization values ; #P < 0.05 compared with values at 7 days post-embolization. PrE=pre-embolization, d=day, PoE=post embolization, m=month, y=year.

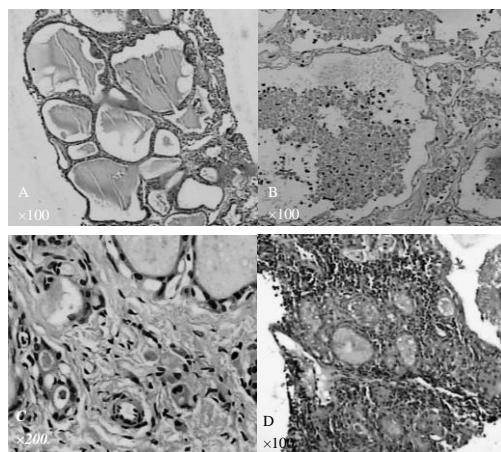


Figure 5.1. Microscopy of thyroid tissue with HE staining in Graves' disease. A. Typical hyperthyroidism before arterial embolization. B. Coagulation necrosis is present in the thyroid 7 days following embolization. C. Six months after embolization, fibroplasia and atrophic follicles are present simultaneously in the thyroid gland. D. Three years later, atrophic follicles and fibrosis of the interstitial tissues are demonstrated in the thyroid.

On the seventh day, the primary pathological changes are acute infarction and necrosis of the glandular epithelium and the interstitium (Fig.5.1 B), consistent with short-term pathological changes caused by arterial embolization. Later on, the thyroid gland starts a process of repair with presence of chronic inflammation, fibroplasia and some atrophic follicles (Fig. 5.1C). Three years later, the major pathological changes are apparent interstitial fibroplasia, lymphocyte infiltration and follicular atrophy (Fig.5.1D) and, consequently, thyroid function is decreased greatly and even returns to the normal range. These pathological changes within the thyroid gland form the basis for thyroid arterial embolization in treating Graves' disease hyperthyroidism. Some authors performed microscopic analysis of the thyroid embolized tissues from patients who received embolization before surgical removal of the thyroid tissue. The microscopic analysis of the embolized thyroid tissue demonstrated that the average diameter of an embolized capillary or an arteriole arising from the superior or inferior artery varied from 0.12 mm to 0.25 mm. The smallest diameter of unembolized capillaries ranged from 0.04 to 0.11 mm. In the isthmus, there was a slightly different range of arteries affected (0.13-0.15 mm). Foreign granuloma and proliferation of fibrous tissues were observed in the vicinity of the embolized capillaries and arterioles. Infiltration of multinuclear giant cells was noted in the lumen of the embolized arterioles. The follicular epithelium of the thyroid changed to a flat or cuboidal shape, and the volume of colloid in the lumen of the follicles decreased. Furthermore, the interstitium was separated by fibrous tissue, and lymphocytic infiltrates or follicles were observed.

Taken together, these findings suggest that thyroid arterial embolization blocks blood supply to the embolized regions of the thyroid gland, giving rise to an associated inflammatory response, necrosis and later, fibrosis.

5.2. Changes of Thyroid and Autoimmune Function Caused by Embolization

5.2.1. Long-Term Thyroid Function Changes

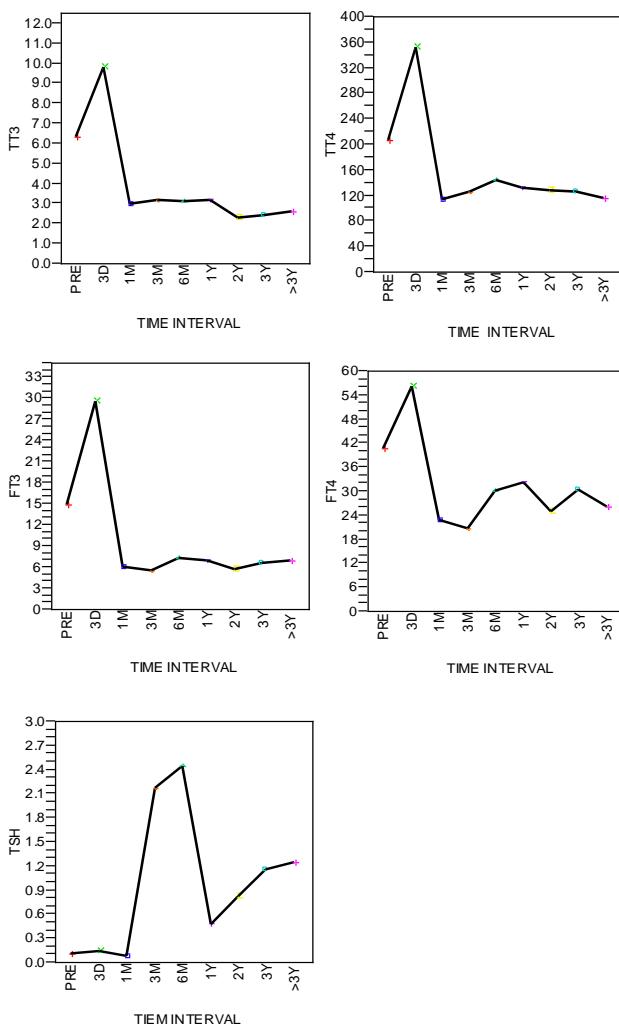
As demonstrated in Table 6 and Fig.7, after embolization, serum thyroid hormones, TSH and autoantibodies have some fluctuations. Compared with the preembolization values, serum concentrations of thyroid hormones increase temporarily on day 3 postembolization, drop greatly to the normal range at 1-2 months after the embolization procedure. TSH increases on day 3, decreases at one month and rises to the normal range 3 months after the embolization.

Immediately after embolization, the patients demonstrate increased levels of thyroid hormones (Table 5.2.1 and Figure 5.2.1), as the embolized thyroid tissue becomes chemically thyroiditic and necrotic, and a great deal of thyroid hormones are released into the blood circulation in a short period of time. This kind of chemical thyroiditis is caused by Pingyangmycin and occlusion of the thyroid arteries. With time, the synthesis and release of thyroid hormone decrease gradually because more than 70% of thyroid tissue becomes dead and fibrinotic, hence resulting in euthyroidism. As expected, the changes in TSH levels are opposite to those of free T₃ and T₄, which is caused by the negative feedback of thyroid hormone. Patients with suppressed TSH levels before embolization have a gradual increase in the TSH index after arterial embolization, and increase of the TSH index occurs in conjunction with the return of free T₃ and free T₄ to normal.

Table 5.2.1. Measurement of thyroid function before and after embolization (\pm SD)

group	TT3(nmol/L)	TT4(nmol/L)	FT3(pmol/L)	FT4(pmol/L)	TSH(uIU/ml)
Normal	1.1-3.4	58-161	2.3-7.7	12.2-28.7	0.4-3.1
3d PrE	6.37 \pm 3.09	206.24 \pm 72.56	14.87 \pm 8.14	40.87 \pm 17.51	0.12 \pm 0.11
3d PoE	9.83 \pm 5.27**	351.9 \pm 95.28**	29.53 \pm 10.14**	56.34 \pm 19.35**	0.15 \pm 0.10
1m PoE	2.97 \pm 1.14**	114.27 \pm 43.54**	6.17 \pm 2.70*	22.76 \pm 6.85*	0.09 \pm 0.10
3m PoE	3.21 \pm 1.16*	125.63 \pm 34.01*	5.61 \pm 2.18*	20.86 \pm 5.69**	2.17 \pm 3.03*
6m PoE	3.14 \pm 1.05*	145.27 \pm 47.12*	7.40 \pm 3.79*	30.02 \pm 14.34*	2.45 \pm 3.36*
1y PoE	3.16 \pm 1.13*	132.46 \pm 34.79*	6.94 \pm 3.31*	32.18 \pm 12.75*	0.47 \pm 0.49
2y PoE	2.32 \pm 0.98*	129.28 \pm 31.64*	5.83 \pm 2.32*	24.97 \pm 9.30*	0.83 \pm 0.95*
3y PoE	2.41 \pm 1.01*	125.29 \pm 31.62*	6.69 \pm 2.17*	30.36 \pm 10.09*	1.16 \pm 1.17*
>3y PoE	2.60 \pm 1.09*	117.08 \pm 40.23*	7.02 \pm 3.51*	26.13 \pm 9.26*	1.25 \pm 1.06*

TT4: total T4; TT3: total T3; FT4: free T4; FT3: free T3; TSH: thyroid-stimulating hormone; d: day; m: month; y: year; PrE: pre-embolization; PoE: post-embolization. * P<0.05 compared to pre-embolization values; ** P<0.01 compared to pre-embolization values.



Note: TT4: total T4; TT3: total T3; FT4: free T4; FT3: free T3; TSH: thyroid-stimulating hormone; d: day; m: month; y: year; PRE: pre-embolization.

Figure 5.2.1. Changes of thyroid function pre- and post-embolization

In evaluation of selective embolization of thyroid arteries as a pre-resective treatment in selected cases of toxic goitre, Marek Dedecjus et al observed a massive increase of thyroglobulin concentrations and a moderate increase of free thyroid hormones, together with a fall of TSH concentration 48 hours after thyroid arterial embolization. They believed that it was resulted from ischemic necrosis of the thyroid gland. Although thyroid arterial embolization reduces thyroid blood supply, the veins are not closed, and blood outflow remains unconstrained. Consequently, colloid from dying thyrocytes (comprising Tg, T3, T4 and probably, other biochemical components) gets into circulation. This creates a potential risk of thyrotoxicosis aggravation, which may be particularly important in elderly people with ischemic heart disease and/or serious arrhythmia. However, the review of the literature did not help elucidate any potential consequences of increased serum Tg concentration.

5.2.2. Long-Term Autoimmune Function Changes

5.2.2.1 Changes of the Serum Level of Thyroid Autoantibodies

The changes of the serum levels and positive rates of thyroid autoantibodies are shown in Table 5.2.2 and Fig.5.2.2-3. Pre-embolization activity and positive rate of TRAb in patients with Graves' disease are much greater than normal subjects, with a statistically significant difference ($P<0.05$). In embolized patients with effective control of hyperthyroidism by arterial embolization, these TRAb values gradually decrease 1 and 3 months following embolization compared with pre-embolization, but with no significant difference ($P>0.05$). Six months later, these values decrease statistically significantly ($P<0.05$) compared with pre-embolization to near the normal level. In patients with recurrence following arterial embolization, the activity and the positive rate of TRAb have no marked change compared with pre-embolization. The activity and positive rate of TSAb are significantly much greater before than any time points after embolization, with the TSAb values gradually decreasing over time. However, the TSAb values return to high levels as before embolization in patients with recurrence. The pre-embolization titre and positive rate of TGAb and TMAb are much greater than in normal subjects, with a statistically significant difference ($P<0.05$). There is no linear correlation between TRAb or TSAb activity and TGAb or TMAb prior to embolization, and no significant correlation exists in TRAb or TSAb activity and TGAb and TMAb titre with FT3, FT4, TT3 or TT4.

TRAb(TSAb), TGAb and TMAb are the three major autoantibodies targeting the major thyroid autoantigens of thyroid stimulating hormone receptor (TSHR), thyroglobulin (TG) and thyroid peroxidase (TPO), respectively, in patients with autoimmune thyroid disease (AITD) including Graves' disease. The exact etiopathogenesis of Graves' disease is unclear up to date but is thought to be caused by the autoantibodies that can stimulate the thyroid gland for hyperblastosis and hyperfunction, especially TSAb. The role of TSAb in the pathogenesis of Graves' disease is critical, including 1) to combine with the major antigen peptide of TSHR for the simulation of TSHR, leading to enzymes activation related to the growth and function of thyroid follicles through a series of pilot processes, and 2) to inhibit the apoptosis of the thyroid cells which may involve many molecules including Fas. Therefore, the thyroid gland grows, swells and releases a great deal of thyroid hormones into blood circulation resulting in hyperthyroidism. Some study had demonstrated that 79%-97% untreated patients with Graves' disease had positive serum TSAb whose value had early diagnostic significance for this disease. In Table 5.2.2, the measurement of TRAb (TSAb) is

through the method of sensitive radioreceptor assay, and the positive rate of TRAb and TSAb of Graves' patients before embolization reach 87.8% and 90%, suggesting higher specificity of TSAb to the diagnosis of this disease. After embolization, the positive rate and activity of TRAb and TSAb decrease significantly greatly ($P<0.05$) compared with before embolization, and will continue to drop with gradual decomposition of the existing TRAb and TSAb and reduction of newly synthesized autoantibodies due to the fact that most thyroid tissue has been embolized and necrotic. Finally, the synthesis and destructive metabolism of the key autoantibodies will gradually reach a balance. In the end, serum TSAb will maintain relatively stable and the immunological disorder will be corrected. If the positive rate and activity of the key autoantibody (TSAb) are still higher than normal level, there may be a possibility of recurrence.

The titre and positive rate of TGAb and TMAb in Graves' patients are all greater than those in normal subjects, but the positive rates of TGAb (48.78%) and TMAb (51.22%) are significantly lower than those of TRAb and TSAb (87.8%, $P<0.05$) in the same group, suggesting that TGAb and TMAb are different indexes of auto-immunology but possibly not critical in the etiopathogenesis of Graves' disease. They play a different role from that of TRAb and TSAb. After thyroid arterial embolization, the titre and positive rate of TGAb and TMAb have a general trend of reduction, even though it is not significant compared with pre-embolization ($P>0.05$).

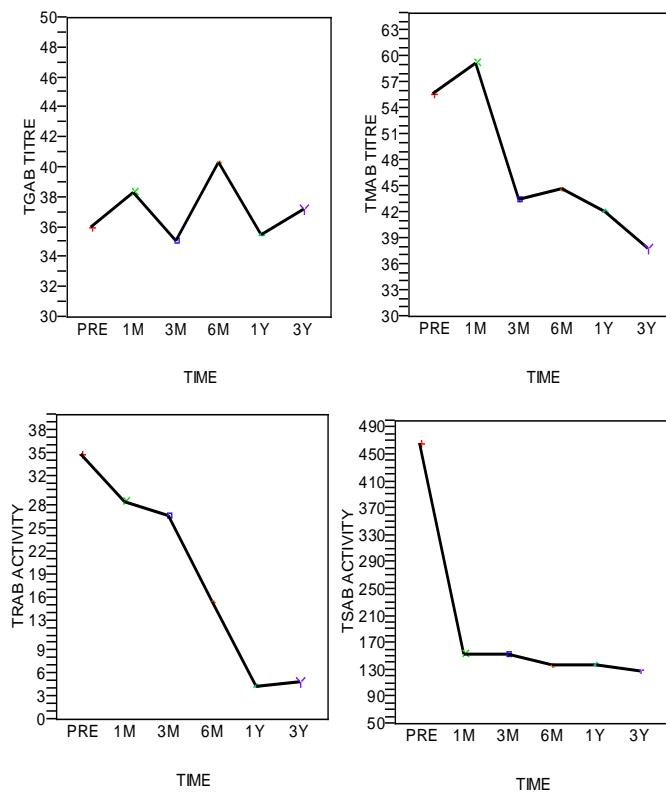


Figure 5.2.2. Titre/concentration of thyroid autoantibodies before and after embolization (PRE: before embolization, M: month, Y: year)

Table 5.2.2. Serum levels of thyroid autoantibodies and positive rate (%) before and after embolization

groups	TGAb		TMAb		TRAb		TSAb	
	Titre (%)	Positive rate (%)	Titre (%)	Positive rate (%)	Activity(U/L)	Positive activity rate (%)	activity	Positive rate(%)
Normal	12.29±4.15	8.00	13.85±17.21	16.00	3.82±3.11	16.00	/	/
PrE	35.96±21.46 **	48.78 **	55.69±48.51 **	51.22 **	34.92±36.78 **	87.80 **	467.3±59.1	90
PoE 1m	38.30±27.99 **	46.87 **	59.22±41.50 **	56.25 **	28.57±32.02 **	71.88 **	154.3±16.4 ##	43.2 ##
3m	35.10±24.85 **	41.94 **	43.50±33.71 **	45.16 **	26.85±25.62 **	70.97 **	152.8±18.7 ##	41.9 ##
6m	40.33±19.62 **	37.93 **	44.83±37.12 **	48.28 **	15.42±10.33 #	62.07 ** #	136.5±42.9 ##	44.8 ##
1y	35.51±20.78 **	41.67 **	42.17±44.60 **	41.67 **	4.30±3.65 ##	54.17 ** #	137.4±47.3 ##	37.5 ##
3y	37.25±26.31 **	36.36 **	37.94±34.92 **	40.91 **	4.94±4.73 ##	22.73 ##	128.7±38.5 ##	36.4 ##
Recurrent	36.29±23.48 **	42.86 **	58.53±47.31 **	57.14 **	42.36±38.17 **	85.71 **	417.6±47.7	87.5

Note: *P<0.05 and **P<0.01 compared to values in the control group; #P<0.05 and ##P<0.01 compared to values in the pre-embolization group. TGAb: thyroglobulin antibody, TMAb: thyroid microsome antibody, TRAb: thyrotropin receptor antibody, TSAb: thyroid stimulating antibody, PrE: pre-embolization, PoE: post-embolization, m: month, y: year.

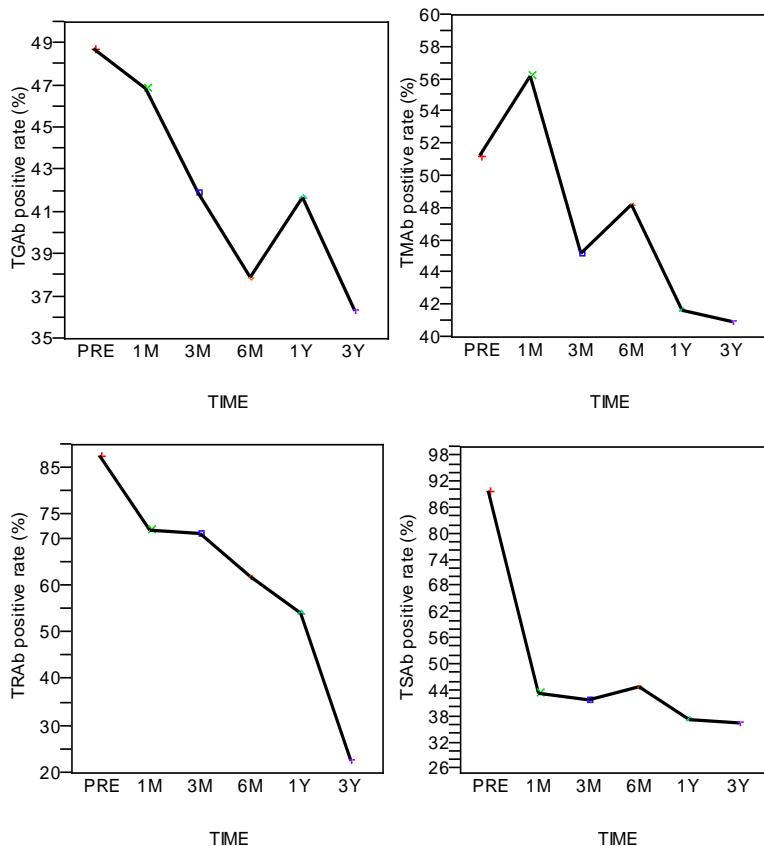


Figure 5.2.3. Positive rate of thyroid autoantibodies before and after embolization (PRE: before embolization, M: month, Y: year)

5.2.2.2. Measurement of Subgroup Peripheral Blood Lymphocytes

The serum levels and changes of the lymphocytes before and after embolization are demonstrated in Table 5.2.2.2. In patients with Graves' disease before embolization, the peripheral blood CD3⁺CD8⁺ decreases while the CD4⁺/CD8⁺ ratio increases statistically significantly compared with the normal subjects ($P<0.05$). CD16⁺CD56⁺, CD3⁺ and the ratio of CD3⁺CD4⁺ in pre-embolization patients are not significantly different from those of the control normal subjects. The level of CD16⁺CD56⁺ is greater 1 and 3 months following embolization than before embolization with a statistically significant difference ($P<0.05$) and decreases gradually to reach the normal level 6 months later. The level of CD3⁺CD8⁺ gradually increases 1, 3 and 6 months following embolization with no significant difference ($P>0.05$) compared with before embolization and continues to rise to nearly the normal level 1 and 3 years later. The ratio of CD4⁺/CD8⁺ decreases 1 and 3 months after embolization but with no significant difference ($P>0.05$) compared with before embolization; however, it decreases to normal range 6 months later with a statistically significant difference ($P<0.05$). There is no significant ($P>0.05$) linear correlation between CD3⁺CD8⁺ or CD4⁺/CD8⁺ and TRAb activity or serum concentration of FT3, FT4, TT3 and TT4.

Table 5.2.2.2. Measurements of peripheral blood lymphocytes before and after embolization

Groups	CD16+CD56 + (%)	CD19+(%)	CD3+(%)	CD3+CD4+ (%)	CD3+CD8+ (%)	CD4 ⁺ /CD8 ⁺
Normal subjects	24.84±5.70	11.51±5.17	65.42±14.23	39.36±11.59	23.93±7.83	1.31±0.53
3d PrE	23.36±6.08	10.72±6.34	60.70±19.46	42.47±14.85	16.58±5.62**	1.77±0.73*
PoE (effective)	1m	32.42±5.61**	9.81±4.68	64.58±13.70	38.05±10.37	16.73±7.21**
	#					1.72±0.60*
	3m	35.35±7.20**	11.06±7.13	61.89±14.15	37.23±9.56	17.33±7.86**
	#					1.64±0.42*
	6m	25.89±6.92	12.72±6.92	64.64±20.57	37.31±10.13	19.57±6.30*
	1y	24.10±6.35	10.33±5.24	69.27±15.83	40.54±12.67	21.43±8.50#
recurrent	3y	21.62±5.81	13.67±5.59	65.47±19.67	35.81±10.78	22.14±9.42#
		26.64±6.78	11.43±6.65	59.05±19.59	41.80±13.32	15.93±4.79*
						1.31±0.40#
						1.81±0.75*

Note: * $P<0.05$ and ** $P<0.01$ compared to values in the control group; # $P<0.05$ and ## $P<0.01$ compared to values in the pre-embolization group. PrE: pre-embolization, PoE: post-embolization, d: day, m: month, y: year.

Lymphocytes are the major cell colony to constitute the body's immune system, accounting for 20%-45% of the total peripheral blood leucocytes. Generally, CD3, CD19 and CD16/CD56 are used as the surface markers of T, B and NK (natural killer) cells. Among T (CD3⁺) cells, CD3⁺CD4⁺ and CD3⁺CD8⁺ represent help T cell (Th) and suppressor T cell (Ts), respectively. NK cells mainly participate in non-specific immune reaction, while Graves'

disease is an organ-specific autoimmune disease. Therefore, the level of peripheral blood NK cells has no apparent increase before embolization. However, thyroid arterial embolization caused in the thyroid tissue acute ischemic necrosis which alters the antigen characteristics of the necrotic tissue to become “non-autoantigen”, thus leading to the rise of NK cell level to eliminate the necrotic thyroid cells. With the elimination of the necrotic thyroid cells, the NK cells decrease to normal range over 3 months following embolization.

B cells are the only cell within the body to produce antibodies, and based on the presence of CD5 antigen on the B cell surface, B cells can be further divided into two subgroups of B1 (CD5+CD19+) and B2 (CD5-CD19+) cells. The level of CD19⁺ in Graves' patients is not significantly different ($P>0.05$) in pre-embolization group compared with the normal control group, and there is no significant difference ($P>0.05$) in every period following embolization compared with pre-embolization. However, it has been reported that the B cells secreting thyroid autoimmune antibodies mainly exist in the thyroid gland itself and that there is no significant difference ($P>0.05$) in the level of CD5-CD19+ in patients with Graves' disease compared with healthy adults. In this case, the assay of active B cells in peripheral blood has limited significance in predicting the prognosis of disease following embolization.

Although the level of Th in patients with Graves' disease is normal, the Ts level decreases, leading to the increase of the ratio of Th/Ts. Some reports have proved this immune abnormality and the close involvement of Ts and Th in the pathogenesis of Graves' disease. In patients with effective control of hyperthyroidism by arterial embolization, the level of Ts gradually increases following embolization and reaches the normal level at one year. The ratio of Th/Ts decreases to the normal range 6 months following embolization, corresponding to the change of TRAb and TSAb. In patients with recurrence after arterial embolization, the level of Ts and the ratio of Th/Ts have no significant difference compared with pre-embolization, suggesting that the level of Ts and the ratio of Th/Ts can both predict the index for prognosis. The ratio of Th/Ts has a closer relationship with the level of TRAb and is more efficient in predicting the effect of treatment. There is no significant difference ($P>0.05$) in the disorder of T cells between the effective and the recurrent groups of patients prior to embolization, and the T cell disorder is corrected following embolization in most patients who are improved or restored to euthyroidism. However, it is not clear why T cell disorder can not be corrected in some patients who are recurrent later on. How does embolization cause the change of the level of Ts and the ratio of Th/Ts? These issues remain mysterious and need further studies.

To conclude, human body is a complicated entirety, immunity involves numerous immune organs, cells and molecules, and the process of immune response is extremely complex. The study of the effect of thyroid arterial embolization on the immunological function of Graves' patients relates to the disease immunological mechanism which is not clear up to date. Further studies are needed to elucidate the effect of embolotherapy on the immunological function. However, it should be pointed out that thyroid arterial embolization is to eliminate most enlarged thyroid tissue and get rid of hyperfunction through the blockage of two to three thyroid arteries, thus achieving the same goal as surgical subtotal thyroidectomy. Thyroid arterial embolization indirectly corrects the abnormal immunological function of the body through destruction of most thyroid tissue.

5.3. Angiogenic Changes in the Thyroid Caused by Thyroid Arterial Embolization

Angiogenesis is the sprouting of new blood vessels from pre-existing capillaries, and requires multiplication of endothelial cells, their migration, remodeling of the extracellular matrix, tube formation and recruitment of surrounding structures to maintain the newly formed vessels. Angiogenesis is tightly controlled and occurs rarely in the adult vasculature except with wound healing, the menstrual cycle, and in pathological conditions including diabetic retinopathy, tumour formation, hyperplastic goitre and Graves' disease in which increased vascularity presents. Neovascular growth is regulated through a balance of soluble angiogenic stimulators and inhibitors, with promoters of angiogenesis including VEGF, acidic FGF, bFGF, insulin-like growth factor, transforming growth factor, NO, angiotensin-II. VEGF and bFGF are two major promoters of angiogenesis. Accumulating evidence shows that VEGF and its receptors are important in the thyroid in Graves' disease, thyroiditis, goitre and cancer. bFGF alone may act as an angiogenic factor in the thyroid, with direct effects on both endothelial and follicular cell growth.

Before embolization, the expression of VEGF is greater in regions of active proliferation of follicular epithelial cells and lymphocyte infiltration. After thyroid arterial embolization, expression of VEGF is enhanced within 6 months but reduced over one year compared with before embolization. Expression of bFGF is heavily enhanced both before and within 6 months after embolization but weakened over one year following embolization. Microvessel density (MVD) is strong and intense in regions of active proliferation of follicular epithelial cells and in regions of lymphocyte infiltration. After thyroid arterial embolization, MVD is enhanced within six months but reduced over one year compared with before embolization (Fig. 5.3.1-3). A positive correlation exists between VEGF and bFGF and between VEGF or bFGF and MVD.

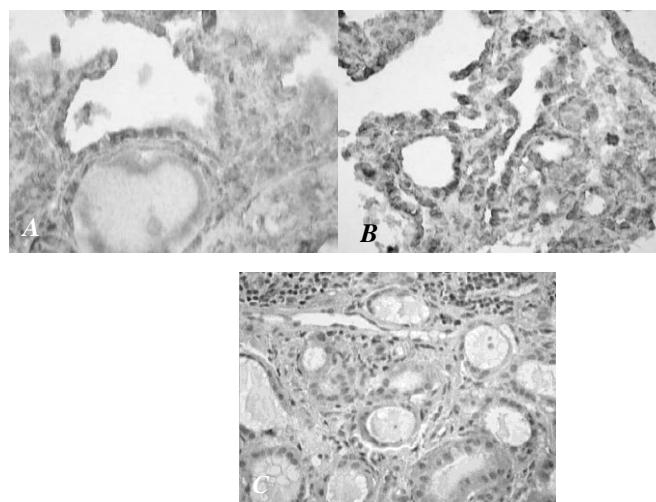


Figure 5.3.1. Expression of VEGF at different time points, $\times 400$. A. Before embolization. B. Three months after embolization. C. Three years after embolization.

The changes of VEGF, bFGF and MVD after thyroid arterial embolization may be caused through many mechanisms. Embolization of the thyroid arteries leads to acute occlusion of small arteries and, consequently, acute aseptic inflammation and necrosis of the thyroid tissue. Acute occlusion of arteries causes thyroid hypoxia which can upregulate the expression of VEGF. Moreover, hypoxia can enhance the stability of VEGF and prevent its breakdown, thus increasing its concentration after embolization. Acute necrosis of thyroid tissue may stimulate expression of VEGF through cytokines and inflammation factors such as insulin-like growth factor, transforming growth factor etc. Consequently, within 6 months after thyroid arterial embolization, expression of VEGF is increased to enhance production and hyperplasia of MVD within the thyroid gland. However, over one year later when the ischemic and necrotic tissues within the thyroid gland have been repaired by hyperplastic tissue and the amount of thyroid follicular cells have been reduced because of ischemia and necrosis, the total amount of oxygen needed by the thyroid gland decreases because of decreased secretory function. Subsequently, expression of VEGF is reduced and returns to normal. MVD within the thyroid is also reduced with decreased expression of VEGF.

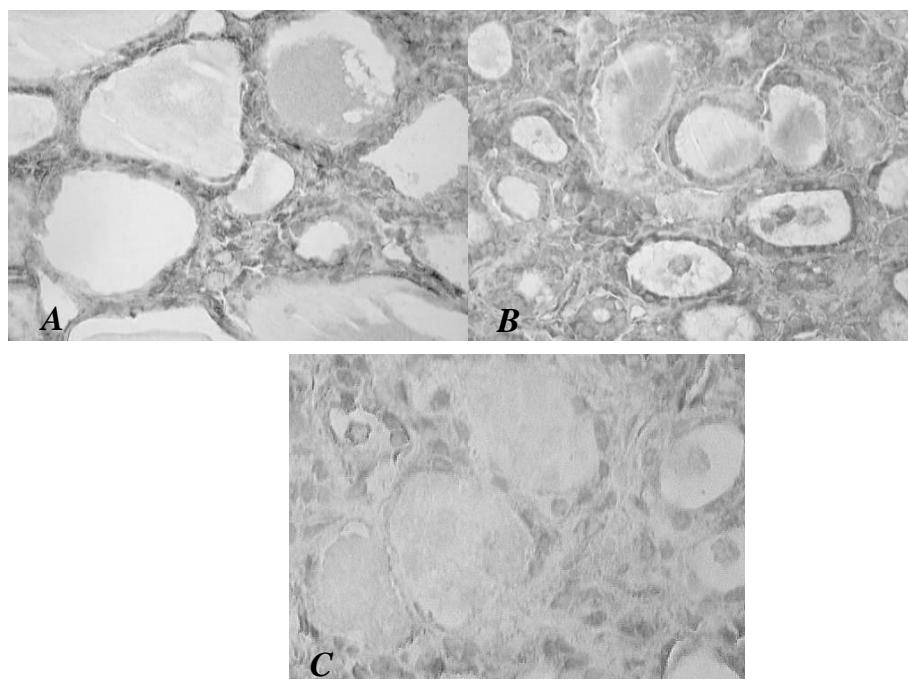


Figure 5.3.2. Expression of bFGF at different time points, $\times 400$. A. Before embolization. B. One month after embolization. C. One year after embolization.

As for bFGF, its expression is not different within six months after embolization but decreases over one year. This suggests that bFGF may not play a major role in angiogenesis shortly after embolization. In the long run, bFGF expression is also reduced together with decreased expression of VEGF and MVD. The mechanism for the change of bFGF after embolization is not clear but may be related to the repair and decreased function of the thyroid gland. Moreover, interaction with VEGF may also play a role in the changes of the

expression of bFGF because there is a complex interdependent relationship between VEGF and bFGF.

In conclusion, thyroid arterial embolization can decrease the expression of VEGF and bFGF and reduce MVD in Graves' disease thyroid in the long term, thus decreasing abnormal growth of the thyroid tissue and converting hyperthyroidism to euthyroidism.

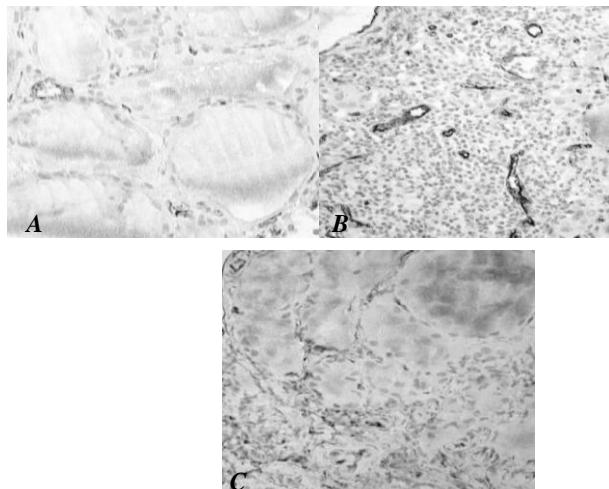


Figure 5.3.3. MVD marked by CD34, $\times 400$. A. Before embolization. B. Six months after embolization. C. Three years after embolization.

5.4. Apoptotic Changes

Apoptosis is critical in the development and homeostasis of multicellular organisms. An increased rate of apoptosis is involved in the pathogenesis of several degenerative diseases while inhibition of apoptosis has been implicated in autoimmune diseases and carcinogenesis. The percentage of apoptotic thyrocytes *in situ* is increased in Hashimoto's thyrocytes, but decreased in Graves' disease, suggesting the importance of apoptotic cell death of thyrocytes in the regulation of functions and numbers of these cells in autoimmune thyroid diseases.

Zhao et al studied apoptosis in the thyroid of Graves' disease treated with thyroid arterial embolization, using semi-quantitative analysis for the expression of Fas, FasL, Bax, Bcl-2 and p53 on thyrocytes before and after arterial embolization. Before arterial embolization, the positive staining of Fas and FasL restricted to the cytoplasma and cellular membrane in the thyroid cells is very low and sparsely distributed. After embolization, significant expression of Fas and FasL is demonstrated within the thyroid tissue, with both the positive cellular number and staining degree greater than before embolization ($P < 0.05$). The expression of Bax and Bcl-2 is both within the cytoplasma, with no apparent enhancement in the positive cellular number and degree before embolization and less than one year after embolization ($P > 0.05$), but is significantly enhanced over one year after arterial embolization both in the positive cellular number and degree ($P < 0.05$). P53 is usually negative or distributed like small patches before embolization, whereas after embolization, the P53 positive cellular number

and staining degree increase with time and are significantly higher than before embolization ($P<0.05$) (Fig. 5.4.1-5).

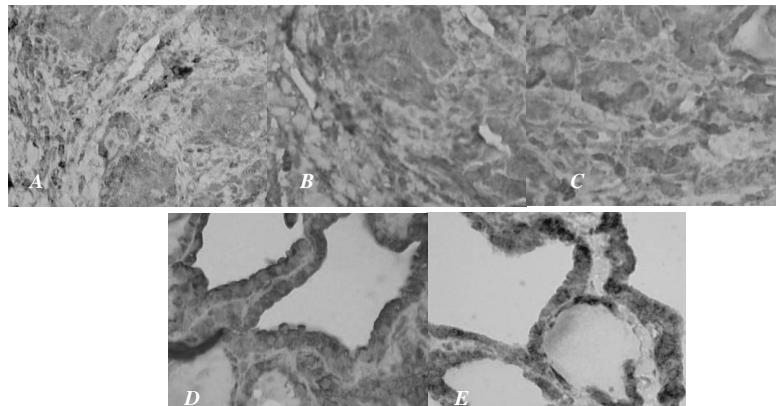


Fig. 5.4.1. The expression of Fas increases with time ($\times 400$). A. Before embolization, the expression of Fas is like small patches within the Graves' disease thyroid tissue. B, C&D. Seven days (B), 3 months (C) and 6 months (D) following embolization, Fas expression is gradually enhanced. There are some atrophied follicles and some non-specific staining. E. At the end of one year, Fas expression greatly increases in both positive cellular number and staining degree, with the staining mainly within the cytoplasma and on the cell membrane and partly in the nuclei ($\times 400$).

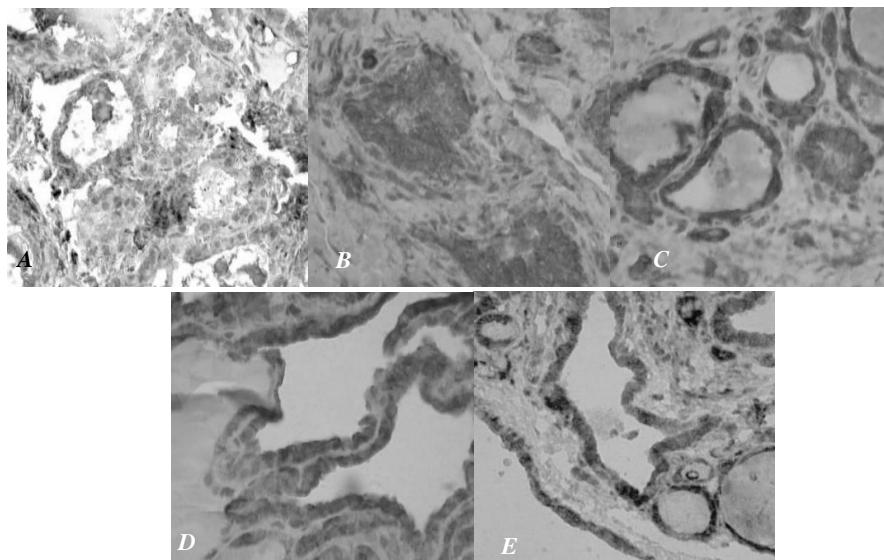


Fig. 5.4.2. The expression of FasL increases over time ($\times 400$). A. Before embolization, FasL is sparsely expressed within the cytoplasma and partly in the nuclei. B, C&D. Seven days (B), 3 months (C) and 6 months (D) following embolization, Fas expression is gradually enhanced. There are some atrophied follicles and some non-specific staining. E. Three years later, FasL expression is greatly enhanced and is mainly expressed within the cytoplasma and on the cellular surface and partly in the nuclei.

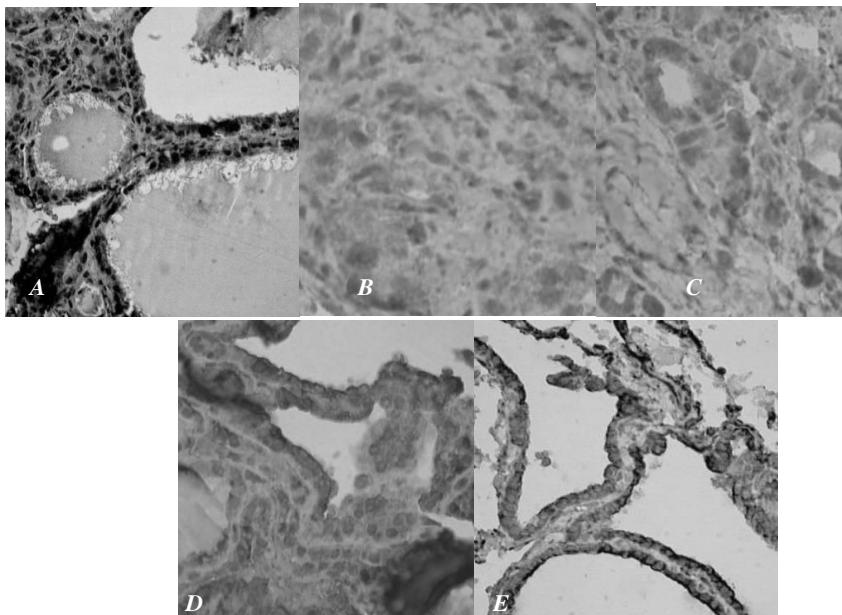


Fig.5.4.3. The expression of Bax is significantly enhanced more than one year following embolization compared with before embolization ($\times 400$). A. Before embolization, Bax is sparsely expressed within the cytoplasma and the nuclei of Graves' disease thyroid tissue. B, C&D. Seven days (B), 3 months (C) and 6 months (D) following embolization, Bax expression is gradually enhanced, but no significant differences exists at less than one year after embolization compared with before embolization. There are some atrophied follicles and some non-specific staining. E. Three years later, Bax expression is significantly greatly enhanced and was mainly expressed within the cytoplasma and partly in the nuclei.

In Graves' thyroid glands treated with arterial embolization, the cellular number and staining degree of positive Fas and FasL are significantly higher ($P < 0.05$) than before embolization, which suggests that thyrocyte apoptosis caused by embolization may overpass thyroid hyperplasia. Although the positive cellular number and degree of pro-apoptotic Bax one year following embolization are not significantly different from before embolization ($P > 0.05$), those at greater than one year are significantly higher than before embolization ($P < 0.05$). In contrast, the positive cellular number and staining degree of anti-apoptotic Bcl-2 have no significant difference before and after arterial embolization ($P > 0.05$), namely no increased expression of Bcl-2 following embolization. Increased Bax expression implicates enhanced thyrocyte apoptosis. The number and degree of positive p53 cells are significantly higher than before embolization ($P < 0.05$), and through the regulation of its downstream genes, increased p53 expression means promotion of thyroid cell apoptosis.

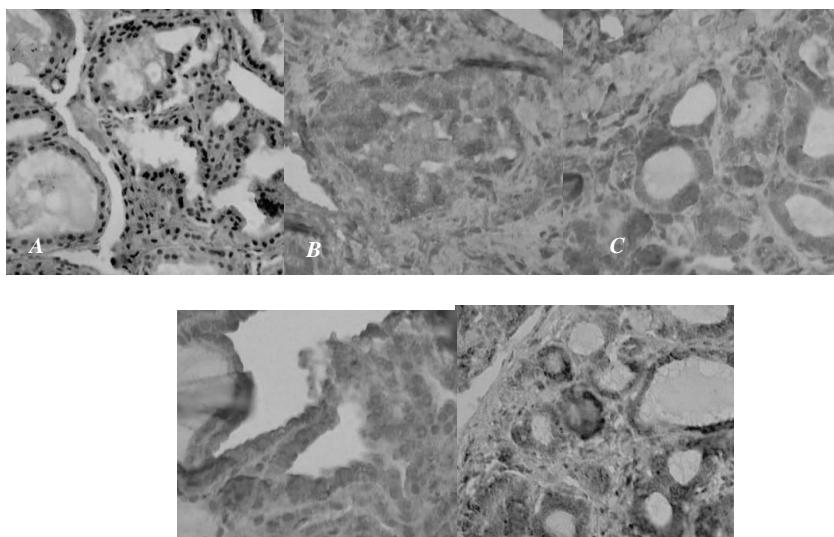


Fig.5.4.4. The expression of Bcl-2 is not significantly different before embolization and at different time points after embolization ($\times 400$). A. Before embolization, Bcl-2 expression is mainly within the nuclei and partly within the cytoplasm. B, C, D&E. Seven days (B), 3 months (C), 6 months (D) and 3 years (E) after embolization, Bcl-2 is sparsely expressed mainly within the cytoplasm but partly within the nuclei.

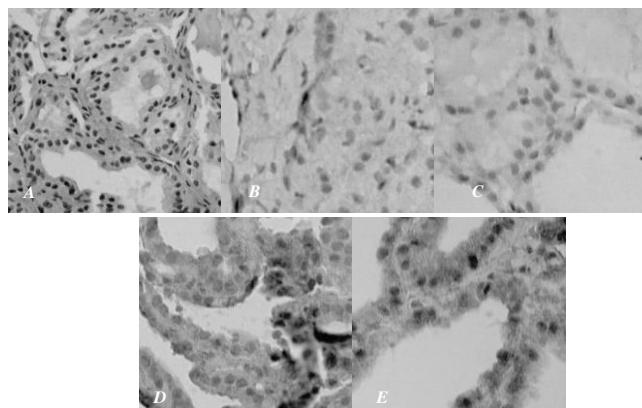


Fig.5.4.5. The positive cellular number and staining degree of p53 increase with time and are significantly higher than before embolization ($P < 0.05$) ($\times 400$). A. Before embolization, the expression of p53 is negative in Graves' disease thyroid tissue. B&C. Seven days (B) and 3 months (C) following embolization, slight expression of p53 is present in the nuclei. D. Six months later, the p53 expression in the nuclei is stronger than at the 3rd month, and furthermore, p53 expression also presents in the cytoplasm. E. At the end of one year, the expression of p53 is continuously enhanced compared with at 6th month and is located in the cytoplasm and the nuclei.

In general, following thyroid arterial embolization, the pro-apoptotic genes expressed in Graves' thyroid are continuously enhanced, leading to the thyroid cell apoptosis greater than thyroid hyperplasia and consequently reduced hyperfunctional follicles of the thyroid. The regulation process of apoptosis is consistent with the improvement of the body immunity as demonstrated by the decrease of TSAb antibody. Because a therapy directed at the

pathogenesis of Graves' disease is impossible, current treatment modalities all aim at inhibiting the synthesis and release of thyroid hormones or reducing and destroying the thyroid tissue for secretion of thyroid hormones. However, the improvement of regulation of the whole body immunity should also be stressed at the same time. In conclusion, thyroid arterial embolization can enhance apoptosis of the thyroid cells which may be able to improve the autoimmunity of the thyroid.

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Chapter 5

New Targets and Approaches to Autoimmune-Induced Salivary Hypofunction

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The pathogenesis of autoimmune diseases is multifaceted, and the complexity of symptoms, diagnosis and treatment options associated with these diseases reflects this. Sjogren's syndrome (SS) is an autoimmune disorder affecting up to 4 million Americans, and results from autoimmune reactions in secretory glands (primary SS) and other tissues (secondary SS). Current treatment options for SS are aimed largely at amelioration of symptoms, but without functional restoration of the secretory glands. Prescribed medications for salivary hypofunction or xerostomia are often associated with severe side effects, typically due to the effects of systemically administered muscarinic cholinergic receptor agonists.

Recently published data indicate that in addition to inflammation, hyperproliferation of glandular cells and reduction in antioxidant enzyme levels are associated with SS salivary gland pathology. Initial findings in SS patients strongly support the idea that the pathogenesis of SS involves a combination of inflammation, hyperproliferation, and reduced antioxidant capacity. This combination can also be found in other autoimmune diseases such as psoriasis, and provides a novel target for the development of new diagnostic tools.

Importantly, animal models for SS and psoriasis have shown that these pathological abnormalities could be improved with non-toxic agents, such as green tea polyphenols, that inhibit inflammation and hyperproliferation, and elevate the antioxidant capacity in autoimmune-affected tissues.

These newly published observations warrant further exploration of new targets and novel agents for treatment of autoimmune disorders.

Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disorder characterized by dysfunction of multiple different exocrine glands (although other epithelial tissues, such as kidneys, can also be affected). Glands producing protective secretions that coat externally exposed epithelia, in particular oral and ocular surfaces, are commonly affected (Fox, 2005; Mathews et al 2008). Thus, salivary gland exocrinopathy leads to a reduction in saliva fluid; in turn it results in the sensation of dry mouth (xerostomia) and in the loss of delivery of protective functions to the mouth. This eventually leads to health problems such as rampant caries and an increase in opportunistic infections such as candidiasis. Similarly, loss of lacrimal gland function reduces tear secretion, resulting in dry eyes (keratoconjunctivitis sicca) and ocular problems. Both oral and ocular dryness causes considerable discomfort and a decline in quality of life (Kassan and Moutsopoulos, 2004; López-Jornet & Camacho-Alonso, 2008; Stewart et al., 2008a). When other tissues, such as the lungs, are involved, serious health complications can result (Fox 2005).

In primary SS, the autoimmune attack is confined mainly (but not necessarily exclusively) to the salivary and lacrimal glands, associated with inflammatory cell infiltrates. In secondary SS other systemic autoimmune diseases affecting connective tissues (particularly systemic lupus erythematosus (SLE), rheumatoid arthritis and scleroderma) occur in parallel with—or precede—the SS. SS affects between 1-4 million people in the U.S., or about 1% of the population (Venables, 2004), and therefore represents a significant public health issue. It can occur at any age, although most cases are diagnosed between 40 and 50 years of age, with preponderance in females (9:1 female-to-male incidence) (Mathews et al 2008; Delaleu et al 2005; Fox 2005; Hsu and Dickinson 2006).

Diagnosis of SS

The diagnosis (and therefore the epidemiology) of SS has in the past been complicated by the use of different diagnostic criteria in different countries (Fox, 2005; Margaix-Muñoz et al., 2009). In 2003, a set of diagnostic criteria was recommended by an international consensus group: diagnosis of primary SS required fulfillment of 4 out of 6 possible criteria; certain ocular symptoms (at least one present), certain oral symptoms (at least one present), objective ocular signs (e.g., Rose Bengal staining score ≥ 4), objective evidence of salivary gland involvement (e.g., unstimulated saliva flow $< 1.5 \text{ ml/ 15 min}$), histopathology (lymphocytic infiltrates in a minor salivary gland biopsy), and laboratory abnormality (certain autoantibodies present).

When dry mouth or dry eye is clearly present along with anti-nuclear antibodies (ANA) and anti-SS-A (Ro) or anti-SS-B (La) autoantibodies the diagnosis of SS is not controversial, but with milder cases, or when the autoantibody profile is less specific, diagnosis is more challenging. For example, a positive ANA score is more confirmatory than useful for screening (Lightfoot 1997). There is no characteristic autoantibody for SS, and different autoantibody profiles may be present in different individuals, although the presence of anti-SS-B antibodies correlates strongly with organ involvement and cytopenias (Locht et al, 2008).

A histopathological diagnosis of SS requires the presence of more than one periductal lymphocytic aggregate (referred to as foci), with each focus comprising at least 50 lymphocytes when viewed at magnification of 10 HPF (High Power Field). This is also equivalent to two or more foci per 4 mm². Standard histopathologic evaluation requires an examination of at least 4 minor salivary gland lobules routinely obtained via incisional biopsy of minor salivary glands of the lower lip. Gland biopsy and accurate histopathologic assessment requires experience to avoid false positives (Fox 2005; Stewart et al., 2008b). An additional challenge in interpreting histopathologic finding is heterogeneous distribution of foci within the gland (Al-Hashimi et al., 2001; Morbini et al., 2005). This has led to the utility of the histopathology results from minor lip gland biopsy being questioned by some investigators. Nevertheless, where sicca symptoms are confirmed and positive serology are present, there is a strong correlation with a positive biopsy, suggesting that when clinical and serologic criteria are evidently strong a biopsy may be unnecessary, especially if immunosuppressive therapy is present (Bamba et al., 2009). An additional complication of labial minor salivary gland biopsy is the risk of permanent sensory loss in the lip mucosa (Pijpe et al., 2007). Recently, incisional biopsy of the parotid gland has been suggested as an alternative to labial biopsy that can overcome disadvantages of the latter, and has certain advantages such as the opportunity for repeat sampling (although it does require specific surgical training, and may have other sequelae such as injury to the facial nerve; Pijpe et al., 2007). Less invasively, sialography can be used to assess gland damage, although acute sialadenitis is a potential complication (Obinata et al., 2010; Thanou-Stavraki & James, 2008).

Since saliva (the product of one set of affected glands) can be acquired non-invasively, the use of salivary components as a diagnostic tool has been explored. However, saliva is less sensitive than serum for the detection of anti-SSA and anti-SS-B antibodies (Hammi et al., 2005). More recently, proteomic and genomic analysis of saliva and salivary glands has been employed in an effort to identify profiles that could be used diagnostically (reviewed in Baldini et al., 2008). DNA microarray analysis of gene expression profiles of minor salivary glands from SS patients revealed characteristic differences that allowed 95% (19/20) of the samples to be correctly identified using the top 200 differentially expressed genes (Hjelmervik et al., 2005). Surface-enhanced laser desorption/ionization time-of flight (SELDITOF) and 2D difference gel electrophoresis (2D-DIGE) were used to compare protein expression profiles in stimulated parotid saliva from SS and normal individuals (Ryu et al., 2006). Relative to controls, in SS patients inflammatory proteins were found to be increased (as might be expected), and certain protein products of acinar cells (where many normal protective products of the gland are synthesized) decreased. Interestingly, there was no correlation with focus score. A broader, and only partially overlapping set of protein changes are observed when whole saliva is examined (Giusti et al., 2007; Hu et al., 2007), although in all cases carbonic anhydrase VI (an enzyme critical for saliva buffering capacity) is decreased. An initial analysis of the proteome of primary SS and normal minor salivary glands has been performed (Hjelmervik et al., 2009). Alpha-defensin-1, involved in viral defense, and calmodulin, involved in inflammation, was only found in primary SS glands. Other quantitative differences in several proteins were seen. More broadly, the effects of SS on salivary protein expression appear to be complex and the use of salivary protein profiles as a diagnostic tool is still in its infancy, but has potential, especially if markers can be found for early stages of the disease.

Current Treatment Strategies

At present there are no specific treatments for SS, and the primary approach is to ameliorate the symptoms of dryness caused by lack of fluid production, and to treat the clinical effects of the decrease in provision of protection these fluids normally afford (reviewed in Delaleu et al., 2005; Mavragani et al 2007; Thanou-Stavraki & James, 2008). Good oral hygiene and preventative dental care is important to minimize damage to the teeth by caries, and mucosal infections, particularly candidiasis. Fluoridated dentrifices, varnishes or gel treatments help reduce caries incidence. The common symptoms of dry mouth and/or dry eyes are treated with a variety of fluids to act as saliva and tear substitutes and provide lubrication. For the oral cavity, mechanical stimulation of secretion using sugar-free chewing gum can be beneficial. Where possible, the use of pharmacologic agents that cause dryness, such as antihistamines, should be avoided. Commercial substitute fluids differ primarily in the agent used to provide viscosity (e.g., methylcellulose, sodium hyaluronate), the preservative, and the form (e.g., gel, solution, spray, etc.) (e.g., Alpöz et al., 2008, and references therein).

The second line of approach to treating SS is to stimulate the remaining glandular tissue to produce more fluid secretion using cholinergic agents, particularly pilocarpine or cievmaline, which are muscarinic receptor agonists. Although effective, these drugs have significant side effects, including headaches, nausea and excessive sweating, to an extent that patients often cease their use (Johnson et al., 1993).

Despite the involvement of inflammatory infiltrates in SS, topically applied steroid and non-steroidal anti-inflammatory agents are of relatively little value for dry eye treatment (Samarkos & Moutsopoulos, 2005). However, topical cyclosporin A has been shown to relieve objective and subjective symptoms of ocular dryness. Thus far, attempts to use systemic immunomodulatory agents (e.g., methotrexate) have met with little success in objective improvement of salivary or lacrimal gland flow rates (reviewed in Mavragani et al., 2007; Thanou-Stavraki & James, 2008). Similarly, systemic pharmacological inhibitors of TNF- α (e.g., infliximab, etanercept) have failed to provide any clinically useful benefit (Mathews et al., 2008; Motsopoulos et al., 2008). Collectively, strategies designed to broadly and relatively non-specifically intervene in the (auto)immune component of SS have not yet provided a treatment.

Pathogenesis of SS

A third—and in principle best—approach to treating SS would be to restore normal function in the affected glands. This strategy is impeded by our current lack of a detailed understanding of the pathogenesis of SS.

The exocrine glands affected in SS all have the same general architecture and mode of operation (Redman, 2008). In simplified terminology, a progressively more finely branched network of ducts terminates in secretory endpieces, or acini. Acini are clusters of cells that release secretory proteins and inorganic cations (primarily chloride and bicarbonate ions) into the lumen of the acinus in response to autonomically regulated signaling. The elevated negative ion concentration draws counterbalancing sodium ions through the interstitial spaces.

The increased osmotic pressure thereby draws in water, augmented by aquaporin transporters (Kontinen *et al.*, 2006; Matthews *et al.*, 2008). As the fluid passes through the ductal system, the ion composition is modified, primarily by absorption of ions, so a hypo-osmotic, protein-rich fluid is gathered in the main collecting ducts and delivered to the relevant surface.

Early studies of SS focused on the observation that secretory glandular tissue appeared to be replaced with fibrotic tissue in association with lymphocytic infiltration (reviewed in Dickinson *et al.*, 2008). In earlier stages, the infiltrates are primarily periductal, and comprised mainly of activated T cells, while in more advanced stages B cells predominate, accompanied by macrophages and dendritic cells, and the infiltrates extend into the parenchyma (Delaleu *et al.*, 2005; Mitsias *et al.*, 2006a). Further, the rates of apoptosis in SS-affected glands from patients, and in animal models of SS, appeared to be elevated (although reported rates varied considerably)(reviewed in Esch 2001; Dickinson *et al.*, 2008; Nguyen & Peck, 2009). Also, several lines of evidence are consistent with a genetic component to the development of SS, although the specific genes involved remain to be identified (Bolstad & Jonsson, 2002; Scofield, 2009). These earlier observations led to a straightforward model for the loss of fluid production: on a particular genetic background, an autoimmune inflammatory response would begin to target the glands, leading to the lymphocyte foci that would release cytokines and toxic molecules that trigger apoptosis of glandular tissue (reviewed in Dawson *et al.*, 2006a; Dickinson *et al.*, 2008). The progressive loss of secretory cells would lead to the decline in fluid production that characterizes SS. A weakness of this model was exposed by the realization that for many patients (and in animal models of SS), the loss of fluid production greatly exceeded the loss of functional tissue in a gland, strongly indicating that the remaining, histologically more normal, glandular tissue was no longer functioning appropriately; moreover, the extent of apoptosis with respect to loss of glandular function was called into question (Esch, 2001; Dawson *et al.*, 2006a).

An alternative model for the decreased glandular secretion—even after muscarinic activation—was provided by the subsequent discovery of autoantibodies that bind to, but fail to activate, muscarinic receptors (particularly the type 3 receptor most involved in secretion), and which also block activation by agonist (Dawson *et al.*, 2006a, b; Fox, 2005; Nikolov and Illei, 2009). Binding of antibodies in a serum fraction from primary SS patients monospecific for the muscarinic type 3 receptor to the receptor in basolateral membranes of minor salivary gland acinar cells has been demonstrated (Kovács *et al.*, 2008). In this model, an initial trigger event (e.g., a developmental anomaly in the glands, or a viral infection), combined with an appropriate genetic background, leads to presentation of autoantigens to a susceptible immune system. Presentation could perhaps occur as a consequence of a modestly increased level of apoptosis in target cells (Nguyen & Peck, 2009). Salivary gland epithelial cells are themselves antigen-presenting cells (Mitsias *et al.*, 2006b). The resultant autoantibodies, including anti-muscarinic receptor antibodies, help drive the inflammatory response and loss of glandular function, augmented by local inflammatory cytokine production. The result is a self-sustaining feedback loop and eventually, sufficient cell death would lead to an irreversible loss of function.

Another proposed mechanism of inhibition of secretion associated with lymphocytic infiltration is inhibition of neurotransmitter release by pro-inflammatory cytokines such as IL-1 β (Zoukheri *et al.*, 2002 and references therein). This has been documented in several different systems, including normal mouse (BALB/c) lacrimal gland. In labial salivary glands

from primary SS patients, infiltrating mononuclear cells and glandular epithelial cells express high levels of IL-1 β mRNA (Boumba et al., 1995).

A feature of SS glandular exocrinopathy requiring explanation is the association of the characteristic lymphocytic infiltrates with the ducts, rather than the acini (the presumptive major targets for loss of glandular function). The SS-A/Ro antigen is a complex of 52kd and 60 kd proteins (SS-A/Ro 52 and SS-A/Ro 60) with a small cytoplasmic RNA. Expression of both SS-A/Ro 60 and SS-B/La protein (but not SS-A/Ro 52) is elevated in minor salivary gland ductal cells in SS patients (Barcellos et al. 2007). Therefore, dysregulation of autoantigen expression in ductal cells could be involved in targeting the inflammatory response, and possibly in initiation of SS. Supporting this idea, anti-SS-A/Ro 60 autoantibodies were found to commonly occur in preclinical samples from individuals who subsequently developed SLE (in some cases years later), suggesting that autoantibody formation is an early event in SLE pathogenesis, potentially resulting from cross-reaction with Epstein-Barr virus Nuclear Antigen-1 (Heinlen et al., 2010).

Several reports are consistent with activation of genes regulated by type 1 interferons (IFNs) in SS (reviewed in Delaleu et al., 2005; Nikolov & Illei, 2009). In salivary glands, recruited plasmacytoid dendritic cells have been identified as the principal source of IFN α (Gottenberg et al., 2006). One gene upregulated by IFN α is B cell-activating factor (BAFF), which promotes B-cell survival. BAFF has been shown to be upregulated in salivary glands of SS patients, where it is expressed by epithelial, T and B cells (Gottenberg et al., 2006; Daridon et al., 2007). IFN α (as well as viruses and toll-like receptor (TLR) agonists) upregulates BAFF production in human salivary gland ductal cells (HSG cells)(Ittah et al., 2008). This raises the possibility that BAFF might be involved in the association of lymphocytic foci with the ducts and in maintaining the inflammatory response. Consistent with this, a BAFF knock-in mouse that overproduces BAFF develops autoimmune diseases similar to systemic lupus erythematosus and SS (Groom et al., 2002). Etanercept was found to activate the IFN α signaling pathway and increase BAFF levels in SS patients treated with etanercept, which could explain (at least in part) the lack of benefit of this drug in SS (Motsopoulos et al., 2008).

In association with the inflammatory infiltration seen in SS, characteristic abnormalities called lymphoepithelial lesions (formerly epimyoepithelial lesions) develop in the striated ducts of glands (Ihrler et al., 1999; Herrera-Esparza et al., 2008). These lesions are characterized by aberrant proliferation (shown by expression of proliferating cell nuclear antigen (PCNA) and Ki67) and metaplastic differentiation of basally located cytokeratin Ks8.12-positive ductal cells. However, it is not known if these lesions are entirely the result of a response to the lymphocytic infiltration, or if they represent an early event in gland dysregulation that is sustained and perhaps amplified by inflammation.

Normal salivary glands show a moderate rate of cell turnover and replacement (Redman, 2008). The abnormal proliferation in SS glands is reminiscent of abnormal wound healing seen in some other conditions associated with injury and inflammation, and suggests an aberrant regenerative process (Dickinson et al., 2008; Ihrler et al., 2002). Supporting this idea, the REG I α gene was found to be widely expressed in association with proliferative, Ki67-positive ductal cells in minor salivary glands from primary SS patients, but not in primary SS glands with a low proliferation index or in those from normal individuals (Kimura et al., 2009). REG I α is thought to play a role in tissue regeneration; it is likely that

inflammation drives REG I α expression, and *in vitro* the protein promotes epithelial cell proliferation and resistance to apoptosis (Kimura *et al.*, 2009 and references therein).

Normal tissue development, and regeneration and wound healing, involve complex interactions between cells and extracellular matrix (ECM). Adhesion of cells to ECM components *via* cell surface integrin receptors is particularly important (Schultz & Wysocki, 2009). In salivary glands, interaction between acinar cell integrins $\alpha 1\beta 1$ and $\alpha 2\beta 1$ and basement membrane laminin $\alpha 1$ chains is required for acinar maintainance (Hoffman *et al.*, 1996). In SS, the components of this critical signaling system are reduced in acini (Laine *et al.*, 2004, 2008; Konttinen *et al.*, 2006). Interestingly, androgens (particularly DHEA) induce integrin $\alpha 1\beta 1$ and $\alpha 2\beta 1$ expression in salivary gland cells *in vitro* (Porola *et al.*, 2010). Patients with SS have lower serum levels of androgens, and the prohormone DHEA, and abnormalities in salivary gland androgen-processing enzymes required for local sex steroid synthesis (Spaan *et al.*, 2009; Konttinen *et al.*, 2006). Human lacrimal and meibomian glands and corneal and conjunctival epithelial cells also contain the enzymes necessary for local synthesis and metabolism of sex steroids (Schirra *et al.*, 2006), and in murine lacrimal glands, testosterone similarly affects the expression of about 1000 genes in ovariectomized and orchietomized animals (Sullivan *et al.*, 2009). However, a modest number of genes showed sex-specific differences in response to testosterone. That is, at least in mice, there are some genes that respond differently in males and females in response to hormonal changes. This sex steroid-mediated regulation of integrin receptor and their gene expression could explain, at least in part, the female predilection for SS.

Collectively, this evidence is consistent with an intial trigger (such as a viral infection) initiating a series of events leading to dysregulation of gene expression in glandular epithelial cells, and to formation of inflammatory infiltrates that release cytokines; these further dysregulate gene expression. Consequences of these changes and of interaction between these cell populations include apoptosis, autoantigen upregulation and presentation, cell proliferation, and a decline in secretion. Moreover, the effect of androgens on these systems would explain the high female:male ratio of SS incidence. This interpretation would allow glandular abnormalities to precede large-scale lymphocytic infiltration, or infiltration to drive the majority of the abnormalities. However, regardless of the order, a feedback loop would be created between infiltration and dysregulation, driving disease progression.

Animal Models for SS Pathogenesis

Several mouse models for SS have been developed (recently reviewed in Chiorini *et al.*, 2009; Jonsson *et al.*, 2007; Lee *et al.*, 2009; Nguyen & Peck, 2009). Although it is important to acknowledge the fact that an animal model may not be an exact version of human SS (and indeed, cannot be established as such until we know the etiology of the human disease), nonetheless, important clues to potential mechanisms underlying SS exocrinopathy can be gleaned that can then be examined in human tissues. Further, animal models can be used to test novel potential treatment strategies targeting different candidate mechanisms of pathology. An important advantage of animal models is they allow disease progression to be followed from its inception; this could augment the development of early salivary markers for diagnosis considerably.

All mouse models are characterized by some form of glandular exocrinopathy, but differ in the presented combination of lymphocytic infiltration, formation of autoantibodies (including ANA and antimuscarinic receptor antibodies), and impairment of secretion. The non-obese diabetic (NOD) mouse and its genetic derivatives are the most commonly used mouse models for SS (reviewed in Lee et al., 2009). The NOD mouse develops autoimmune type I diabetes, and an SS-like exocrinopathy characterized by glandular lymphocytic foci, autoantibodies (including ANA and anti M3R antibodies), and loss of secretion. Early signs of periductal inflammation are seen in some animals by 8 weeks of age, and by 17 weeks in all animals (Jonsson et al., 2006). However, the decline in secretion occurs between 17 and 24 weeks; that is, after the infiltrates develop, and correlating with an increase in salivary TNF α levels (Jonsson et al., 2006). The NOD strain also shows elevated apoptosis, which persists in NOD-scid mice that lack T and B lymphocytes and do not develop inflammatory infiltrates, indicating an underlying abnormality in the glandular epithelial cells (Kong et al., 1998). Consistent with the weak association of human SS with major histocompatibility (MHC)-associated genes, exchange of the NOD MHC I-A g7 type I diabetes susceptibility locus with the C57BL/10 MHC I-A b non-susceptibility locus does not affect the SS-disease phenotype in the resultant NOD.B10-H2b mice, but they do not develop the insulinitis or type I diabetes that can complicate interpretation of results in NOD mice (Wicker et al., 1992; Robinson et al., 1998).

A limitation to the use of NOD and NOD.B10-H2b strains is their complex genetic background, resulting in a lack of a suitable normal congenic control. Transfer of two susceptibility loci (*Idd3*, *Idd5*) onto a C57BL/6 background produced the C57BL/6.NOD-Aec1Aec2 strain (where *Aec1* corresponds to *Idd3* and *Aec2* corresponds to *Idd5*) that retains the SS-like phenotype (Brayer et al 2000). This strain also shows salivary gland abnormalities, such as increased apoptosis and expression of matrix metalloproteinases, which precede formation of foci and loss of secretion, again consistent with the idea that gland dysfunction can precede, and perhaps initiate, the subsequent autoimmune response (Killedar et al., 2006). Importantly, NOD mice show proliferation of glandular ductal and acinar cells, as seen in human primary SS salivary glands (Dickinson et al., 2008; Gillespie et al., 2008).

In this strain, T_H17 effector cells have been implicated in tissue destruction in glands through secretion of potently pro-inflammatory IL-17 cytokines that can induce expression of other molecules involved in inflammation, including pro-apoptotic TNF- α , matrix metalloproteinases, and the signaling molecule nitric oxide (NO)(reviewed in Lee et al., 2009). T_H17 cells have been shown to be important in other autoimmune diseases (Korn et al., 2009).

NZB/NZW F1 females spontaneously develop autoimmune diseases with characteristics of SLE and SS, including lymphocytic foci in salivary glands, the presence of SS-A and ANA autoantibodies in the serum, and a decrease in secretion. However, when treated with poly(I:C), the animals develop a rapid but reversible loss of salivary function in the absence of inflammatory infiltrates (Deshmukh et al., 2009). Poly(I:C) mimics a viral infection and activates Toll-like receptor 3 (TLR3). TLR3 is expressed by murine salivary gland ductal and acinar epithelial cells. Activation of TLR3 leads to activation of NF- κ B and interferon regulatory factor 3 signaling pathways, and to production of type I interferons (Alexopoulou

et al., 2001; Matsumoto & Seya, 2008), consistent with the upregulation of type I IFN responsive genes observed in SS salivary glands (see above).

In further agreement with the lack of correlation between infiltration and loss of function, the MRL/lpr mouse model develops a periductal lymphocytic infiltration of salivary and lacrimal glands. However, it fails to develop anti M3R autoantibodies and retains glandular secretory function, consistent with a role for M3R antibodies in loss of secretion and also demonstrating that lymphocytic infiltration alone does not cause a decrease in secretion. Similarly, NOD.IL4^{-/-} and NOD.B10-H2b.IL4^{-/-} IL4 knockout mice develop lymphocytic infiltration of their salivary glands, but do not develop secretory dysfunction (Gao et al., 2006). This may be explained by their failure to develop IgG1 isotype M3R autoantibodies.

These models, in combination with human data, have led to the development of a concept for SS autoimmune exocrinopathy involving three main stages (Dawson et al., 2006a; Delaleu et al., 2005; Looney et al., 2007; Lee et al., 2009). In the first stage, a combination of genetic factors, perhaps in association with environmental factors, leads to glandular abnormalities promoting expression and presentation of autoantigens, perhaps associated with a modest increase in apoptosis. One possible trigger in humans is viral infection and activation of TLR3. In the second stage, leucocytes are recruited to the ductal regions of the gland, leading to local production of inflammatory cytokines that further alter glandular function. In the third clinical phase, production of anti-M3R autoantibodies contributes to a decline in secretion, compounded by cell death (not necessary by apoptosis) associated with IL17 release by T_H17 cells.

Dysregulation of Glandular Epithelial Cells: a Potential Role for Reactive Oxygen and Nitrogen Species

Reactive oxygen species (ROS) such as the superoxide anion (O_2^-), and reactive nitrogen species (RNS) such as nitric oxide (NO), are important multifunctional molecules involved in a range of normal physiological functions, including cell signaling and inflammation (Valko et al., 2007; Veal et al., 2007). However, being for the most part highly reactive radicals, high levels of ROS or RNS can damage cellular components; this damage is referred to respectively as oxidative and nitrosative stress. Cells normally tightly control the levels of these molecules by regulation of their production and of their removal by antioxidants and enzymes. Therefore, stress is the result of an imbalance between these processes. ROS and RNS have been implicated in a variety of pathologies, including autoimmune diseases, and recently, attention has focused on a role for ROS and RNS in the development of SS.

Superoxide in cells is produced mostly by the mitochondria as a result of premature “leakage” of electrons to oxygen, but it is also produced by xanthine oxidase during purine catabolism and by NAD(P)H oxidases (Valko et al., 2007). During the respiratory burst, neutrophils and macrophages release toxic amounts of O_2^- generated by an NAD(P)H oxidase activated in response to inflammatory signaling. Peroxisomes produce hydrogen peroxide (H_2O_2), which can form the hydroxyl radical (OH) under certain conditions.

Glutathione (GSH) and glutathione disulfide (GSSG, the oxidized form) represent the main redox buffer system in cells, and together with the protein thioredoxin (TRX), provide

protection against oxidative stress. The high ratios of reduced to oxidized GSH and TRX are maintained by GSH and TRX reductases, respectively. There are several enzyme antioxidants in cells: superoxide dismutase (SOD) converts O_2^- to H_2O_2 , which in turn is cleaved by glutathione peroxidase (GPX)(using GSH as an electron donor) to oxygen and water. Catalase catalyzes the same reaction.

Nuclear factor- κ B (NF κ B) is a transcription factor that regulates the expression of a variety of genes mediating cellular responses involved in stress response, inflammation, innate immunity, and growth (Wan & Lenardo, 2010). It is retained in the cytoplasm in a latent form by association with a member of the I κ B protein family. Activation of the I κ B kinase complex (IKK) by a variety of pathways results in phosphorylation of I κ B proteins and their targeting for degradation by the proteasome. The released NF κ B traffics to the nucleus, and activates a complex set of genes. NF κ B is also a redox sensing factor, and antioxidants (e.g. N-acetylcysteine) suppress its activation (Suzuki & Packer, 1993; Beauparlant & Hiscott, 1996). Thus, increased levels of ROS in SS as a result of inflammation could lead to activation of NF κ B in glandular epithelial cells, and expression of genes downstream (see below).

Consistent with a role for oxidative stress in SS, chemical signatures for ROS damage to DNA, lipids and proteins were observed in labial salivary gland ductal cells from patients with SS, but not in healthy controls (Kurimoto *et al.*, 2007). Presumably as a protective response to oxidative stress, levels of TRX were also increased in SS ductal cells; importantly, salivary flow rates were found to vary inversely with TRX levels, suggesting that the decrease in flow rate was coupled to increased oxidative stress (Kurimoto *et al.*, 2007). In addition to the inflammatory infiltrate, a second source of oxidative stress in glandular epithelium could be alteration in levels of pro- and anti-oxidant enzymes: in the conjunctival epithelium of patients with SS, xanthine oxidase levels are increased, and levels of SOD, catalase and GPX decreased (Cejkova *et al.*, 2009). Interestingly, SS-A/Ro 52, an autoantigen in SS and SLE with an as yet unknown function, is translocated from the cytoplasm to the nucleus in response to hydrogen peroxide treatment (*via* ERK MAPK pathway signaling), suggesting it might be a component in an oxidative (hydrogen peroxide) stress response (Nobuhara *et al.*, 2007).

NO is synthesized from L-arginine by nitric oxide synthase enzymes (Murad 1998, 2006). There are 3 isoforms: eNOS (NOSIII) is constitutively expressed in vascular endothelium (where NO is involved in vasodilation); nNOS (NOSI) in nerves (where NO is involved in neural signaling); and iNOS (NOSII). iNOS is an inducible enzyme expressed in different cell types in response to pro-inflammatory (and other) signals, such as IL-1 and TNF α , acting (in part) through NF- κ B activation (Bryan *et al.*, 2009). In macrophages and neutrophils, full iNOS induction leads to release of cytotoxic and microbicidal amounts of NO. NO also reacts very rapidly with the O_2^- that is released during the respiratory burst to form the highly toxic peroxynitrite anion (ONOO $^-$)(Valko *et al.*, 2007). The effects of NO on cells are mediated primarily (but not exclusively) by activation of guanylate cyclase and production of the second messenger cGMP.

In aqueous solution, NO reacts rapidly to form nitrite (NO_2^-) and nitrate (NO_3^-), which can be readily measured as surrogate markers for NO production. Serum and salivary nitrite levels are elevated in patients with SS, consistent with increased NO production (Konttinen *et al.*, 1997; Wanchu *et al.*, 2000). iNOS expression, and a substantial increase in NO

production, is induced in cultured rabbit lacrimal gland acinar cells by IL-1 β in cultured lacrimal acinar cells (Beauregard et al., 2003).

NO has a biphasic effect on mouse and human salivary acinar cells (Caulfield et al., 2009). Brief exposure to NO causes a typical guanylate cyclase mediated increase in cGMP, leading to an amplification of an acetylcholine-stimulated intracellular Ca²⁺ signal. However, more prolonged exposure to NO (as would be the case in SS), reduced the induced Ca²⁺ signal significantly by a cGMP-independent mechanism. This could explain, at least in part, why SS minor salivary glands are less sensitive to stimulation.

The induction of nitric oxide formation by pro-inflammatory cytokines is influenced by the levels of sex steroids: androgens, DHEA, and 17 β -estradiol inhibit IL-1 β induction of NO production in rabbit lacrimal gland acinar cells (Beauregard et al., 2004). This effect could also contribute to the marker bias for occurrence of SS in females.

Proteasome Defects in SS

The proteasome is involved in regulation of various cell cycle and cell death regulators, such as p53 and p27^{Kip}. I κ B α is targeted to the proteasome during activation of NF κ B, which also requires proteasome mediated cleavage of a precursor protein for subunit formation. Minor salivary glands from patients with SS show a significant increase in proteasomal LMP7 (β 5i) catalytic site subunit expression relative to normal glands (Egerer et al., 2006) suggesting alterations in glandular epithelial proteasomal processing occur in SS.

The proteasome is also involved in antigen processing, and the proteasomal LMP2 (β 1i) catalytic site subunit is essential for the formation of self-peptides for T cell self-tolerance. NOD mice have a defect in the production of LMP2 protein, and as a result they have a defect in NF- κ B signaling and have autoreactive T cells (Hayashi & Faustman, 1999). Remarkably, injection of NOD mice with matched normal splenocytes and complete Freund's adjuvant led to a complete recovery of salivary fluid flow (Tran et al., 2007). The basis of this strategy is reselection of naïve pathogenic T cells by display of self peptides on normal splenocytes, combined with TNF α induced killing of activated pathogenic T cells, which are more susceptible to apoptosis due to the proteasomal mediated dysregulation of NF- κ B signaling. This argues strongly for a role of T cells in the pathogenesis of SS-like disease in the NOD mouse.

Interestingly, patients with SS show altered expression of the proteasomal catalytic site BMP2 (β 1i) subunit in peripheral blood monocytes and in minor gland lymphocytic infiltrates, consistent with a proteasomal abnormality underlying at least some aspects of SS pathogenesis (Hjelmervik et al., 2005; Krause et al., 2006; Morawietz et al., 2009).

Potential Future Treatments

Advances in our understanding of SS and affected tissues, and other related autoimmune disorders such as SLE, have led to the investigation of several novel strategies as potential avenues to clinical treatment of SS. Since B lymphocytes, and their excessive activation, as

well as different T lymphocyte populations, have been implicated in the complex immune response observed in both SS patients and animal models (Looney, 2007; Nguyen & Peck, 2009), most strategies focus on components of the immune system, and seek to systemically down-regulate activity of T and/or B cells (Perl, 2009; Tobón *et al.*, 2010). The majority are still in the early stages of testing.

Mizoribine is an immunosuppressant used to control graft rejection. It is a competitive inhibitor of inosine monophosphate dehydrogenase (an enzyme in *de novo* purine biosynthesis), and has suppressive effects on both B and T lymphocytes, such as reducing proliferation. It has been tested in an open label human clinical trial for safety and efficacy as a treatment for SS (Nakayamada *et al.*, 2007). Over a 16 week treatment period, there was a significant improvement in the volume of the salivary secretion and in the patients and investigators assessments of dry mouth and dry eyes. However, 30.5% of the patients reported an adverse drug reaction, and treatment was discontinued in 10% (6/59 enrolled).

Mycophenolic acid is another inhibitor of inosine monophosphate dehydrogenase that is an effective treatment for SLE and other autoimmune disorders, and has been tested in a small open-label pilot study for efficacy in primary SS patients refractory to other immunosuppressive agents (Willeke *et al.*, 2007). The drug caused a significant reduction in hypergammaglobulinemia over the 6 month study period, but overall did not produce an improvement in salivary or lacrimal gland function in the patient cohort. However, a substantial improvement in glandular function was seen in two patients with a disease duration under 3 years, perhaps due to retention of more functional tissue (although histology was not examined). Alternatively, B cells may only be needed in the early stages of the disease, until long-lived plasma cells and autoimmune T cells have developed (Looney, 2007).

Rituximab is a human/murine chimeric monoclonal antibody that binds to the CD20 transmembrane protein on B cells, leading to a major depletion of peripheral B cells through several potential mechanisms, including induction of apoptosis (reviewed in Gürcan *et al.*, 2009; Tobón *et al.*, 2010). It has been the subject of several small clinical trials to assess safety and efficacy for the treatment of primary SS (Pijpe *et al.*, 2009; reviewed in Looney, 2007; Gürcan *et al.*, 2009; Tobón *et al.*, 2010). Rituximab provides a modest improvement in objective measures of salivary function in a subset of patients with either early disease (as seen for mycophenolic acid) or a less severe loss of glandular function, but many patients showed no objective improvement, although the subjective patient assessment of treatment indicated marked improvement. A disadvantage is that it must be delivered by infusion. However, rituximab could have benefit for patients who have extraglandular symptoms related to B cell activity. Interestingly, rituximab treatment also reduced the elevated acinar cell proliferation seen in primary SS parotid biopsy samples to near normal, and caused a reduction or even disappearance of the lymphoepithelial lesions, consistent with a role for B lymphocytes in promoting abnormal cell proliferation (Pijpe *et al.*, 2009). Further, these observations suggest that this aspect of epithelial cell behavior could be normalized under appropriate conditions.

An obvious concern with systemic immunosuppressive agents is the increased risk of infection. Indeed, 30% of patients treated with rituximab for lymphoma develop an adverse infectious event, of which 4% are serious (Kimby, 2005). In addition, infusion related reactions are relatively common, and can be severe in about 10% of patients. The benefits of

rituximab for cancer treatment far outweigh the risks, but it is less clear if that is the case for treating SS.

As noted above, muscarinic receptor agonists are routinely used to enhance glandular secretion in SS patients. Nizatidine may offer a complementary approach. This drug is an H₂ receptor antagonist that also appears to inhibit acetylcholinesterase, thereby increasing the availability of acetylcholine for signaling through muscarinic receptors in salivary glands: it has been shown to stimulate salivary secretion in healthy volunteers (Adachi et al., 2002). In a preliminary open-label clinical trial, the drug was well tolerated without adverse effects, and provided a significant increase in salivary secretion and some improvement in subjective reports of symptoms over the 8 week treatment period (Kasama et al., 2008). Famotidine, another H₂ receptor antagonist, failed to provide an improvement, suggesting the benefits of Nizatidine were not due to a placebo effect.

If indeed glandular abnormalities precede the major clinical manifestations of autoimmune disease, and are sustained by inflammation, then one alternative strategy to treat SS would be to attempt to normalize the biology of glandular epithelial cells, thereby reducing the trigger event(s) and breaking feedback loops promoting disease progression. One approach would be to target signaling pathways mediating glandular dysregulation. At present, our knowledge of these pathways is rudimentary, but we have some potential pharmacological clues. Peroxisome proliferator-activated receptor (PPAR) α and γ agonists have been shown to partially inhibit NO production in cultured rabbit lacrimal gland acinar cells treated with IL-1 β (Beauregard & Brandt, 2003). Given that cytokine-induced NO production seems to be involved in glandular signal dysregulation and/or damage (see above), it would be of interest to test PPAR agonists in mouse models for SS.

Evidence from the study of green tea polyphenols (GTPs) suggests a different approach to modulation of exocrinopathy in SS. GTPs (also known as green tea catechins) are a group of polyphenols present in the leaves of *Camellia sinensis*. The leaves of this plant are used to make tea. This beverage is consumed by over two thirds of the world's population, attesting to the relative lack of toxicity of GTPs. Epidemiological evidence shows an inverse relationship between green (i.e., unoxidized leaf) tea and rheumatoid arthritis, and a body of *in vitro* and *in vivo* experimental data demonstrate a range of anti-inflammatory effects for GTPs, of which the most potent is (-)-epigallocatechin-3-gallate (EGCG)(reviewed in Hsu & Dickinson, 2006). For example, EGCG effectively inhibits IL-1 β induction of MMP-1 and MMP-13 in human chondrocytes, and inhibits this cytokine's pro-inflammatory signal transduction in respiratory epithelial cells, mediated in part by GTP modulation of the MAPK pathways (Singh et al., 2003; Ahmed et al., 2004; Wheeler et al., 2004). In macrophages, EGCG blocks NF κ B activation, thereby reducing production of TNF α (an important mediator of inflammation) as well nitric oxide synthase iNOS gene expression; in mice, GTPs (*via* gavage) will completely inhibit LPS-induced death (Yang et al., 1998; Lin & Lin, 1997). In the MRL/lpr mouse model for SLE, GTPs (fed as a green tea powder diet) prolonged survival and reduced levels of anti-DNA antibodies (Sayama et al., 2003). In rats with chronic lipopolysaccharide-induced inflammation, dietary GTPs reduced bone loss, perhaps due to the observed inhibition of TNF α expression (Shen et al., 2010). Consistent with these anti-inflammatory effects, in the mouse NOD model dietary EGCG reduces the size of the lymphocytic infiltrates in the earlier (16 week) but not later (22 week) stages of the clinical

disease (Dickinson *et al.*, 2008; Gillespie *et al.*, 2008). It would be of interest to examine the effects of EGCG on BAFF expression in the NOD mouse submandibular gland.

GTPs also have anti-apoptotic effects. *In vitro*, EGCG has been shown to protect human salivary acinar and duct-derived cells from radiation and chemotherapeutic drug-induced apoptosis (Yamamoto *et al.*, 2004), and to protect human salivary acinar-derived cells from TNF α -induced cytotoxicity (Hsu *et al.*, 2007). In the latter case, protection was blocked by inhibition of the p38 MAPK pathway. EGCG has also been shown to be an effective inhibitor of TNF α -mediated activation of the I κ B kinase complex (IKK) in intestinal epithelial cells (Yang *et al.*, 2001). IKK phosphorylates I κ B α (a member of the I κ B family of proteins that act as inhibitors of NF κ B), leading to activation of NF κ B. Therefore, EGCG could also reduce the response to inflammatory signals in epithelial cells through inhibition of NF κ B activation.

Despite these anti-apoptotic effects, in the submandibular gland of the 16 and 22 week old NOD mouse (when clinical signs of SS have manifested), EGCG has no significant effect on the modest levels of apoptosis (relative to BALB/c controls) seen in the submandibular gland parenchyma, although it does reduce apoptosis in the lymphocytic infiltrates (Dickinson *et al.*, 2008; Gillespie *et al.*, 2008). The identity of the cells in the infiltrates undergoing apoptosis has not yet been established; it is possible that at least some are acinar cells, and that EGCG protects them from apoptosis induced by local lymphocytes. This remains to be tested.

GTPs have several other effects relevant to SS. Significantly, treatment with 100 μ M EGCG (a concentration physiologically achievable by oral administration) inhibited transcription and translation of major autoantigens, including SS-B/La, SS-A/Ro and fodrin, in normal human primary epidermal keratinocytes and a human salivary acinar cell-derived cell line (Hsu *et al.*, 2005). Of particular interest, in the NOD mouse model dietary EGCG reduces the abnormal proliferation of ductal and acinar cells to near normal (BALB/c mouse control), as determined by Ki67 and PCNA immunostaining (Dickinson *et al.*, 2008; Gillespie *et al.*, 2008). The effects of EGCG on expression of the REG I α protein or salivary gland integrins, proteins associated with the abnormal proliferation in SS, have not been reported.

Yet more complexity is added to the potential actions of GTPs by the discovery that EGCG is a potent inhibitor of the chymotrypsin-like activity of the 20S proteasome subunit (reviewed in Shay & Banz, 2005). Treatment of Jurkat and prostate cancer cells with EGCG caused accumulation of p27 Kip and I κ B α , and G1 growth arrest (Nam *et al.*, 2001). This mechanism may explain the decrease in cell proliferation seen in the NOD mouse model. It is possible that EGCG could reduce lymphocytic infiltration in the NOD mouse salivary glands through an effect on lymphocyte proteasomes, and may modulate epithelial cell gene expression the same way.

GTPs are potent antioxidants. Given the potential role of ROS in autoimmunity and glandular dysfunction, this could represent an additional mode of action, perhaps *via* an effect on NF- κ B signaling. However, it seems unlikely it is a dominant mechanism behind the beneficial effects of GTPs in the NOD mouse model, as clinical trials of other antioxidants (N-acetylcysteine, vitamin C) for SS treatment have shown little, if any, benefit (Walters *et al.*, 1986; McKendry, 1982). The effects of GTPs (direct or indirect) on NO production in salivary glands have not been assessed, although it is anticipated that it would be reduced.

Overall, GTPs represent a category of agents that have little, if any toxicity, and a broad spectrum of effects consistent with a normalization of glandular tissue in the NOD model for SS (at least in earlier stages of the disease), including reduction in autoantigen expression and abnormal proliferation, and a temporary reduction in lymphocytic infiltration. As yet, clinical trials of GTPs for the treatment of SS have not been reported.

GTPs mediate some of their beneficial effects *via* p38 MAPK signaling. However, this signaling pathway is also involved in immune cell function. Based on this, p38 inhibitors have been developed as potential immunomodulators. Unfortunately, the outcomes of clinical trials have largely been disappointing, perhaps because it is not just the immune cells that are dysregulated in disease, but also the target tissues, and the latter require p38 MAPK signaling for normal function (reviewed in Hsu and Dickinson, 2009).

These candidate approaches to treating SS all rely on the existence of sufficient remaining functional glandular tissue to provide adequate fluid production (and emphasizing the need for the development of tools for early diagnosis of SS). How might late stage patients with extensive loss of acinar tissue be treated? Regenerative medicine seeks to replace lost tissue by the use of cells having an appropriate capacity for proliferation and differentiation (e.g., a suitable stem cell), either by transplantation into injured tissue, or in combination with growth factors and mechanical scaffolds. At present, a number of animal studies suggest this approach is plausible (reviewed in Kagami et al., 2008; Redman, 2008), but we need to know more regarding the biology of salivary gland stem cells, and it is uncertain if the cost and potential risks (e.g. if immunosuppressive drugs are required) warrant use of this treatment for a generally non-lethal disease.

Summary

Progress has been made in our understanding of the remarkably complex and interacting mechanisms underlying development of SS and glandular exocrinopathy. However, we still lack information on early events that initiate disease, although viral infection, epithelial cell dysregulation (perhaps involving ROS and NOS signaling systems required for normal function, as well as NF- κ B and MAPK pathways), apoptosis, and autoantigen upregulation and presentation (in part determined by the genetic background) are all likely to be involved. Knowledge of early events could provide the basis for improved diagnostic tests that would allow early intervention, and perhaps a better prognosis, particularly if a genetic susceptibility profile could be developed that would identify at-risk individuals. The advent of low cost whole genome sequencing will facilitate this. Current treatments are primarily palliative, and strategies based on global immunosuppression will have to balance risks against benefits. The observation that benign natural products such as green tea polyphenols can have broad beneficial effects, at least in animal models for SS, raises the possibility that agents can be identified that normalize glandular epithelial function efficiently. In conjunction with other approaches, a treatment for SS with minimized side-effects appears to be biologically possible.

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Chapter 6

Systemic Lupus Erythematosus: Definition, Pathogenesis and Treatment

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Abstract

Systemic lupus erythematosus (SLE) is a multisystemic disease characterized by profound alterations of the immune system that contribute to inflammation and tissue damage. The diverse presentations of SLE range from rash and arthritis through anemia and thrombocytopenia to serositis, nephritis, seizures, and psychosis. SLE should be part of the differential diagnosis in virtually any patient presenting with one of these clinical problems, especially in female patients between 15 and 50 years of age. Since 90% of patients with SLE are female, an important role for sex hormones seems likely, but a protective role for male hormones is also possible. Pathogenic auto-antibodies are the primary cause of tissue damage in patients with SLE. The production of these antibodies arises by means of complex mechanisms involving every key facet of the immune system. There are no diagnostic criteria, only classification criteria. In order to consider SLE for research practice, the patient must present at least four of 11 criteria established by the American College of Rheumatology (ACR). Many different elements of the system are potential targets for therapeutic drugs in SLE. The treatment involves immunosuppressive medications like high-dose of corticosteroids, azathioprine, and cyclophosphamide. Mycophenolate mofetil and rituximab have been used in association with corticosteroids (oral or intravenous pulse therapy) in SLE patients. Besides, anti-malarial medications are used not only to control disease, but also to improve survival

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and to reduce the risk of thrombosis. The aim of this review is to describe symptoms of SLE that may vary from rash and arthritis through seizures, and psychosis. We will also include a review on current treatment and new medications.

Keywords: SLE, pathogenesis, diagnosis, treatment.

Introduction

Systemic lupus erythematosus (SLE) is a chronic, multisystemic autoimmune disease. The characteristic tissue damage is a result from antibody and complement-fixing immune complex deposition. The pathogenic immune responses probably result from environmental triggers acting in the setting of susceptibility genes (1).

Pathogenic autoantibodies are the primary cause of tissue damage in patients with SLE. The production of these antibodies arises by means of complex mechanisms involving every key facet of the immune system. Many different elements of the system are potential targets for therapeutic drugs in patients with lupus (2).

In this review we will describe symptoms of SLE that may vary from rash and arthritis through seizures, and psychosis. We will also include a review on current treatment and new medications.

Incidence and Prevalence of SLE Around the World

Incidence rates of SLE range from approximately 1 to 10 per 100, 000 person-years (Table 1) and prevalence rates generally range from 20 to 70 per 100, 000 inhabitants (3). Childhood incidence and prevalence rates of SLE are considerably lower than adult rates. The annual incidence rate of SLE in children (<16 years) was less than 1 per 100, 000 persons in studies from Europe and North America (4). In Taiwan, the prevalence of childhood SLE was estimated as 6.3 per 100, 000 (5).

SLE has been described in Europe, North America, South America, Africa, Asia, and Australia. It is rare in Africa but common in African descendants around the world; however, some degree of under-ascertainment cannot be ruled out (6). Incidence and prevalence rates in Africans or Asians are approximately 2 to 3 times higher than in white populations (7-12). The disease is also more common among Aboriginal than non-Aboriginal Australians (13, 14), and in some First Nations or Native American groups in Canada (16) and the United States (15).

Sle Classification Criteria

The American College of Rheumatology (ACR) classification criteria was described in 1982. In 1997, the immunologic disorder criterion was revised by a committee (Table 2) (17).

Although widely accepted and used, these criteria have their limitations. One of the most important laboratory tests, for example, hypocomplementemia, is not included in any classification criteria. In addition these criteria have been validated against other rheumatic diseases and not against infectious or neoplastic disease. New criteria are needed, emphasizing weighting or recursive partitioning (18). There are no validated diagnostic criteria for SLE, however the classification criteria are often used for diagnosis.

Table 1. Incidence of SLE in Adults, by location, in Studies Spanning 1975 to 2000

Author (Reference), Study Period, Country	Total Rate per 100, 000 per year (n)	Female Rate per 100, 000 per year (n)
Uramoto (30), 1980 to 1992, United States (Minnesota)	5.6 (48)	9.4 (42)
McCarty (7), 1985 to 1990, United States (Pennsylvania)	2.4 (191)	African Americans 9.2 (45) Whites 3.5 (129)
Naleway (133), 1991 to 2001, United States (Wisconsin)	5.1 (44)	8.2 (36)
Peschken (9), 1980 to 1996, Canada (Manitoba)	First Nations ~3.5 (49) Whites ~ 1.2 (177)	Uninformed
Nossent (134), 1980 to 1989, Curaçao	Afro-Caribbean 4.6 (68)	7.9 (60)
Vilar (135), 2000, Brazil	8.7 (43)	14.1 (38)
Stahl-Hallengren (136, 137), 1981 to 1991, Sweden	1981-86 4.5 (38) 1987-91 4.5 (41)	1981-86 5.4 (32) Uninformed
Voss (138), 1980 to 1994, Denmark	1980-84 1.0 (—) 1985-89 1.1 (—) 1990-94 2.5 (—)	Uninformed Uninformed Uninformed
Nossent (139), 1978 to 1996, Norway	2.9 (83)	5.1 (73)
Johnson (8), 1991, United Kingdom (Birmingham),	Total 3.8 (33) Uninformed Uninformed Uninformed	Total 6.8 (31) Afro-Caribbean 22.8 (6) Asian 29.2 (8) Whites 4.5 (17)
Hopkinson (9, 140), 1989 to 1990, United Kingdom (Nottingham)	Total 4.0 (23) Afro-Caribbean 31.9 (3) Whites 3.4 (19)	Total 6.5 (19) Uninformed Uninformed
Nightingale (141), 1992 to 1998, United Kingdom	3.0 (390)	5.3 (349)
Somers (142), 1990 to 1999, United Kingdom	4.7 (1638)	7.9 (1374)
Gudmundsson (143), 1975 to 1984, Iceland	3.3 (76)	5.8 (67)
López (144), 1998 to 2002, Spain	2.2 (116)	3.6 (102)
Alamanos (145), 1982 to 200, 1 Greece	1.9 (178)	Uninformed
Anstey (146), 1986 to 1990, Australia	Aboriginal 11 (13)	Uninformed

Table 2. The 1997 revised criteria for the classification of SLE (17)

Criterion		Definition
1	Malar rash	Fixed malar erythema, flat or raised
2	Discoid rash	Erythematous-raised patches with keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3	Photosensitivity	Skin rash as an usual reaction to sunlight, by patient history or physician observation
4	Oral ulcers	Oral or nasopharyngeal ulcers, usually painless
5	Arthritis	Nonerosive arthritis involving two or more peripheral joints, characterized by tenderness, swelling or effusion
6	Serositis	a. Pleuritis (convincing history of pleuritic pain or rub heard by physician or evidence of pleural effusion) OR b. Pericarditis (documented by ECG, rub, or evidence of pericardial effusion)
7	Renal disorders	a. Persistent proteinuria ($>0, 5\text{g/d}$ or $>3+$) OR b. Cellular casts of any type
8	Neurologic disorder	a. Seizures (in the absence of other causes) OR b. Psychosis (in the absence of other causes)
9	Hematologic disorder	a. Hemolytic anemia OR b. Leukopenia ($<4, 000/\text{mL}$ on two or more occasions) OR c. Lymphopenia ($<1, 500/\text{mL}$ on two or more occasions) OR d. Thrombocytopenia ($<100, 000/\text{mL}$ in the absence of offending drugs)
10	Immunologic disorder	a. Anti-double-stranded DNA OR b. Anti-Smith OR c. Positive finding of antiphospholipid antibodies based on (1) an abnormal serum level of IgG or IgM anticardiolipin antibodies, (2) a positive test result for lupus anticoagulant using a standard method, or (3) a false-positive serologic test for syphilis known to be positive for at least 6 months and confirmed by <i>Treponema pallidum</i> immobilization or fluorescent treponemal antibody absorption test
11	Antinuclear antibody (ANA)	An abnormal titer of ANA by immunofluorescence or an equivalent assay at any time and in the absence of drugs known to be associated with "drug-induced lupus syndrome".

Clinical Presentation

The diverse presentations of SLE range from rash and arthritis through anemia and thrombocytopenia to serositis, nephritis, seizures, and psychosis. SLE should be part of the differential diagnosis in virtually any patient presenting with one of these clinical problems, especially in female patients between 15 and 50 years of age (2).

Constitutional symptoms such as fatigue, weight loss and fever are not life threatening, but have a significant impact on quality of life. Patients with SLE describe overwhelming fatigue and unsatisfying sleep, though the extent to which this tiredness relates directly to lupus disease activity remains controversial (19).

Skin involvement in lupus is also very common, affecting up to 90% of patients. In addition to the classic rash malar and discoid rashes, more generalized photosensitivity is often present. Alopecia is often transient associated with disease activity, but can be scarring when associated with discoid lesions. Recurrent mouth ulcers, especially in the soft palate are also a feature of active disease.

Other oral manifestations include reduced salivary and ocular gland flow, resulting in dry eyes and/or mouth as a result of secondary Sjogren's syndrome (20).

Arthralgia and myalgia occur in the majority of the patients. Arthritis generally affects small joints of the hand and does not progress to erosions. The classical "Jaccoud's arthropathy" although not causing a destructive arthritis, can result in significant deformity and functional impairment. A rheumatoid-like arthritis is seen more rarely, sometimes associated with a positive rheumatoid factor (20).

Renal disease affects about 30% of SLE patients, and remains the most dangerous, life-threatening complication. Patients who will develop lupus nephritis most commonly do so within the first few years of their disease. As renal involvement is often asymptomatic, particularly initially, regular urinalysis and blood pressure monitoring is important. It is characterized by proteinuria (> 0.5 g/24 hours), and/or red cell casts, and early referral for renal biopsy is generally advocated. The histological classification of lupus nephritis has recently been updated (21). The revised classification criteria (21), developed by the International Society of Nephrology and the Renal Pathology Society is shown in Table 3.

The renal biopsy findings are used to assess prognosis and guide management. Response to treatment can be assessed using serial urine protein/creatinine ratios, in addition to other more general measures of disease activity (21).

Neuropsychiatric (NP) manifestations occur in up to 75% of the patients. However the frequency of these manifestations in SLE studies varies widely, depending on the type of manifestations included and the method used for evaluation (22-24). Neuropsychiatric lupus (NPSLE) is often a difficult diagnosis to make. Not only are there 19 different clinical manifestations as described by the ACR (25) (Table 4), but there is also no single, simple diagnostic test. In many cases, a brain biopsy would be the only definitive test, and this is rarely performed. The clinical features vary from central nervous system (CNS) disease causing headache and seizures, or psychiatric diagnoses including depression and psychosis, to peripheral nervous system involvement causing neuropathy.

The investigations of choice will vary with the presentation. CNS disease usually warrants magnetic resonance imaging (MRI) of brain or spinal cord, and examination of the cerebrospinal fluid where appropriate. It must be remembered, however, that normal investigations, and lack of evidence of disease activity in another system, do not rule out the diagnosis of NPSLE (26).

Table 3. The revised classification of glomerulonephritis in SLE (21)

Classification	Description
<i>Class I</i>	Minimal mesangial lupus nephritis: Normal on light microscopy. Mesangial immune deposits on immunofluorescence
<i>Class II</i>	Mesangial proliferative lupus nephritis: Mesangial hypercellularity or matrix expansion, with mesangial immune deposits on immunofluorescence
<i>Class III</i>	Focal lupus nephritis Glomerulonephritis: involving < 50% of glomeruli, typically with sub-endothelial immune deposits.
<i>Class IV</i>	Diffuse lupus nephritis Glomerulonephritis: involving > 50% of glomeruli, typically with sub-endothelial immune deposits. Can be segmental or global.
<i>Class V</i>	Membranous lupus nephritis: Global or segmental sub-epithelial immune deposits
<i>Class VI</i>	Advanced sclerotic lupus nephritis: > 90% of glomeruli globally sclerosed without residual activity

Table 4. Central nervous system manifestation following ACR case definitions (25).

Central nervous system manifestations	Peripheral nervous system manifestations
Aseptic meningitis	Acute Inflammatory Demyelinating Polyradiculoneuropathy
Acute Confusional State	Autonomic Disorders
Anxiety Disorder	Cranial Neuropathy
Cerebrovascular Disease	Mononeuropathy
Cognitive Dysfunction	Myasthenia Gravis
Demyelinating Syndrome	Plexopathy
Headache	Polyneuropathy
Movement Disorder	
Mood Disorders	
Myelopathy	
Psychosis	
Seizures	

Hematological features include anemia, thrombocytopenia and leukopenia. Severe hematological disease can occur, but this is relatively rare (27). Most patients develop anemia at some time during the course of their disease. The anemia usually was normochromic and normocytic, and it appeared to depend partly on the severity and duration of the illness. Anemia in SLE is classified into two categories according to putative mechanisms: nonimmune and immune. The nonimmune-mediated group includes anemia of chronic disease, iron deficiency anemia, sideroblastic anemia, anemia of renal disorder, drug-induced anemia and anemia secondary to another disorder. Immune-mediated anemias include autoimmune hemolytic anemia, drug-induced hemolytic anemia, aplastic anemia, pure red cell aplasia and pernicious anemia (28).

Thrombocytopenia, which is defined as a platelet count of less than 150, 000 cells/mL, is a common finding in SLE. The degree is variable. Mild thrombocytopenia often appears during an exacerbation of SLE without causing bleeding tendency. Profound thrombocytopenia is rarely (29).

Leukopenia is common and may result from active disease or a drug reaction. It has been shown to be significantly associated with a high frequency of skin rash, lymphopenia and elevated anti-DNA titer. Anemia, fatigue, arthritis and elevated sedimentation rate also were more common in patients with leucopenia (30).

Pleuritis, causing chest pain, cough and breathlessness is the most common pulmonary manifestation of SLE (31). Although pleuritic symptoms may relate directly to active lupus, pulmonary embolism must always be considered, particularly in those who have antiphospholipid antibodies. Pleural effusions are usually exudates, have low levels of complement, and test positive for ANA (20). Infections are common, and any parenchymal lesion must be treated as infectious until proven otherwise.

The cardiac complications include pericarditis, valvular diseases with Libman-Sacks vegetation, myocarditis, cardiomyopathies, coronary artery diseases and conduction abnormalities (32). 25% of SLE patients have cardiovascular involvement at some point in the course of their disease, and cardiovascular disease is the third major cause of death in

these patients, although not all cardiovascular disease or cardiovascular deaths are inflammatory in nature; in fact, a significant proportion are due to atherosclerosis (33). Myocarditis is a rare but potentially fatal manifestation of SLE (34). It is often subclinical in nature, but 5–10% of all SLE patients develop symptomatic myocarditis (35, 36). Recent studies indicate that the risk of thrombotic events is more strongly associated with lupus anticoagulant (LAC) than with anticardiolipin (aCL) (37, 38).

Gastrointestinal involvement (39) most commonly results in non-specific abdominal pain and dyspepsia though it can be unclear whether such pain results from the illness itself or from drug side-effects. Hepatosplenomegaly is recurrent, depending of the disease activity. Mesenteric vasculitis is very rare, but can be life-threatening, especially if it leads to perforation.

SLE is associated with a variety of vascular manifestations. Raynaud's phenomenon, caused by microangiopathy, is observed in up 35% of patients (40). Arterial and venous thrombosis affected up to 10% of the cohort, particularly in association with the secondary antiphospholipid syndrome. In the last decade, it has become clear that patients with SLE are at increased risk of atherosclerosis. Chronic inflammation and the use of corticosteroids contribute to this risk, and also the higher levels of the Tumor Necrosis Factor (TNF).

Other observed manifestation is the vasculitis. It is a clinicopathologic process characterized by inflammation and necrosis of blood vessels. In SLE is most commonly due to the local deposition of immune complexes, particularly those containing anti-DNA in blood vessel walls (41).

Lupus and Pregnancy

In pregnancy, lupus tends to flare and the puerperium (42), most flares being mild. The frequency of exacerbations is lower in women with mild and well controlled disease (43). Maternal flares are associated with increased prematurity, and active nephritis has been shown to be an independent factor for fetal mortality (44). Antiphospholipid syndrome (APS) is a recognized cause of pregnancy complications, including miscarriage, fetal death, and pre-eclampsia. Several prospective series have shown the impressive improvement in the obstetric outlook in treated pregnant women with APS (45).

Neonatal lupus erythematosus (NLE) is a syndrome consisting of congenital heart block (CHB), transient cutaneous lupus lesions, cytopenia, hepatic, and other systemic manifestations in children born to mothers with SLE, Sjogren's syndrome, or other rheumatic diseases with a positive anti-Ro or anti-La antibodies (46). CHB is the most common manifestation of the NLE syndrome. The estimated incidence of CHB in the general population is around one in 20 000 live births (0.005%) (47).

Drugs used for disease control during pregnancy require attention. High dose aspirin and NSAIDs should be avoided in the last few weeks of pregnancy. Corticosteroids and hydroxychloroquine have not been shown to be teratogenic. Azathioprine and cyclosporin A may be considered during pregnancy when intense immunosuppression is deemed necessary and finally, cyclophosphamide should be avoided because of its power teratogenic (48-50).

Pathogenesis

Individuals who develop lupus have two major characteristics: they can produce pathogenic subsets of autoantibodies and immune complexes or they cannot regulate the clearance of them.

Genetic susceptibility: Genes contribute on the development of SLE. Genes of the human leukocyte antigen (HLA), particularly HLA-A1, B8, and DR3, have been associated to lupus (51). The response of a T lymphocyte to an antigen is triggered when a receptor molecule on the surface of the T cell recognizes a complex formed by the antigen and a HLA peptide on the surface of an antigen-presenting cell (APC). Cells like B lymphocyte, macrophages (MO), and dendritic cells (DC), can function as APCs. The HLA genotype determines which HLA molecules are available to the antigens that are present and thus how well the antigens can be recognized by T cells. Because of this reason, particular HLA genes are associated with a risk of an immune response to self antigens and hence a risk of diseases such as lupus (2, 51) (Figure 1).

Environmental triggers: Ultraviolet (UV) light and some infectious agents (e.g. virus). There is good evidence that exposure of the body to UV light alters the DNA's chemistry and location as well as the availability of Ro and RNP antigens (52). Viruses like Epstein-Bar (EBV) and Cytomegalovirus (CMV) have awaking interest. Mechanisms might include molecular mimicry between the self and the external antigens, epitope spreading, nonspecific activation of T or B lymphocytes in a host that cannot down regulate those responses or damage caused to tissue by the infection resulting in release of altered, more immunogenic self-antigen (53) (Figure 1).

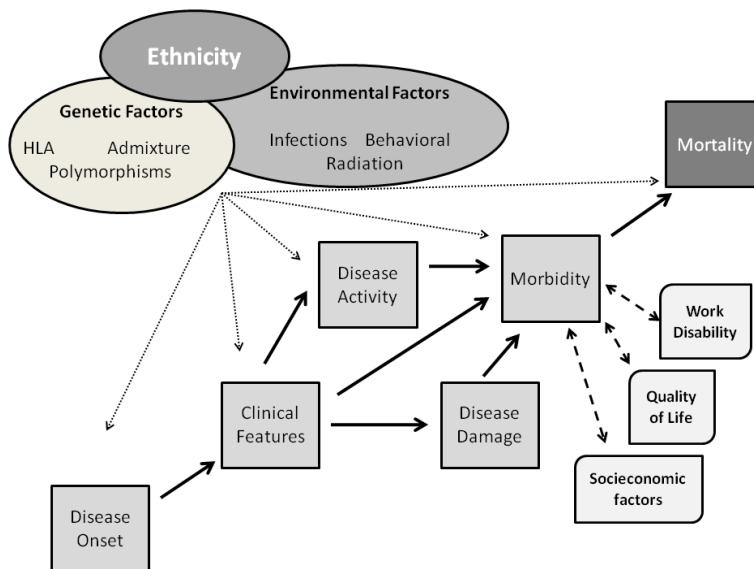


Figure 1. Disease course and outcome in SLE: genetic and non-genetic factors account for differences observed between ethnic groups (adapted from (3))

The source of autoantigens in SLE: The mechanism of activation-induced cell death, a programmed cell death known as “apoptosis”, is probably a major source of autoantigens (54, 55). A cell undergoing apoptosis develops surface vesicles resulting from antigen moving from cytoplasm into cell membrane. Near the surface of cells, the antigen can activate immune response. Autoantigens as nucleosomes, Ro 62, Ro 50, La, anionic phospholipids and U1 RNP can be found in these vesicles. Moreover, in cells that have been activated, the antigenic “heads” of phospholipid molecules flip from inward to outward orientation. These “heads” can be recognized by immune system. All of us have cells undergoing apoptosis continually, but in SLE patients this mechanism become pathogenic. The answer for this may be simply increased in quantity and duration of apoptotic cells and bodies. There is substantial evidence that lymphocytes from people with SLE undergo increased levels of apoptosis upon stimulation (54, 56) and apoptosis’ clearance is impaired in SLE patients (57).

Sex hormones and SLE: The strongest risk factor for SLE is clearly gender: SLE is much more frequent among women than men (3), an important role for female hormones seems likely, but a protective role for male hormones or an effect of genes on the X chromosome is also possible. Some trials of hormonal treatments for lupus, such as dehydroepiandrosterone, have been disappointing (58). SLE patient (women and men) metabolize testosterone more rapidly than normal and estrogenic metabolites of estradiol persist longer in women with this disease. There is an evidence that estradiol prevents tolerance of some autoreactive B cells (59), thus setting the stage for release of autoantibody, which can mature into pathogenic subsets. It is still inconclusive how sex hormones could promote or protect from lupus (60).

Neuroendocrine system: Abnormalities in hypothalamic and/or pituitary function contribute to the pathogenesis of SLE. It was observed that some patients have hyperprolactinemia and others have inappropriate levels of antidiuretic hormone (61).

B- and T-cell hyperactivity: Mechanisms for B- and T-cell hyperactivity involves sustained presence of autoantigens; it is related to increased apoptosis in lymphocytes, impaired clearance of apoptotic bodies and self antigens provided continually by tissue damage. Epitopes can spread in both B- and T-cell populations; a single antigen initiates a response but in the absence of appropriate control mechanism, the immune response continues to mature, to engage more T and B cells with specificities somewhat related to the initiating antigen, until both T and B cells are activated by multiple antigens, many of which are self. Another important mechanism about hyperactivity is the increased expression of surface molecules participating in cell activation in both cells populations (T and B cells) (62).

Autoantibodies can active T cells and help to continue their production. The production of cytokines is abnormal in SLE patients. There is a defect in the production of interleukin 12 (IL-12) (required to develop regulatory T cells), transforming growth factor beta (TGF β) (mediates suppression). There is an overproduction of IL-6 and IL-10 (promote B-cell maturation and immunoglobulin (Ig) secretion) (62).

Autoantibodies in SLE: Antinuclear antibodies (ANA) are serological hallmark of SLE, originally described in 1957 using an immunofluorescent assay with rodent liver tissue as the substrate (63). Over 90% of patients with SLE have positive ANA. Significant titers are

accepted to be of 1, 80 or greater. ANA although sensitive, is far from specific for SLE. A positive ANA is also seen in many other illnesses including polymyositis and systemic sclerosis, as well as some chronic infections. All patients should be screened for extractable nuclear antigens (ENA). Different ENAs are associated with different disease manifestations (20).

The affected organs in SLE that have been studied most intensively are the kidneys and the skin. In both cases, there is inflammation caused by deposition of antibodies and complement. In 1967, kidneys from patients with lupus nephritis were shown to contain antibodies that bound native, double-stranded DNA (anti-dsDNA) (64). These are autoantibodies; they bind a normal constituent, in this case, double-stranded DNA of the patient's cells and tissues. Anti-dsDNA is highly specific for lupus; they are present in 70% of patients but in less than 0.5% of healthy people or patients with other autoimmune diseases (65). Among patients who have both elevated levels of anti-dsDNA and clinically quiescent disease, 80% have disease that becomes clinically active within 5 years after the detection of elevated levels of these antibodies (66).

In a study of renal-biopsy specimens obtained from patients with SLE at autopsy (67), Mannik et al. detected IgG that bound to a number of non-DNA antigens, including Ro (a ribonucleoprotein complex), La (an RNA-binding protein), C1q (a subunit of the C1 complement component), and Sm (nuclear particles consisting of several different polypeptides). The detection of antibodies to these antigens in autopsy specimens does not prove that they play a role in the development of lupus nephritis. Rather than cause the inflammation, these autoantibodies may establish themselves in tissue only after the apoptosis of cells in inflamed kidney tissue exposes nuclear antigens. The strongest evidence concerning the mechanism of lupus nephritis relates to anti-dsDNA, anti-nucleosome, and anti- α -actinin antibodies (2).

The presence of anti-Ro antibodies, anti-La antibodies, or both in pregnancy confers a 1 to 2% risk of fetal heart block. Ro antigens are exposed on the surface of fetal (but not maternal) cardiac myocytes as the heart undergoes remodeling by apoptosis, and maternal anti-Ro antibodies that cross the placenta interact with these antigens (68, 69). The absence of an effect on the mother's heart shows the importance of both the autoantibody and exposure of the antigen on cardiac tissue.

Antibodies against the N-methyl-d-aspartate (NMDA) receptor may be important in CNS (70). NMDA is an excitatory aminoacid released by neurons. A recent study showed that in patients with lupus, the serum with anti-dsDNA and NMDA receptors caused cognitive impairment and hippocampal damage when given intravenously to mice. It also showed that anti-NMDA-receptor antibodies are present in the brain tissue of patients with cognitive impairment (70).

Ribosomal P protein is a pentamer consisting of 3 different phosphoproteins forming monomer P0 and dimers P1 and P2. It plays an important role in all stages of protein synthesis. Anti-P antibodies recognize at least 1 epitope common for all 3 phosphoproteins and are reactive with linear structure of antigenic determinant within their homologous C-terminal 22 amino acid sequence (71). The presence of anti-P antibodies might be associated with CNS impairment (72-75), kidneys (76-78) and/or liver damage (79-81).

Anti-Ro and anti-nucleosome antibodies may play a role in cutaneous lupus. Anti-Ro antibodies are associated with an increased risk of the development of a photosensitive rash

(82). Anti-nucleosome antibodies have been detected in skin biopsy specimens obtained from a minority of patients with active renal lupus, and these patients had no rash (83).

Differential Diagnosis

The list of possible differential diagnoses is broad, and will vary with the presentation of each case. The non-specific clinical characteristics of widespread pain and fatigue mean that in some cases fibromyalgia and other chronic pain syndromes may be appropriate differentials. Indeed, it is important to note that fibromyalgia and SLE can co-exist in the same patient (20).

Some of patients will present with a cluster of features suggestive of an autoimmune rheumatic disease, though at initial presentation the final diagnosis appears unclear. A proportion of these undetermined patients will go on to develop full blown SLE, or other diseases such as systemic sclerosis (20).

Some malignancies, particularly lymphoma and leukemia, which are relevant to this age-group, can be, clinically, similar. There is significant overlap with the presentation of some infections, notably, tuberculosis, HIV/AIDS and bacterial endocarditis. In view of the immunosuppressive nature of the required drugs, it is clearly crucial to exclude underlying infection before starting treatment for SLE (20).

SLE Activity Indices

Defining the degree of disease activity is the first step in trying to quantify changes in patients, standardizing differences between them, and evaluating clinical responses to therapy (84). Most studies of the usefulness of the various available indices have focused on the British Isles Lupus Assessment Group (BILAG) scale, the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), the Systemic Lupus Activity Measure (SLAM), and the University of California, San Francisco/John Hopkins University Lupus Activity Index (LAI) (84).

The BILAG system was developed by clinical investigators from four centers in United Kingdom and one in the Republic of Ireland and rates the activity of SLE in eight organ systems (85). The SLEDAI was developed at the University of Toronto. Several clinicians rated the importance of 37 variables in defining SLE activity (86). Possible scores using this index vary from 0 to 105. The manifestations must be present in the 10 days preceding evaluation (86).

MEX-SLEDAI and SELENA-SLEDAI are two modified indices from the SLEDAI. MEX-SLEDAI was developed for use in Third World countries where immunologic and complement assays are costly and/or unavailable (87). SELENA-SLEDAI was adapted from the SLEDAI for use in a multicenter safety study of estrogens in women with SLE; it has been validated through its prospective use in the ongoing study (88).

The SLAM which was developed at Brigham and Women's Hospital, lists 33 clinical and laboratory manifestations of SLE, and each manifestation is assessed as either active or inactive (89). The LAI is a five-part scale. Part one is the physician's global disease activity

assessment on a 0- to 3-point visual analogue scale (VAS). Part two is an assessment of four symptoms, each on 0- to 3-point VAS. Part three scores the activity of four organ systems, each on 0- to 3-point VAS. Part four involves medication. Part five scores for three laboratory parameters (90).

Damage Index

The SLICC/ACR Damage Index (DI) for SLE was developed by the Systemic Lupus International Collaborating Clinics and accepted by ACR as a valid measure of damage in patients with SLE (91). SLICC/ACR-DI is an unweighted index composed of 41 items grouped in 12 domains, with a maximum possible score of 47. As previously established, damage was considered when the irreversible lesions were present for at least 6 months unrelated to active inflammation and had occurred after SLE diagnosis (91).

The assessment of morbidity has been greatly improved by the use of the widely accepted SLICC/ACR. Studies utilizing this index have demonstrated that damage predicts further damage (92) and that longer disease duration is associated with higher damage scores (93).

Treatment

SLE is a relapsing and remitting disease, and treatment aims managing acute periods of potentially life-threatening ill health, minimizing the risk of flares during periods of relative stability, and controlling the less life-threatening, but often incapacitating day to day symptoms. Our limited understanding of the precise pathogenesis of SLE, the lack of reliable outcome measures, the propensity of lupus patients to have bad outcomes and to react to medicines in unusual ways and the heterogeneity of the patient population means that the majority of treatments is still broadly immunosuppressive in action, and hence carries a significant risk of adverse effects (20).

It is necessary first to identify and treat potential aggravating factors such as hypertension, infection and metabolic abnormalities and second, symptomatic therapy should be considered, such as anticonvulsants, antidepressants and antipsychotic medications, when necessary (94).

Salicylate and Nonsteroidal Therapy: Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most commonly prescribed drugs in the world. NSAIDs are able to produce analgesia, inhibit platelet aggregation, and reduce fever and inflammation. Despite the fact that the Food and Drug Administration (FDA) has not approved the commercial promotion of NSAIDs in the management of SLE, these agents have been used for the treatment of fever, arthritis, pleuritis and pericarditis. NSAIDs can interact with other medications; NSAIDs blunt the antihypertensive effects of loop and thiazide diuretics (95).

Antimalarial Therapies: Unlike other disease-modifying therapeutic agents that are used to treat SLE, antimalarials do not suppress the bone marrow or increase the risk for opportunistic infections (table 5) (96). Chloroquine is 90% absorbed by the gastrointestinal

tract. Renal excretion (50%) is increased by acidification and decreased by alkalinization. The drug is bound by plasma proteins and largely deposited into tissues. It is used to cutaneous lesions, arthritis-arthralgias, fatigue, and serositis. Hydroxychloroquine has a hydroxyl group at the end of a side chain, therefore differs from chloroquine, but it has similar pharmacokinetics. The only serious complication of the chloroquines is retinotoxicity, which is observed in 10% of patients in chloroquine and in 3% of those on hydroxychloroquine (97).

Glucocorticoid Therapy: The biologic effects of glucocorticois (GC) are multiple, affect all tissues and are essential for body homeostasis during normal or stress conditions. Although in clinical medicine GCs are used to suppress inflammation and pathologic immune responses (61, 98). It seems that endogenous GCs have an important overall regulatory role in modulating immune responses that develop to such stressors as infections (61).

The most common anti-inflammatory effects of GCs are mediated via their receptors and correlate with dose and duration of treatment. At the level of blood vessels, GCs inhibit vasodilatation and vascular permeability, limiting therefore erythema, plasma exudation, and swelling. Neutrophils are affected primarily in their ability to migrate to inflammatory area. Consequently, GCs inhibit chemokine synthesis and adhesion molecule expression. There is inhibition of synthesis of inflammatory mediators such as eicosanoids by downregulating phospholipase A₂ and COX-2. An alteration between cytokines anti-inflammatory cytokines (Interleukin 10, Transforming growth factor β) and proinflammatory cytokines (TNF- α , Interleukin 1 β) happens during the treatment with GCs (99).

Immunosuppressive effects also happen. They include lymphopenia, inhibition of signal transduction events critical for T-cell activation, downregulation of cell surface molecules (important for full T-cell activation and function), inducing of T-cell apoptosis and inhibition of antigen-presenting cell function (100).

Table 5. Mechanism of actions of antimalarial drugs (96)

Mechanisms
Absorbs ultraviolet light
Antimicrobial effects
Antioxidant actions block superoxide release
Antiproliferative effects
Blocks antigen processing by raising intracytoplasmic pH
Blocks graft <i>vs</i> host disease
Decreases estrogen production
Decreases macrophage-mediated cytokine production
Dissolves circulating immune complexes
Induction of apoptosis
Inhibits phospholipase A ₂ and C
Inhibits platelet aggregation and adhesion
Produces hypoglycemia
Quinidine-like cardiac actions
Stabilizes lysosomal membranes

Immunosuppressive Drug Therapy: Immunosuppressive agents are widely used to treat SLE despite the relative paucity of controlled trials showing their efficacy, especially with regard to prolongation of survival. Cyclophosphamide is inactive when administrated. It is metabolized by mitochondrial cytochrome P-450 enzymes in the liver to a variety of active metabolites, an increasing number of which have been shown to have both therapeutic and toxic effects. This medication can cause hematologic alterations as lymphopenia (dose-related). It is toxic to the granulose cell and, as consequence, reduces serum estradiol levels and progesterone production, inhibits the maturation of oocytes and reduces the number of ovarian follicles, resulting in ovarian failure (101). Patients who are receiving cyclophosphamide may develop transient amenorrhea. The risk of osteoporosis is increased by amenorrhea regardless of its cause. It is also a potent teratogen, which can cause severe birth defects after administration of as little as 200 mg during early pregnancy (102, 103).

Azathioprine has been used for a variety of nonrenal indications in active SLE. Moreover, it has been reported to be effective in severe cutaneous lupus and to have a steroid-sparing effect (104).

Cyclosporine has complex immunologic effects, mainly inhibition of T-cell gene activation, transcription of cytokine genes and lymphokine release. Besides, it inhibits the recruitment of antigen-presenting cells (105). A major adverse effect is nephrotoxicity. Reduction of glomerular filtration may be underestimated because of compensatory hyperfiltration and the increasing contribution of tubular secretion of creatinine to the measured creatinine clearance as renal function declines (106).

Methotrexate (MTX) appears to have multiple anti-inflammatory effects including increased adenosine levels at the local of inflammation, inhibition of leukotriene B₂ formation, interleukin-1 (IL-1) effects, fibroblast proliferation, and preferential cyclooxygenase-2 inhibition. Side effects of MTX are hepatotoxicity and cytopenias (107).

Mycophenolate Mofetil (MMF) has established itself as a successful immunosuppressive drug in multiple applications and has a unique mode of action that may be particularly applicable to control of SLE. MMF is the 2-morpholinoethyl ester derivative of mycophenolic acid (MPA), a weak organic acid produced by several *Penicillium* species (108). MMF has excellent oral bioavailability of 94.1% in healthy volunteers (109). After absorption, MMF is rapidly converted to its active metabolite, MPA by various plasma, liver and renal esterases. Several factors including renal dysfunction, hypoalbuminemia, accumulation of glucuronide and hemoglobin levels have been shown to affect MPA pharmacokinetics and pharmacodynamics (109).

MMF has several effects on the immune system. The best described of these is its selective inhibition of inosine monophosphate dehydrogenase (IMPDH), an enzyme involved in purine biosynthesis. IMPDH exists in two isoforms – type I, which is seen in most cell types and type II, which has greatly increased expression in activated lymphocytes (109). MMF inhibits the type II isoform nearly 5 times as much compared with the type I isoform, hence conferring its specificity for activated lymphocytes (110).

The principal adverse effects include gastrointestinal symptoms particularly diarrhea, nausea and vomiting and abdominal cramps. There is a suggestion that the gastrointestinal side effects may occur more frequently in the transplant setting compared with its use in inflammatory disease (111).

Biologic Therapy: Rituximab, a chimeric monoclonal antibody that selectively targets CD20-positive B cells while sparing stem cells and plasma cells (112-114), is approved for the treatment of non-Hodgkin's lymphoma. The results of several small uncontrolled trials have suggested that rituximab might have potential efficacy and be steroid-sparing in SLE (113-116). It has now been successfully tested in double-blind, placebo-controlled trials for a wide variety of autoimmune diseases, including rheumatoid arthritis (RA), multiple sclerosis, type 1 diabetes mellitus and antineutrophil cytoplasm antibody (ANCA)-positive vasculitis (117-119).

A recent experience has been done with Belimumab. It is a fully human antibody that binds to and inhibits the action of soluble human B lymphocyte stimulator (BLyS) (120). Besides, new trials with Epratuzumab are being done. This is an anti-CD22 molecule which reduces B-cell numbers by approximately 35–44% (121).

SLE Prognosis

Studies have shown a greater reduction in mortality in SLE patients than in the general population (30, 122). The 5-year survival rate among 99 patients seen at Johns Hopkins University from 1949 to 1953 was 50% (123). In contrast, since the mid-1970s, most studies in Europe, the United States, Canada, and Latin America have demonstrated 5-year survival rates among newly diagnosed patients of over 90%, and 15 to 20 years survival rates of around 80%. Identification of milder cases, or cases earlier in the disease process, made possible by improvements in diagnostic testing, may contribute to the observed improvements in survival (3).

Improved survival over time has been noted in analyses of period-specific survival rates since 1980 within adult patients around the world (30, 122-124). Although the improvement in survival in SLE has been greater than that observed in the general population (126), life expectancy in SLE patients is still below that of comparable demographic groups (122, 127). The improvement in survival over time has become evident when examining all causes of death together, but not when examining those deaths attributed to cardiovascular disease (124). It is also important to note the importance of economic development and socioeconomic factors in the prognosis of SLE.

Relatively large studies are needed to characterize potential gender-related differences in mortality risk, because only about 10% of SLE patients are male. Virtually all studies that examine mortality rates for specific ethnic groups (128-132) reported higher mortality risks among the black, Hispanic, and First Nations groups compared with white (or majority) populations. This differential risk by ethnicity was not seen, however, in several of the studies that adjusted for socioeconomic status (130-132). This is an important consideration in the interpretation of the role of ethnicity in mortality, given that ethnicity reflects socioeconomic variables and psychological factors that could directly affect mortality risk and that may be amenable to interventions.

Despite their improved survival, SLE patients still die at a rate that is three times that of the general population (122). Causes of death may be divided into those related to the SLE disease process itself, those related to therapy and those from unclear causes.

Urowitz et al. in 1976 (147) demonstrated a bimodal mortality pattern in SLE. Of 81 patients studied, 11 died and 6 of these died within a year of diagnosis, usually from complications of active lupus or sepsis. All were on high-dose steroids. The remaining 5 patients died a mean of 8.6 years after diagnosis. The mean dose of steroids was minimal, and 4 patients had myocardial infarctions.

Abu-Shakra et al. (148) also reviewed the causes of death in 124 SLE patients who died during follow-up at the University of Toronto Lupus Clinic between 1970 and 1994. The results were similar. Patients who died within the first 5 years of diagnosis were more likely to die of active disease, whereas patients who died late in the course of their disease tended to die of atherosclerotic complications, thus demonstrating the bimodal mortality once again.

Accelerated atherosclerosis has been identified as a major cause of mortality and morbidity in SLE. Studies revealed that at any point in time 10% of the patients will have features of clinical atherosclerosis either manifesting as angina, myocardial infarction, and peripheral vascular disease alone or in combination (149).

Conclusion

SLE is a chronic disease with severe involvement of the immune system. Symptoms range from rash and arthritis through anemia and thrombocytopenia to serositis, nephritis, seizures, and psychosis. Incidence rates of SLE range from approximately 1 to 10 per 100,000 person-years and prevalence rates generally range from 20 to 70 per 100,000 inhabitants. The onset of SLE may be due to a genetic susceptibility associated with environmental triggers. There are no criteria for SLE diagnosis, only classification criteria.

Our limited understanding of the precise pathogenesis of SLE means that the majority of treatments is still broadly immunosuppressive and steroids in action, and hence carries a significant risk of adverse effects. Mortality in SLE follows a bimodal pattern. Patients who die early in the course of their disease, die with active lupus, receive large doses of steroids and have a remarkable incidence of infection. In those who die late in the course of the disease, death is associated with inactive lupus, long duration of steroid therapy and a striking incidence of myocardial infarction due to atherosclerotic heart disease.

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Chapter 7

Diagnosis and Pathogenesis of Neuropsychiatric Syndromes of Systemic Lupus Erythematosus (NPSLE)

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Abstract

Neuropsychiatric syndromes of systemic lupus erythematosus (NPSLE) is a life-threatening disorder and early diagnosis and proper treatment are critical in the management of this neuropsychiatric manifestation in lupus. Symptoms of NPSLE are extremely diverse, ranging from depression, psychosis, and seizures to stroke. The origin of minor clinical symptoms, such as headaches and mood swings are not specific to NPSLE. In fact, SLE patients may be under the influence of other conditions capable of causing neuropsychiatric symptoms, such as infections, severe hypertension, metabolic complications, steroid psychosis, and other drug toxicities. Without proper treatment, neuropsychiatric involvement in SLE is known to increase morbidity and mortality. The availability of beneficial treatments increases the need for the early recognition of neuropsychiatric manifestations in lupus. Currently, tests for diagnosing NPSLE include brain magnetic resonance imaging (MRI), electroencephalogram (EEG), neuropsychological tests, and lumbar puncture. In addition to the conventional diagnostic tools, increased levels of proinflammatory cytokines and chemokines have been reported in the cerebral spinal fluids (CSF) of patients with NPSLE, and some reports have shown cytokines such as interleukin-6 (IL-6), IL-1, IL-8, IL-10, tumor necrosis factor (TNF)- α , interferon (IFN)- γ , monocyte chemotactic protein 1 (MCP-1)/CCL2, interferon-gamma inducible protein-10 (IP-10)/CXCL10 and Fractalkine/CX3CL1 to be elevated

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intrathecally, thereby allowing these cytokines to be used as diagnostic tools. Cytokines and chemokines are also considered to be therapeutic targets in NPSLE. Based on the number of recently published studies, this review focuses on the diagnosis, pathophysiology and therapeutic strategies for NPSLE.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by widespread immunologic abnormalities and multi-organ involvement, including the skin, joints, and kidney, as well as the peripheral and central nervous systems (CNS). Neuropsychiatric syndromes of systemic lupus erythematosus (NPSLE) may occur at any time during the course of the disease, and symptoms are extremely diverse, ranging from depression, psychosis, and seizures to stroke (1). The origin of minor clinical symptoms, such as headaches and mood swings are not specific to NPSLE. In fact, SLE patients may be under the influence of other conditions capable of causing neuropsychiatric symptoms, such as infections, severe hypertension, metabolic complications, steroid psychosis, and other drug toxicities (2). Without proper treatment, neuropsychiatric involvement in SLE is known to increase morbidity and mortality, and therefore, the availability of beneficial treatments increases the need for the early recognition of neuropsychiatric manifestations in lupus. Along with more specific diagnostic tools and an effective method of monitoring disease activity, therapeutic responses are crucial in the management of NPSLE. Tests for diagnosing NPSLE include brain magnetic resonance imaging (MRI), electroencephalogram (EEG), neuropsychological tests, and lumbar puncture. These findings are reported to be abnormal in some, but not all patients and therefore, none of the findings are specific for NPSLE. The large discrepancy in the reported frequency of neuropsychiatric involvement in SLE patients (14%-75%) further proves there is no single confirmatory diagnostic tool (3,4). This article focuses on the diagnosis, pathophysiology and therapeutic strategy for NPSLE.

Diagnosis of NPSLE

1.1. Neuroimaging

As neuroimaging tools, conventional MRI, magnetic resonance spectroscopy (MRS), magnetization transfer imaging (MTI), diffusion weighted imaging (DWI), perfusion weighted imaging (PWI), single photon emission computed tomography (SPECT), and positron emission tomography (PET) have provided large amounts of information regarding NPSLE. Conventional MRI still remains the modality of choice because of its availability and accessibility. The most frequent MRI pathologic pattern of NPSLE is that of small, hyperintensive, T2-weighted focal lesions in the subcortical and periventricular white matter. However, the sensitivity of MRI detected lesions for acute NPSLE is only 50-55% and is higher for focal lesions than for diffuse lesions. These lesions also present in non-NPSLE neuropsychiatric disorders and in SLE patients without neuropsychiatric symptoms (5). In

addition to MRI, neuroimaging techniques for the assessment of perfusional, metabolic and microstructural brain alterations have been studied. PET is a nuclear technique used to detect both brain glucose metabolism and cerebral blood flow. Multiple areas of hypometabolism are frequently found in NPSLE. SPECT detects brain perfusion and the most frequent findings in NPSLE are diffuse, focal or multifocal areas of hypoperfusion. SPECT is highly sensitive (80-100%) but has low specificity (10-50% of SLE patients without neuropsychiatric symptoms). MTI is a quantitative MRI technique that is sensitive to macroscopic and microscopic brain tissue changes. MTI measures the magnetization transfer ratio (MTR) and displays data as histograms. MTI has been reported to be useful for the diagnosis of NPSLE and the MTR peak height is lower in NPSLE patients than in healthy controls. In addition, the MTR peak height was associated with cognitive dysfunction but not with the other neuropsychiatric syndromes (6). MRS is an MRI application which explores the *in vivo* biochemical profile of brain tissue and provides quantitative and qualitative information about brain metabolisms displayed as spectra. Changes of spectra have been reported in NPSLE but they are not specific. In a recent study, combining Single Photon Emission Computed Tomography (SPECT) with MRI was useful for the diagnosis of NPSLE (7).

Major improvements in neuroimaging techniques have provided large amounts of information towards understanding the pathology of NPSLE. However, no single technique to date has diagnostic specificity for NPSLE. Further studies exploring a multimodality approach by coupling a morphological and a functional imaging technique are required for the specific diagnosis of NPSLE.

1.2. Cytokines

Cytokines, small substances secreted by specific cells of the immune system which carry signals locally between cells and have important roles in the development and functioning of both the innate and adaptive immune response. Chemokines are chemo-attractant cytokines which play key roles in the accumulation of inflammatory cells at the site of inflammation. Increased levels of proinflammatory cytokines and chemokines have been reported in the cerebral spinal fluids (CSF) of patients with NPSLE, and some reports have shown cytokines such as interleukin-6 (IL-6), IL-1, IL-8, IL-10, tumor necrosis factor (TNF) - α , interferon (IFN)- γ , monocyte chemotactic protein 1 (MCP-1)/CCL2, Interferon-gamma inducible protein-10 (IP-10)/CXCL10 and Fractalkine/CX3CL1 to be elevated intrathecally, thereby allowing these cytokines to be used as diagnostic tools (8-12). Cytokines and chemokines are considered to be therapeutic targets in several chronic inflammatory disorders such as SLE. Based on the number of recently published studies, this review focuses on the use of cytokines and chemokines as biomarkers as well as the pathogenic factors involved in NPSLE. The role of TNF- α in lupus is still controversial. TNF- α may be protective in patients with lupus, since low TNF- α activity is associated with increased disease activity. In some patients with rheumatoid arthritis who were treated with anti- TNF- α antibodies, anti-double-stranded DNA antibodies were detected and lupus developed in a few of these patients. By contrast, TNF- α may promote the pathogenesis of lupus, since the level of TNF- α messenger RNA was high in kidney-biopsy specimens from patients with lupus nephritis

and there is a report showing that giving the anti- TNF- α antibody infliximab to six patients with lupus led to resolution of joint swelling in three patients with arthritis and a 60% reduction of urinary protein loss in four patients with renal lupus (13,14). Serum levels of interferon- α (IFN- α) are also elevated in patients with active lupus and microarray studies showed that 13 genes regulated by IFN were up-regulated in peripheral-blood mononuclear cells from patients with lupus, as compared with healthy controls (14). Serum levels of interleukin-10 (IL-10) are consistently high in patients with lupus, and they correlate with the activity of the disease. IL-10 has a number of biologic effects, including stimulation of polyclonal populations of B lymphocytes. Consequently, blocking this cytokine could reduce the production of pathogenic autoantibodies (14). Furthermore, several cytokines such as IL-4, IL-6, IL-8, IL-10, TNF- α IFN- α and IFN- γ have been reported to be elevated in the cerebrospinal fluid (CSF) from patients with NPSLE.

Among reported cytokines, IL-6 has been shown to have the strongest positive association with NPSLE. IL-6 levels in the CSF of NPSLE were reported to be elevated without damage of the blood-brain barrier. In addition, the expression of IL-6 mRNA was elevated in the hippocampus and cerebral cortex, suggesting that IL-6 expression was increased within the entire CNS of NPSLE (15,16). There is a report studying the expression of IL-4, IL-10 TNF- α and IFN- γ in both peripheral blood lymphocytes (PBLs) and CSF from NPSLE patients whereby the authors found that mRNA for IL-10, TNF- α and IFN- γ were increased in PBLs while only IL-10 and IFN- γ were elevated in CSF (9). An exhaustive study of cytokines and chemokines recently reported that IL-6 and IL-8 were elevated in NPSLE compared with non-NPSLE and non-autoimmune disease patients. This study also found that IL-2, IL-4, IL-10, TNF- α , and IFN- γ were low in all groups examined (17). In other reports, no association was found between the levels of IL-2, IL-6, IL-10, TNF- α and IFN- γ and NPSLE (18,19). A recent study has shown that the sensitivity and specificity of CSF IL-6 for diagnosis of lupus psychosis was 87.5% and 92.3%, respectively, indicating that CSF IL-6 might be an effective marker for the diagnosis of lupus psychosis (20). The TNF family ligands BAFF (B-cell activating factor of TNF family) and APRIL (a proliferation-inducing ligand) are essential for B-cell survival and function. Elevated serum levels of BAFF and APRIL have been reported in patients with SLE. Recently BAFF and APRIL were studied in CSF of NPSLE patients. They found that levels of APRIL and BAFF in CSF were more than 20 and 200-fold higher respectively in NPSLE patients than those of healthy controls. Comparing the levels of APRIL in CSF between NPSLE and non-NPSLE patients, enhanced levels of APRIL were noted in NPSLE. Moreover, they found that CSF levels of APRIL correlated with BAFF but not with IL-6 (21). There is a report regarding the association between cytokine levels and acute confusional state (ACS) of NPSLE. The authors performed a prospective study using a cohort of 59 patients with SLE and compared those with and without ACS as well as associations between ACS and each CSF test [IL-6, IL-8, IFN- α , IgG index, and Q-albumin]. In this study, ACS was diagnosed in 10 patients (ACS group), SLE-related CNS syndromes except ACS in 13, and no CNS syndromes in 36 (non-CNS group). CSF IL-6 levels in the ACS group were significantly higher than those in the non-CNS group ($p < 0.05$) and a positive IgG index ($p = 0.028$) was significantly associated with ACS. No other test showed a significant association with ACS. The positive and negative predictive values for the diagnosis of ACS in SLE were 80% and 85% for elevated CSF IL-6 levels ($> 31.8 \text{ pg/ml}$), and 75% and 83% for the IgG index, respectively. From these results, the

authors concluded that no single CSF test had sufficient predictive value to diagnose ACS in SLE, although CSF IL-6 levels and the IgG index showed statistical associations with ACS (22).

The summary of the reported results is shown in Table 1.

Table 1 Cytokines in CSF of NPSLE

Cytokine	NPSLE	Control group	Reference
IL-1	Increased	none	48
IL-1 β	Same	neurological symptoms without neurological diseases	19
soluble IL-1	Increased	neurological symptoms without neurological diseases	19
IL-6	Increased	non-NPSLE, HI	18
	Increased	HI, neurocysticerosis	49
	Increased	non-NPSLE	15
	Increased	cerebral infarction	16
	Increased	none	48
	Increased	CNS inflammation, non-inflammatory CNS diseases	50
	Increased	non-NPSLE	8
	Increased	HI	51
	Increased	non-NPSLE, SM, non-AID	17
IL-8	Increased	non-NPSLE	8
	Increased	non-NPSLE, SM, non-AID	17
	Increased	MS, other AID	42
IL-10	Same	non-NPSLE, HI	18
	Increased	HI	51
	Same	non-NPSLE, SM, non-AID	17
TNF- α	Increased	HI	51
	Same	non-NPSLE, SM, non-AID	17
	Same	neurological symptoms without neurological diseases	19
IFN- γ	Same	non-NPSLE, SM, non-AID	17
IFN- α	Increased	non-NPSLE, HI	18
	Increased	non-NPSLE	52
	Increased	non-NPSLE	53

AID: autoimmune diseases, HI: healthy individuals, MS: multiple sclerosis, SM: septic meningitis

1.3. Chemokines

Chemokines in humans comprise more than 50 small (8-to-10-kDa) heparin-binding proteins with 20 to 70 percent homology in amino acid sequences. Chemokines were

originally identified by their chemotactic activity on bone marrow-derived cells (23,24). They are classified into at least four families according to the location of their cysteine residues. The four chemokine groups are CC, C, CXC, and CX3C, where C is a cysteine and X any amino-acid residue. Chemokine receptors are consequently classified as CCR, CR, CXCR, and CX3CR. The chemokine receptors are bound to the cell membrane through seven transmembrane helical segments coupled with a G-protein which transduces the intracellular signal. The two major subclasses include the CC chemokines where the cysteines are neighboring and the CXC chemokines where the cysteines are separated by one amino acid. The CXC chemokines mainly act on neutrophils and lymphocytes, whereas the CC chemokines mainly act on monocytes and lymphocytes without affecting neutrophils (25). Lymphotactin, in the C chemokine family, is similar to members of both the CC and CXC chemokine families but lacks two of the four cysteine residues and is a potent attractant for T cells, but not for monocytes or neutrophils (26). Fractalkine, in the CX3C family, is a cell-surface-bound protein, in which the first two cysteine residues are separated by three amino acids. Fractalkine has potent chemoattractant activity for T cells and monocytes (27). One characteristic feature of chemokines is the redundancy of the system. Several chemokines bind to more than one receptor and the majority of chemokine receptors have multiple ligands leading to the generation of multiple pathways directing similar cellular responses. Until recently, chemokines have been named randomly, with no clear system being used. Some have been included in the same group as interleukins (for example, IL-8), while others have been given names describing a function, for example macrophage chemoattractant proteins. In an attempt to clarify the confused and complex nomenclature associated with chemokines, the nomenclature of the chemokine system has been revised. The name referring to a specific biologic function has been replaced by the chemokine subfamily name followed by a number (for example, MCP-1 is CCL2) (28,29). In this review, chemokines are described with common names as well as the revised names according to the new nomenclature. Several chemokines such as IL-8/CXCL8, IP-10/CXCL10, fractalkine/CXCL1, RANTES/CCL5 and MCP-1/CCL2 have been reported to be elevated in the CSF from patients with NPSLE (Table 2).

Monocyte chemoattractant protein (MCP)-1/CCL2 (a ligand of CCR2) can attract monocytes, T cells, NK cells, and basophils (10,30). It is a high-affinity ligand for the CCR2 chemokine receptor that is constitutively expressed in monocytes but is expressed on lymphocytes only after stimulation by IL-2. Expression of CCR2 on monocytes can be down-regulated by lipopolysaccharides. We and others have reported that CSF MCP-1/CCL2 levels are higher in NPSLE patients than in non-NPSLE patients (10,17). In addition, we reported that levels of MCP-1/CCL2 decreased after immunosuppressive treatment. Furthermore, we compared the levels of MCP-1/CCL2 among various neuropsychiatric symptoms. However, as some patient groups have very few cases, we were unable to conclude which type of symptom was associated with the increase of CSF MCP-1/CCL2 levels in our study (10).

(RANTES)/CCL5 (a ligand of CCR1, CCR3, and CCR5) is another CC chemokine which attracts monocytes, memory T cells and NK cells and is implicated in the pathophysiology of SLE, rheumatoid arthritis (RA) and multiple sclerosis (MS) (31). Chemokine receptor CCR5 is preferentially expressed on helper T1 (Th1) lymphocytes and has been reported to have an important role in the pathogenesis of RA. It has been reported that systemic administration of a small molecular weight antagonist of CCR5, SCH-X, suppressed the development of collagen-induced arthritis (CIA) in a monkey model of RA (32). We also provided evidence

showing that systemic administration of TAK-779, a nonpeptide compound with a small molecular weight (aka small molecule)??, inhibits the development of adjuvant-induced arthritis in rats (33). Recent reports suggest that CSF levels of CCL5 are increased in NPSLE patients compared with non-NPSLE patients (17).

Table 2**Chemokines and chemokine receptors in NPSLE**

Chemokines	Chemokine receptors	Cell Types
IL-8/CXCL8	CXCR1	Neutrophils, monocytes
IP-10/CXCL10	CXCR3	Th 1 cells, mast cells, mesangial cells
fractalkine/CXCL1	CX3CR1d	Macrophages, endothelial cells, smooth muscle cells
RANTES/CCL5	CCR1	T cells, monocytes, eosinophils, basophils
RANTES/CCL5	CCR3	Eosinophils, basophils, mast cells, Th2 cells
MCP-1/CCL2	CCR2	Memory T cells, monocytes, immature dendritic cells

Chemokines in CSF of NPSLE

Chemokines	NPSLE	Control group	References
MCP-1/CCL2	Increased	non-NPSLE	10
	Increased	non-NPSLE	17
RANTES/CCL5	Increased	non-NPSLE	17
IL-8/CXCL8	Increased	non-NPSLE	37
	Increased	non-NPSLE, SM, non-AID	17
	Increased	MS, other AID	42
IP-10/CXCL10	Increased	non-NPSLE	12
Fractalkine	Increased	non-NPSLE	40
	Same	non-NPSLE	41
IP-10/MCP-1 ratio	Increased	non-NPSLE	11

AID: autoimmune diseases, MS: multiple sclerosis, SM: septic meningitis

Interferon-gamma inducible protein-10 (IP-10)/CXCL10 (a ligand of CXCR3) is expressed and secreted by monocytes and fibroblasts following stimulation with IFN- γ (34). IP-10/CXCL10 is a high-affinity ligand for the CXCR3 chemokine receptor which is mainly

expressed on Th1 cells. The predominance of Th1 versus Th2 cells in NPSLE patients remains unresolved. We and others have reported that IP-10/CXCL10 was up-regulated in the CNS fluid of NPSLE (12,17). IL-8/CXCL8 (a ligand of CXCR1 and CXCR2) was the first chemokine identified to be involved in leukocyte chemotaxis such as polymorphonuclear neutrophils and specific T cells (35, 36). There are several reports showing that IL-8/CXCL8 levels in CSF are elevated in NPSLE (37).

The C chemokine family is represented by two chemokines, lymphotactin/XCL1 and SCM-1 β /XCL2, whereas the CX₃C chemokine family contains only one member, called fractalkine/CX₃CL1 (38). Fractalkine/CX₃CL1 is synthesized by endothelial cells as a type 1 transmembrane protein which is then cleaved by proteolysis, possibly mediated by TNF- α -converting enzyme (TACE) and ADAM 10, thereby yielding the soluble form of Fractalkine/CX₃CL1 (sFKN). Fractalkine/CX₃CL1 binds to a receptor known as CXCR1, and signals via the G protein pathway in NK cells, macrophages and a certain proportion of T cells. Fractalkine/CX₃CL1 plays important roles in the pathogenesis of RA by attracting pro-inflammatory cells, such as activated macrophages and T cells (39). There is a report showing that levels of sFKN/s CX₃CL1 were elevated in the CSF of NPSLE. In this report, both serum and CSF sFKN/s CX₃CL1 levels declined with treatment (40). However, our group did not find a significant increase of sFKN/s CX₃CL1 in CSF from NPSLE patients compared with that of non-NPSLE patients (41).

We reported that the IP-10/MCP-1 ratio is a useful marker to detect NPSLE (11). In our study, the IP-10/MCP-1 ratio in the NPSLE group was significantly higher than that in the non-NPSLE group ($P=0.0000014$, Mann-Whitney's U test). The discriminative ability (area under the curve) of each chemokine was 0.63111(95% confidence interval [95% CI] 822.4064-7787.1975) (IP-10/CXCL10), 0.67626([95% CI] 418.6142-1761.8262) (MCP-1/CCL2) and 0.82672 ([95% CI] 1.222729-5.011448) (IP-10/MCP-1 ratio). There was no correlation between the levels of MCP-1 in CSF and that in serum ($r=-1332$, $p=0.2481$) and the levels of IP-10 in CSF and that in serum ($r=-1445$, $p=0.3842$). There was also no correlation between the levels of MCP-1 and IL-6 ($r=-0.1229$, $p=0.462$), IL-8 ($r=-0.1585$, $p=0.349$) and IFN alpha ($r=-0.0818$, $p=0.179$); and between IP-10 and IL-6 ($r=-0.1435$, $p=0.384$), IL-8 ($r=-0.1553$, $p=0.332$) and IFN alpha ($r=-0.1021$, $p=0.213$). We concluded that CSF IP-10/MCP-1 ratios are higher in NPSLE patients than in non-NPSLE patients and that this index is a useful diagnostic marker of NPSLE (11). The summary of the reported results are shown in Table 2.

2. Pathophysiology and Therapeutic Strategies of NPSLE

Although some cytokines are important biomarkers of NPSLE, the mechanism for the elevated levels of cytokines is thus far unknown. As SLE is an autoimmune disorder characterized by numerous autoantibodies, a pathogenetic role for autoantibodies is theoretically suspected. Immune complexes in SLE can stimulate IFN- α and there is strong evidence in humans and in mice that IFN- α can cause neuropsychiatric manifestations. Santer DM et al. used a bioassay containing plasmacytoid dendritic cells to demonstrate that NPSLE CSF induced significantly higher IFN- α compared with CSF from patients with multiple

sclerosis or other autoimmune disease controls. When normalized for IgG concentration, NPSLE CSF was 800-fold more potent at inducing IFN- α compared with paired serum due to inhibitors present in serum. In addition to IFN- α , immune complexes formed by CSF autoantibodies produced significantly increased levels of IP-10/CXCL, IL-8, and MCP-1. From these results they proposed a two-step model of NPSLE whereby CSF autoantibodies bind to antigens released by neurocytotoxic antibodies or other brain cell injury, and the resulting immune complexes stimulate IFN- α and proinflammatory cytokines and chemokines (42). Recently, our group has shown that IgG anti-NR2 glutamate receptor antibodies (anti-NR2) from SLE patients directly activated endothelial cells by the activation of NF- κ B signaling, resulting in the up-regulation of adhesion molecules and cytokine production (43). Several novel brain antigens have been identified in a proteomic analysis of patients with NPSLE. The anti-Rab guanosine diphosphate dissociation inhibitor alpha (α GDI) antibody has been reported to be a diagnostic marker of psychosis associated with NPSLE. α GDI functions to control the activity of the small GTPases of the Rab3 proteins available for synaptic vesicle cycling and neurotransmitter release (44). Another systemic proteomic analysis identified additional NPSLE-specific brain antigens such as MAP-2B, triosephosphate isomerase, and septin 7 and suggested that stability of neuronal microtubules might be involved in the pathogenesis of NPSLE (45). Further immunological studies are expected to show how autoantibodies in SLE patients work to promote the cytokine storm associated with the pathophysiology of NPSLE.

There is growing evidence showing that chemokines not only recruit specific subsets of lymphocytes and inflammatory cells but also determine the type of immune response at the site of inflammation, and that chemokines have pivotal roles in the development and progression of autoimmune disorders. In the course of an immune response, distinct subsets of effector T cells and regulatory T cells are generated in the lymphoid compartments after the differentiation of naive T cells under the influence of specific cytokines. Chemokines guide these T cell subsets out of the lymphoid compartment and into sites of inflammation and infection. Before the commitment of antigen-specific T cell subsets, innate T cells such as NKT and $\gamma\delta$ T cells work as the first line of host defense and also are guided to the sites of inflammation by chemokines. Naive T cells differentiate into Th1 cells under the direction of the cytokine IL-12 and transcription factor T-bet. Th1 cells are characterized by the production of IFN- γ and have a role in the protection against intracellular pathogens. On the other hand, IL-4 and the transcription factor GATA-3 are critical in the differentiation of naive T cells into Th2 cells. Th 2 cells are characterized by the production of IL-4, IL-5 and IL-13 which mediate protection against parasites and allergic responses. Th1 cells preferentially express CCR5, CXCR3 and CXCR6, whereas Th2 cells preferentially express CCR4 and CCR8.

We reported that CSF MCP-1/CCL2 and IP-10/CXCL10 levels are higher in NPSLE patients than in non-NPSLE patients, indicating possible involvement of this chemokine in the pathogenesis of NPSLE. The receptor of IP-10/CXCL10, CXCR3, is predominantly expressed on natural killer cells and activated T cells, especially on Th1 cells. On the other hand, the receptor of MCP-1/CCL2, CCR2 is expressed not only on activated T cells and natural killer cells but also on monocytes, basophils and dendritic cells. CD4+ T cells populations that upregulate expression of the transcription factor ROR γ t can differentiate into IL-17 producing CD4+ T cells (Th17 cells) that differ in phenotype and function from Th1

and Th2 cells. Th 17 cells are thought to protect against bacteria and fungi and these cells are also involved in the pathogenesis of autoimmune diseases (38). Interestingly, CCR2 is expressed on a subpopulation of Th17 cells which produce a large amount of IL-17 but little IFN- γ (46). These results implicate the differential contribution of both CXCR3 and CCR2 signaling in the pathogenesis of NPSLE, especially on effector T cells such as Th1, Th2 and Th17 cells.

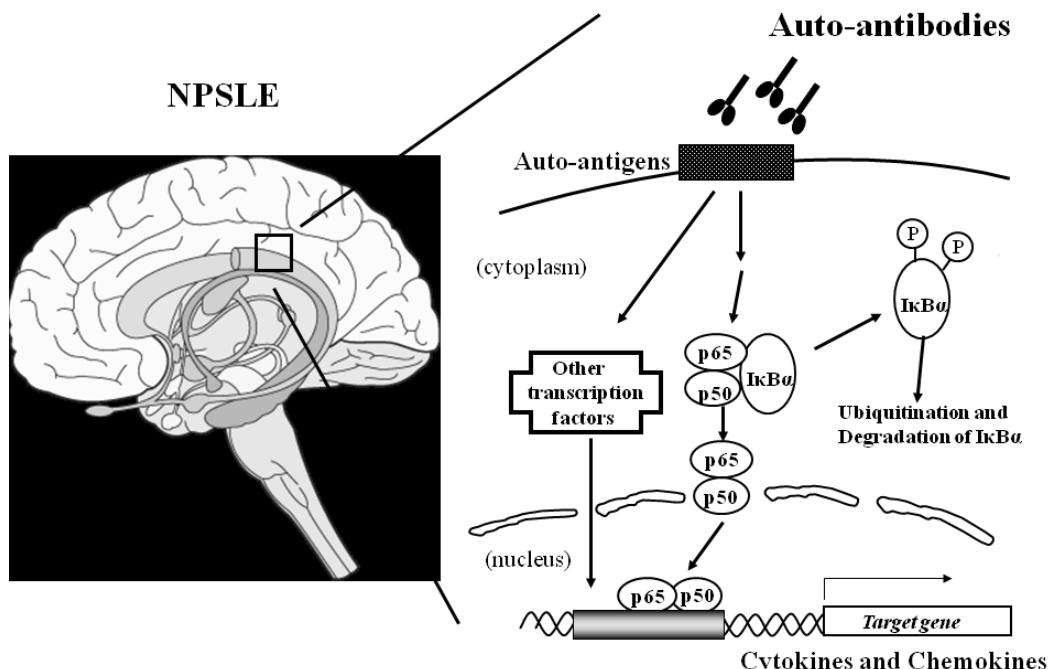


Figure 1.

3. Conclusion

This review, summarizes the diagnosis and pathology of NPSLE. Although a large number of studies have been performed, the precise pathophysiology of NPSLE is not completely understood thus far. As I presented here, neuroimaging techniques are improved and some of them, especially the combination of a morphological and a functional imaging techniques are promising for the diagnosis of NPSLE. In addition, various cytokines and chemokines are highly expressed in the brain of NPSLE patients, and it is believed that these small molecules have important roles in the pathogenesis of NPSLE. However, the molecular mechanisms by which these molecules work in the course of the development of NPSLE have not yet been completely revealed. Cytokines and chemokines are expressed by the stimulation of NF-κB signaling and signal transduction pathways involving other transcription factors (47). As mentioned above, our group has shown that IgG anti-NR2 glutamate receptor antibodies (anti-NR2) from SLE patients direct NF-κB signaling in endothelial cells, resulting

in the up-regulation of adhesion molecules and cytokine production. Therefore, autoantibodies which are characteristic features of SLE bind to corresponding autoantigens on the cell surface and these interactions may stimulate signaling cascades, resulting in the activation of certain transcription factors (Figure 1). The activation of signal transduction pathways involving these transcription factors might activate transcription and expression of cytokines and chemokines, resulting in a cytokine/chemokine storm and the development of NPSLE pathophysiology. Further molecular studies are required to prove this proposed mode of action for cytokines and chemokines. As other brain antigens are studied more extensively, molecular studies are required to elucidate the mode of action of these molecules and the roles they play in the pathogenesis of NPSLE. In addition, cytokines and chemokines are considered to be therapeutic targets in NPSLE. As most of the cytokines and chemokines involved in NPSLE have pleiotrophic roles in other biological processes, inhibition of these cytokines and chemokines might invite unexpected side effects *in vivo*. Therefore, a cooperative contribution of both clinical studies and molecular biological studies is required for the development of ideal therapeutic strategies against NPSLE.

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Chapter 8

Cytomegalovirus-Induced Autoimmunity

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Abstract

Human cytomegalovirus (HCMV) is a ubiquitous pathogen that causes severe infections in immunocompromised patients. During active infection, the virus is able to modulate the host immune system in immunocompetent as well as immunocompromised individuals. HCMV-infected patients often develop signs of immune dysfunction, such as autoimmune phenomena. Furthermore, case reports suggest a link between primary HCMV infection and onset of autoimmune disorders. Signs of active viral infection have also been identified in a number of autoimmune diseases, which further highlights the potential role of HCMV in the genesis and/or maintenance of immunopathological phenomena. Mechanisms by which HCMV could induce host immunopathology, inflammation and autoimmunity will be discussed as well as the opportunity to administer antivirals in selected patients.

1. The Virus

Human cytomegalovirus (HCMV) is a highly species-specific herpes virus that infects and is carried by the majority of the human population [1]. The clinical importance of HCMV has risen during the last decades due to an increasing number of AIDS patients and patients

undergoing immunosuppressive therapy following organ or bone marrow transplantation. In addition, the development of novel and highly sensitive techniques has led to more frequent detection of active HCMV infection in immunosuppressed patients and to identification of viral protein expression in patients with cancer and autoimmune diseases [2,3].

The HCMV genome has a high G+C content (57.2%) and viral proteins are expressed at immediate early (IE), early (E), and late (L) phases of infection. Characterization of clinical HCMV strains suggests that the viral genome has 252 open reading frames that may encode potential proteins [4]. However, genome analyses and mutagenesis studies show that only 45 to 57 of the viral genes are committed to essential tasks of replication. Hence, the vast majority of viral proteins are possibly developed during the evolution and devoted to modulate the cellular and immunological responses of the host.

During the acute phase of HCMV disease, many cell types in virtually any organ system can be infected, including endothelial cells, epithelial cells, smooth muscle cells, fibroblasts, neuronal cells, hepatocytes and monocytes/ macrophages (M ϕ s) (as reviewed in [5]). Although the susceptibility of these cells to HCMV infection has been confirmed *in vitro*, the ability of the virus to grow varies in different cell types. For example, viral replication is considerably less efficient in M ϕ s, dendritic cells (DCs), and epithelial cells than in fibroblasts—the prototype cell for growth of virus *in vitro*.

There are three forms of active HCMV infection, as determined by prior and current experience with the virus; 1) Primary infection occurs when the virus infects a HCMV-naive host; 2) Reactivation occurs when a HCMV-seropositive individual reactivates endogenous virus that is already present in a latent stage; 3) reinfection is caused by infection with a different strain in a HCMV-seropositive individual. This is due to the considerable heterogeneity genetically and antigenically among different isolates of HCMV [6].

In addition to acute infection, virus persistence is established in all infected individuals and appears to be maintained by both a chronic productive infection as well as latency with restricted viral gene expression. The contribution of each of these mechanisms to the persistence of HCMV in the host is presently unknown [7].

A step that is believed to be crucial in the context of autoimmune disorders is HCMV reactivation from latency. Initiation of virus replication is not only caused by immunosuppression but, as for other viruses such as HIV [8], appears to be linked to the activation of the immune system.

Virus reactivation is accomplished by among other factors, tumor necrosis factor (TNF)- α , released in response to an inflammatory process. TNF- α binds to the TNF receptor on latently infected cells, which is then responsible for a signalling process that involves activation of protein kinase C and nuclear factor-kB (NF-kB). The resulting activated p65/p50 NF-kB heterodimer translocates into the nucleus and binds to the HCMV IE enhancer region, which initiates viral replication [9]. In concordance with this molecular mechanism, subclinical reactivation of latent HCMV has been demonstrated in association with elevated serum levels of TNF- α in patients with atopic dermatitis [10] or sepsis [9,11,12].

Furthermore, HCMV infection is commonly reactivated following acute rejection of organ transplants as well as after acute graft-versus-host disease (GVHD) in bone marrow transplant recipients (BMT) in whom elevated levels of TNF- α have been shown to involve an increased risk for HCMV infection [13-16]. In addition, release of proinflammatory prostaglandins stimulates the cyclic AMP mechanism which, in turn, can trigger the virus

reactivation process [17]. Finally, stress catecholamines may also induce increasing concentrations of cyclic AMP, leading to viral reactivation [6,18]. Through some of these mechanisms, chronic inflammation can be very likely responsible for HCMV reactivation.

Peripheral blood monocytes and myeloid progenitors are a major site of carriage of latent HCMV [19,20]. Evidence shows that HCMV reactivates from latency by allogeneic stimulation in monocytes from seropositive donors [21]. Virus reactivation occurs also when mononuclear hematopoietic progenitors that are latently infected with HCMV are stimulated to differentiate to mature DCs [22]. Thus, both inflammation and cell differentiation may represent processes that trigger HCMV reactivation.

2. Clinical Significance of HCMV Infection

2.1. Congenital Infection

HCMV is an important cause of congenital infection occurring in 0.3% to 2% of all live births. More than 10% of congenitally infected neonates have symptoms at birth, such as central nervous system involvement, hematologic abnormalities, hepatosplenomegaly, and 10–15% of the infected newborns without symptoms at birth will develop long-term sequelae [1]. It is generally accepted that symptomatic congenital HCMV infection occurs mainly after primary infection during pregnancy. Nevertheless, increasing evidence shows that the outcome of nonprimary maternal infection may be symptomatic and severe [23,24].

2.2. HCMV Mononucleosis

Primary HCMV infection occurs in 0.1% to 0.6% of blood donors and shows a prolonged course as detected by sensitive virological methods, such as PCR [25,26]. Primary infection is usually asymptomatic in immunocompetent individuals [26,27], but the virus occasionally gives rise to clinical illness, i.e., a self-limited mononucleosis-like syndrome. Mononucleosis due to HCMV is clinically similar to the more common Epstein-Barr virus (EBV) mononucleosis. Malaise, headache and high fever are typical features and may persist for weeks. A variety of other clinical abnormalities have been associated with HCMV infection in the normal host, including the Guillan-Barré syndrome, peripheral neuropathy, meningoencephalitis, myocarditis, hepatitis, pneumonitis, hemolytic anemia, and thrombocytopenia [1].

2.3. HCMV Disease in Immunosuppressed Patients

HCMV is one of the most common opportunistic pathogens that complicate the care of immunocompromised patients. Infection occurs by reactivation of latent virus, by re-infection in patients who are already infected, or by primary infection [1]. HCMV infection in immunocompromised individuals cause different clinical syndromes in different groups of patients and the severity of the infection parallels the degree of the immunosuppression. The

most severe infections are seen in recipients of allogeneic bone marrow or allogeneic stem cell transplant (alloSCT) and in AIDS patients with low CD4⁺ counts. Symptomatic HCMV infections are also often observed in solid organ transplant recipients.

The incidence of HCMV disease has dramatically decreased in HIV-positive individuals with the advent of an effective anti-retroviral therapy, as a result of an induced HCMV-specific immunity coupled with a decrease in HCMV reactivation [28]. The virus is still an important cause of morbidity and mortality in other groups of immunosuppressed patients, such as transplant recipients, and remains the single most important pathogen affecting these subjects.

The effects of HCMV infection in transplant patients can be divided into two categories: the direct effect of the infection, which causes tissue invasive disease, severe symptoms and/or death; and the indirect effects, which include viral mechanisms related to allograft injury and immunosuppression [29,30]. HCMV appears to promote classical rejection, as well as a vasculopathy of the allograft, which has a major impact on the longevity of the allograft (as reviewed in [6]). In fact, evidence from several cohort studies shows that HCMV infection is associated with an increased risk of acute graft rejection in solid organ transplant patients and with long term complications, such as chronic graft rejection and chronic GVHD in solid organ recipients and in alloSCT, respectively [16,31,32].

In addition, evidence suggests that HCMV is a strong immunosuppressive agent, and a rising incidence of bacterial and fungal infections have been observed in both alloSCT and solid organ transplant patients suffering from active HCMV infection [6,33]. The contribution of active viral infection in worsening the iatrogenic-induced immunosuppression and in possibly triggering acute and/or chronic graft rejection implies that HCMV can induce a strong modulation of the host immune system.

2.4. HCMV Infection in Patients with Autoimmune Disorders

Recently, laboratory signs of active HCMV infection have been observed in concomitance with the onset or during the course of autoimmune diseases [3], as extensively reviewed below.

3. HCMV Infection and Autoimmunity

3.1. Induction of Autoantibodies

HCMV-infected patients often develop autoimmune phenomena. For example, natural antiphospholipid and anti-CD13-specific autoantibodies have been found in HCMV-infected BMT [34-36], and anti-CD13 antibodies have been associated with the development of chronic GVHD in these patients [37]. In solid organ transplant recipients, non-organ-specific autoantibodies such as antiendothelial cell autoantibodies, anti-smooth muscle cell autoantibodies, and anti-nucleus autoantibodies, are associated with HCMV infection [38,39], possibly increasing risk for humoral and chronic allograft rejection [40,41]. In addition,

hypergammaglobulinemia, cryoglobulinemia, and autoantibody production are features of HCMV-induced mononucleosis and post-perfusion syndrome [42-44].

3.2. HCMV as a Potential Trigger of Autoimmune Disorders

3.2.1 *Vasculitides and scleroderma*

A temporal link has been observed between active HCMV infection and onset of autoimmune disorders in previously healthy individuals. Interestingly, in nine out of ten cases we are aware of, the presence of HCMV replication has been associated with the development of autoimmune vasculitis or scleroderma, suggesting a virus-induced vasculopathy as trigger of autoimmunity.

In the first case, active HCMV infection has been described in association with a newly diagnosed necrotizing vasculitis that resulted positive for perinuclear antineutrophil cytoplasmic antibodies (p-ANCA). The patient, a 69 year old woman, presented with kidney and pulmonary involvement and polyneuropathy. Serological signs of active HCMV infection were detected and HCMV DNA was found in the sputum of the patient. Antiviral treatment in combination with prednisolone resulted in complete remission, favouring the diagnosis of HCMV-induced vasculitis [45].

In four additional patients, the onset of cutaneous vasculitis has been observed in temporal association with active HCMV infection, as detected by serological and virological methods. In two of these cases, skin manifestations were part of a multiorgan vasculopathy represented by mixed cryoglobulinemia and multiorgan vascular thrombosis. Notably, in three out of four cases, the symptoms responded to therapy with ganciclovir in association or not with prednisone [46].

Furthermore, the development of vasculitis that was positive for ANCA with cytoplasmic pattern (c-ANCA) has been described after HCMV mononucleosis in a previously healthy young woman. When undergoing HCMV mononucleosis, the patient exhibited extraordinarily high plasma levels of some proinflammatory cytokines and autoantibodies. After the onset of vasculitis, HCMV genomes were found in blood and urine, and HCMV antigens were detected in the inflammatory lesions of the kidney. These findings suggest a role for the virus in triggering and maintaining the autoimmune process [44].

Finally, serological signs of HCMV infection have been observed in association with virus RNA in endothelial cells of skin biopsies in three cases presenting with sudden onset of autoimmune sclerosis. Only one patient was treated with ganciclovir; there was, however, no change in her clinical course [46].

3.2.2 *Autoimmune encephalitis*

Abrupt onset of autoimmune encephalitis has been recently observed in a previously healthy young woman that concomitantly suffered of active HCMV infection (Varani et al., unpublished data). The patient was admitted to the hospital with fever, confusion and personality's changes, followed by myoclonies, convulsion and general hyperreactivity to sensorial stimuli. Diagnosis of primary HCMV infection was posed by serological means, and HCMV DNA was detected in the cerebrospinal fluid and blood at the onset of the clinical syndrome. Long-course treatment with ganciclovir combined with intravenous

immunoglobulins and decreasing doses of cortisone resulted in significant amelioration of the neurological status, supporting the diagnosis of HCMV-induced autoimmune encephalitis.

Thus, HCMV can be considered a potential trigger of autoimmune disorders involving the vascular system and the central nervous system. The possibility that active HCMV infection and onset of these cases of autoimmunity were concurrent but causally unrelated events cannot be excluded. However, the temporal coincidence between active viral infection and onset of autoimmunity in these cases and the frequent effectiveness of antiviral therapy, when employed, are evidence in support of the fact that the virus can trigger incipient autoimmunity in predisposed individuals.

3.2.3 Post-transplant diabetes mellitus

The hypothesis that virus infection may provoke type I diabetes in genetically predisposed individuals is generally accepted [47]. Clinical evidence suggests that both asymptomatic HCMV infection and HCMV disease are independent risk factors for early development of new-onset post-transplant diabetes mellitus (PTDM) in renal transplant recipients [48,49]. Furthermore, a HCMV donor-positive/recipient-negative serostatus is a risk factor for the development of PTDM among pediatric renal transplant patients [50] and active HCMV infection has been indicated as a risk factor for the development of PTDM in adult liver transplant patients [51]. Additional suggestive evidence for HCMV playing a role in PTDM shows that the incidence of PTDM has been significantly reduced in the era of institution of preemptive regimens for treatment of HCMV infection [52].

Interestingly, transplant patients with active HCMV infection exhibit a significant lower insulin secretion than controls without infection [48]. HCMV may damage the β -cells by direct viral infection, by cytotoxic effects of infiltrating activated effector lymphocytes or by induction of pro-inflammatory cytokines leading to altered β -cell functionality or apoptosis [53]. However, experimental data indicating the inhibitory role of HCMV on β -cell function are limited and not conclusive. Thus, further studies are needed to prove the potential causal relationship between HCMV infection and PTDM. Nevertheless, the mentioned studies support the concept that HCMV may provoke the development of autoimmunity.

3.3. Active HCMV Infection in Autoimmune Disorders

Current evidence suggests that HCMV reactivates from latency by allogeneic stimulation in monocytes from seropositive donors [21] and that interferon (IFN)- γ and TNF- α are necessary for the development of HCMV-permissive M ϕ s [54]. These findings have important clinical implications since immune-mediated processes involving activation of T cells and production of these cytokines may facilitate the reactivation of latent HCMV from monocytes *in vivo*. Thus, chronic inflammation associated with autoimmune diseases could provide an ideal microenvironment for reactivation of latent HCMV in inflammatory M ϕ s. Inflammation also induces DC maturation, which can provoke virus reactivation from latency [22]. Once reactivated *in situ*, the virus may contribute to the pathogenesis of autoimmune diseases by a number of immunopathological mechanisms as described below (see point 4).

3.3.1. *Ulcerative colitis*

It is known that HCMV can efficiently replicate in epithelial cells of intestinal mucosa [55,56]. In the last years a large body of studies have focused on the possible pathogenic role for HCMV replication in inflammatory bowel diseases (IBDs). Notably, HCMV antigens have been found in 30-90% of biopsies from patients with IBDs [57,58] and the presence of HCMV infection has been associated with more severe disease in patients with ulcerative colitis (UC) [59,60]. In addition, a number of studies have shown the occurrence of HCMV infection in 20-80 % of steroid-refractory active UC [61-71], suggesting a pathogenic role for the virus in worsening inflammation. This hypothesis is further corroborated by the effectiveness of antiviral treatment in patients with steroid resistant UC and active HCMV infection, as described in isolated cases or small groups of patients [61,62,65,67-69,72].

Interestingly, diagnosis of active HCMV infection was posed by detection of HCMV by immunohistochemistry (IHC) and/or PCR in mucosal biopsy specimen from the colon in most of these studies [59-63,65-68,70], while analysis of the virus in the blood, when performed, showed low viral load or absence of viremia [61,68]. These findings imply that HCMV replication mainly occurs in the colon of patients with UC. Furthermore, recent evidence shows that all patients with steroid refractory UC undergoing active HCMV infection were previously HCMV-seropositive, which suggests reactivation of the virus at the site of inflammation during the active phase of the disease [68].

A schematic representation of sequelae during bowel inflammation in HCMV-seropositive patients has been classified into 3 phases; initiation, reactivation, and consolidation phase. During the initiation phase, the mucosal inflammatory responses induce expression of soluble mediators, which trigger the hosts' latently infected cells and migration of monocytes and DCs into the inflamed mucosa. Next is the reactivation phase, in which latently infected monocytes differentiate into tissue M ϕ s and DCs. Finally, during the consolidation phase, active replication takes place predominantly in endothelial cells, and possibly is responsible for further aggravating the inflammatory response [73]. The frequent occurrence of HCMV reactivation in the colonic mucosa from patients with refractory UC and the possible pathogenic role of the virus in worsening inflammation raise the question on whether patients with UC should be screened for HCMV serostatus and whether antiviral protocols should be employed to prevent or treat virus replication in HCMV-seropositive patients suffering of UC [74].

3.3.2 *Autoimmune disorders with major vascular involvement; vasculitis and systemic sclerosis*

Increasing evidence suggests that- apart from hepatitis C virus-other viral infections, such as HCMV, EBV, HIV or Parvovirus B19, may accompany systemic vasculitis [75]. In such disorders, HCMV infection has been temporally associated with the onset of inflammatory disease [44-46] or, as in other inflammatory connective tissue diseases, with the implementation of immunosuppressive therapy [76]. Furthermore, in a recent study that screened 80 patients with vasculitides for the presence of antibodies against viral, bacterial and parasitic infection, IgM antibodies against HCMV were significantly more common among patients with c-ANCA-positive vasculitis than among controls [77]. HCMV infection may therefore exert an active role in initiating and/or maintaining inflammation in vasculitides.

HCMV has also been implicated in triggering vascular damage during systemic sclerosis [78]. Clinical onset of systemic sclerosis has been temporally associated with the presence of active HCMV infection [46]. Furthermore, autoantibodies specific for systemic sclerosis recognize the late HCMV protein UL94 and are associated with the diffuse form of the disease but not with the limited form, suggesting a viral correlation with the severity of systemic sclerosis [79,80]. Intriguingly, such antibodies against the late HCMV protein UL94 induce apoptosis of endothelial cells and activate dermal fibroblasts *in vitro*, thus causing the two hallmark of the disease; vascular damage and fibrosis [81].

3.3.3 Other autoimmune diseases

Laboratory signs of acute HCMV infection and anti-HCMV antibodies have also been observed in other autoimmune diseases. Subclinical systemic HCMV infection has been found in psoriatic patients and has been related to high levels of TNF- α expression [82].

Moreover, HCMV DNA, specific antigens and infectious virus particles have been detected in synovial tissue and fluid from the joints of 10-50% patients with rheumatoid arthritis [83-86]. In such patients, the virus was strictly confined in the inflamed joints, while no signs of systemic active infection were observed (Söderberg-Naucler, unpublished observation).

Active HCMV infection is also frequent in children with systemic lupus erythematosus (SLE) [87], and the virus has been implicated in the development and/or exacerbation of the disease in a number of case reports [88-91]. Serological signs of active HCMV infection have been detected in 10% of patients with SLE and the presence of viral infection/reactivation is associated with higher scores of disease activity [92]. In addition, patients with SLE also exhibit a stronger humoral activity in response to HCMV [93] and in particular to the HCMV-structural protein pp65 [94] as compared to healthy donors or patients suffering of other autoimmune disorders. In a recent study, HCMV was the only infectious agent for which both a higher rate of IgM seropositivity and higher antibody titers were observed in SLE patients versus controls [95].

4. Generation of HCMV-Induced Immunopathology

4.1. Humoral Autoimmune Phenomena

How HCMV manipulates the immune response to induce autoimmune phenomena is not fully understood. One mechanisms that might be responsible for virus-induced humoral autoimmunity is viral mimicry [96]. The HCMV genome encodes a series of genes that are homologous to cellular genes, and host response to viral determinants may cross react with host tissues, eventually leading to autoimmunity (**Figure 1A**). This mechanism likely explains the generation of autoreactive pathogenetic autoantibodies that cross react with HCMV during systemic sclerosis [78].

Humoral autoimmune phenomena may also be generated by nonspecific B-cell activation caused by HCMV. Autoantibody production has been extensively described during infection with EBV [97]. Similarly to EBV, HCMV is a polyclonal B-cell activator *in vitro*, and the B-

cell overresponse does not require viral replication [98]. In addition, HCMV interacts with toll-like receptor (TLR)7 or 9 in human plasmacytoid DCs, leading to high secretion of IFN- α , B-cell proliferation, and antibody production in the presence of IL-2 [99]. This DC-mediated mechanism may potentially contribute to polyclonal B-cell activation and to the development of autoimmune phenomena during HCMV infection in the natural host (**Figure 1A**).

4.2. HCMV-Induced Inflammation

4.2.1 Generation of CD4 $^{+}$ CD28 $^{\text{null}}$ T cells

A peculiar subset of CD4 $^{+}$ T cells lacking the costimulatory molecule CD28 is expanded in patients with autoimmune diseases, such as rheumatoid arthritis (RA), Wegener's granulomatosis, dermatomyositis and polymyositis, multiple sclerosis, and inflammatory bowel diseases [100-103]. These cells display pathogenic properties *in vitro* [104], are a major source of Th1-type cytokines in the lesions of Wegener's granulomatosis [105] and are associated with early atherosclerotic vessel damage in RA patients [106]. In addition, CD4 $^{+}$ CD28 $^{-}$ and CD8 $^{+}$ CD28 $^{-}$ T cells are the predominant T cells infiltrating the inflamed muscles in patients with dermatomyositis and polymyositis and secrete IFN- γ upon HCMV specific antigen stimulation [103]. Interestingly, CD4 $^{+}$ CD28 $^{-}$ T cells appear to exist almost exclusively in HCMV-infected individuals [103,107]. In RA patients and healthy controls, CD4 $^{+}$ CD28 $^{-}$ lymphocytes react specifically with several HCMV epitopes [104]. *In vitro*, end-stage differentiation of these T cell subset is crucially dependent on IFN- α secreted by HCMV-stimulated plasmacytoid DCs [108]. Of note, accumulation of plasmacytoid DCs has been reported in lesions of patients with autoimmune diseases [109-112]. Thus, it has been speculated that chronic HCMV replication in inflammatory lesions, possibly by contribution of plasmacytoid DCs, may be the driving force in the differentiation of CD4 $^{+}$ T cells into pro-inflammatory, pathogenetic CD28-null cells, thereby contributing to local chronic inflammation in autoimmune disorders [104] (**Figure 1B**).

4.2.2. NF- κ B and other inflammation factors

Besides inducing end-stage differentiation of pathogenic T cells, HCMV may sustain chronic inflammation by other means. *In vitro*, HCMV infection rapidly induces translocation of NF- κ B into the nucleus, which then promotes the production of TNF- α leading to further activation of latent HCMV and additional upregulation of the inflammatory response [113]. The interplay between HCMV and TNF- α is supported in clinical studies that correlate circulating TNF- α levels with HCMV replication in patients with atopic dermatitis [10] or sepsis [9,11].

Importantly, HCMV transiently induces cyclooxygenase 2 expression in infected fibroblasts and subsequent release of prostaglandin E2, an important mediator of inflammation [114]. HCMV also stimulates 5-lipoxygenase expression that is crucial for the synthesis of leukotriene B4, a powerful chemoattractant (**Figure 1B**). As a clinical correlation of these *in vitro* findings, a massive leukocyte infiltration has been observed around HCMV-infected vessels in lesions of inflammatory bowel diseases [115].

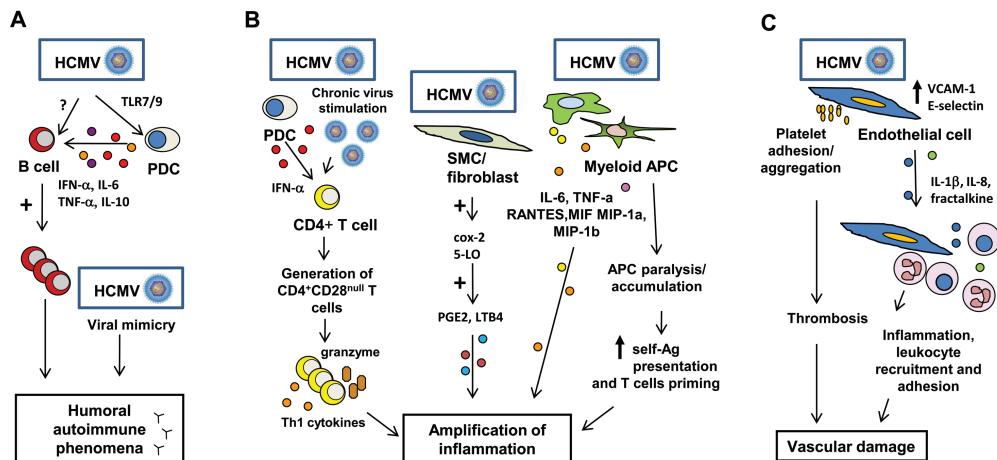


Figure 1. Proposed mechanisms by which HCMV can induce host immunopathology. A; HCMV-induced autoantibody production. B; Enhanced inflammation caused by the virus. C; HCMV-induced vascular damage. TLR7/9; toll-like receptor 7/9, PDC; plasmacytoid dendritic cell, SMC; smooth muscle cell, 5-LO; 5-lipoxygenase, cox-2, cyclooxygenase, PGE2; prostaglandin E2, LTB4; leukotriene B4, MIF; macrophage migration inhibitory factor, MIP-1 α ; macrophage inflammatory protein 1- α , MIP-1 β , macrophage inflammatory protein 1- β , VCAM-1; vascular cell adhesion molecule-1.

4.2.3 Antigen presenting cell paralysis

A number of evidence indicate that HCMV can efficiently inhibit migration of various subsets of myeloid antigen presenting cells (APCs) *in vitro* [116-119]. This may help sustain inflammation in HCMV-infected lesions of autoimmune disorders. In fact, APCs recruited to the area of HCMV replication would be quickly infected [120,121], lose responsiveness to chemoattractants [116-119], and thereby be retained *in situ*. Increasing evidence shows that HCMV-infected APCs can release a number of inflammatory mediators [116,119,122]. Such soluble factors may then induce maturation of accumulated professional APCs, which may in turn overprocess and overpresent viral and cellular antigens to large numbers of T cells, including autoreactive T cells, leading to their activation and to the progression of autoimmunity by epitope spreading [123] (**Figure 1B**). Thus, blocking APC migration and increasing release of proinflammatory mediators is a potential mechanism by which the virus exacerbates chronic inflammation. However, no studies have so far demonstrated such a phenomenon *in vivo*, i.e. in HCMV-infected target tissues of patients with autoimmune diseases.

4.3. Vascular Damage

Viral infections can contribute to the pathogenesis of vasculitides by various mechanisms, such as infection of endothelial cells causing cellular dysfunction or death, immune-mediated injury of the vessel wall, and hemorheological dysfunction due to increased procoagulant activity [124,125].

HCMV infection can cause vasculitis in the gastrointestinal tract, the central nervous system, and skin [126]. Direct pathogenic effects of HCMV on endothelial cells with

characteristic cytological features are mainly seen in immunocompromised patients, such as HIV-infected subjects, patients with underlying malignancies, and transplant recipients [126-129]. Notably, HCMV appears to have a specific tropism for vascular endothelium and can productively infect endothelial cells *in vitro* [130-132]. HCMV-infected endothelial cells appear to be dysfunctional, owing to diminished expression or activity of endothelial nitric oxide synthase [133], augmented release of the neutrophil migration regulator IL-8 [134], increased secretion of the proinflammatory cytokine IL-1 β , and upregulation of adhesion molecules that promote leukocyte adhesion [135] (**Figure 1C**).

The role of virus-induced cytokines and chemokines in the initiation and exacerbation of vascular damage is a growing research area. Host CD4 $^{+}$ T cell responses to HCMV antigen can induce expression of IFN- γ and TNF- α at levels sufficient to induce the expression of fractalkine, a key marker of inflammation in endothelial cells [136]. Once induced by HCMV antigens, fractalkine plays a key role in recruitment and mobilization of natural killer cells and monocytes, which are specifically involved in endothelial cell damage [137]. Thus, HCMV-associated chronic endothelial cell inflammation and damage can also result from a chemokine-mediated immunopathogenetic effects (**Figure 1C**).

Finally, HCMV infection can change endothelium from an anticoagulant to a procoagulant status [138] and induce platelet adherence and aggregation on infected endothelium [139]. In addition, acute and latent phases of HCMV infection have been associated with thrombosis [140,141]. Such an effect could contribute to the vascular damage induced by HCMV and to the genesis of vascular inflammation (**Figure 1C**). Thus, HCMV can induce endothelial cell dysfunction and inflammation by a number of different means.

5. Anti-HCMV Therapy: Effect on Immunopathology

Despite the substantial progresses achieved in the field of transplantation, HCMV continues to be a significant cause of morbidity in transplanted recipients, causing a number of direct and indirect effects. The direct effects of HCMV infection are well managed by treatment with ganciclovir or its pro-drug valganciclovir. Conversely, optimal therapy for treating and/or preventing virus-induced immunopathology causing organ rejection and/or immunosuppression in recipients of allografts still remains to be defined [6]. Similarly, there are no guidelines for the treatment of potential viral induced immunopathology in patients with autoimmune disorders.

When HCMV replication is detected in patients with autoimmune diseases, clinicians are faced with a therapeutic dilemma; should antiviral therapy be initiated and the level of immunosuppression be reduced to facilitate specific antiviral immune response, with the risk of further worsening the autoimmune disorder or should the immunosuppressive agents be increased to suppress inflammatory activity?

As mentioned, in four patients with vasculitis that tested positive for active HCMV infection complete remission of the autoimmune process was achieved upon treatment with ganciclovir alone or in combination with HCMV immunoglobulin and/or cortisone [45,46]. A successful outcome was also reached in a case of autoimmune encephalitis following primary HCMV infection by employing a long-course treatment with ganciclovir associated to

intravenous immunoglobulins and decreasing doses of prednisone (Varani et al; unpublished observation). The positive outcome in these cases suggests that a two-armed treatment including inhibition of viral replication by antivirals and immunomodulation by intravenous IgG [142] and/or prednisone may be warranted when the onset of autoimmune disorders is temporally associated with active HCMV infection.

Increasing evidence shows that HCMV can also worsen the clinical outcome of UC. It has been therefore suggested that antiviral therapy or other medical therapy that could reduce virus replication may be beneficial when HCMV infection is histologically proven at the site of inflammation [68]. In this view, three therapeutic options have been considered; 1. Employment of antiviral compounds; 2. Modulation of immunosuppression; 3. Modulation of inflammation.

In support to the first option, a number of studies has described successful outcome by employing antivirals such as ganciclovir or oral valganciclovir in isolated cases or small groups of patients with steroid refractory UC and active HCMV infection [61,62,65,67-69,143].

As a second option, modulation of the immunosuppressive therapy can lead to a stronger anti-HCMV specific immune response and this option has been successfully employed as the only treatment [61] or in association to antivirals [61,64,69].

Finally, since HCMV reactivation is strictly dependent on inflammation [9], treatments that reduce colonic inflammation, such as employment of anti-TNF- α compounds or leukapheresis can also lead to reduced viral replication in UC patients, as shown by two recent reports [72,144].

Thus, employment of antivirals or indirect blockage of virus replication could be effective means for the treatment of HCMV-positive refractory UC. However, large randomized, controlled studies are needed to further elucidate the potential effectiveness of such treatments in UC patients as well as other patients with autoimmune disorders undergoing active HCMV infection.

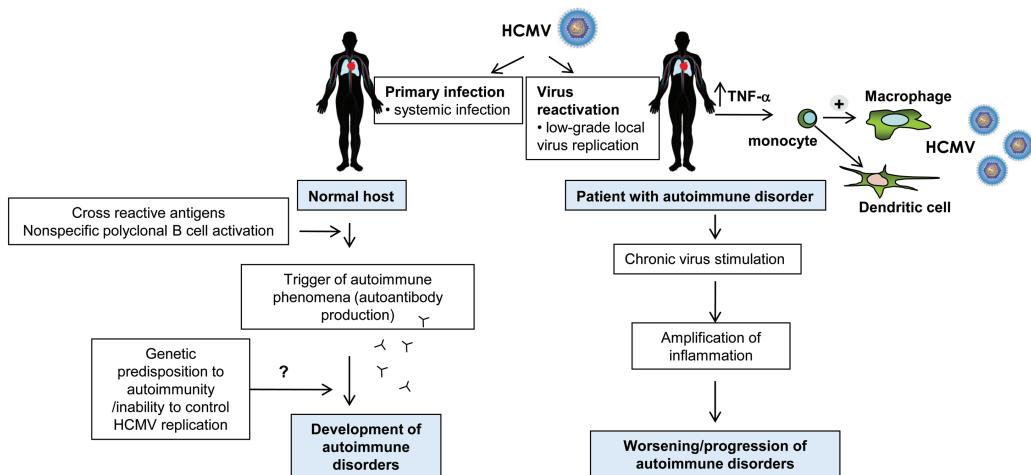


Figure 2. Proposed mechanisms by which HCMV may trigger or enhance autoimmunity in two different groups of patients, i.e. previously healthy subjects and patients with autoimmune disorders.

6. Conclusion

During acute HCMV infection, patients often suffer from immunological dysfunctions. Autoimmune phenomena are common in HCMV-infected patients, and a variety of autoantibodies have been detected in patients with active, systemic HCMV infection [34-36,38,39,44].

In potentially predisposed patients, primary HCMV infection may trigger autoimmune disorders, and development of vasculitides, scleroderma and autoimmune encephalitis has been reported in concomitance or immediately after active HCMV infection [44-46] (Varani et al., unpublished data) (**Figure 2**).

Clearly, various mechanisms can potentially be involved in inducing immunopathology upon HCMV infection. Virus-induced activation of APCs, particularly plasmacytoid DCs, may contribute to T cell-independent activation of B cells and to the generation of autoimmune phenomena. Nonspecific hyperactivation of humoral immunity may also impede the development of specific B cell responses—a potential mechanism of viral immune evasion. However, B cell hyperactivation may be a simple epiphenomenon that does not affect the viral efficiency, but it nevertheless could have important clinical implications for infected patients. This has been demonstrated in transplant recipients, where autoantibodies can contribute to the development of GVHD in HCMV-infected alloSCT patients and to graft rejection in solid organ recipients [36,40,41].

In addition to acute systemic HCMV infection, low-grade HCMV replication appears to be a frequent event in autoimmune diseases [3]. The virus may contribute to the progression of autoimmune disorders by worsening inflammation and mimicking autoimmune tissue destruction (**Figure 2**). Indeed, HCMV can induce inflammation by different means. For example, it can induce cyclooxygenase 2, and 5-lipoxygenase expression and downstream prostaglandin E2 and leukotriene B4 synthesis in infected cells [114,115]. Moreover, a chronically active HCMV infection may induce a pro-inflammatory, pathogenic T cell response [104] that generally cannot eliminate the virus and may consequently amplify inflammation in a sustained way. Furthermore, accumulation of HCMV-infected myeloid APCs may sustain inflammation by epitope spreading and bystander activation [123]. Thus, enhancement of inflammation may be a distinctive signature of low-grade local virus replication that is commonly detected in inflamed lesions of autoimmune diseases.

Finally, HCMV could contribute to the pathogenesis of vasculitides by various mechanisms, such as infection of endothelial cells causing, cell lysis, cellular dysfunction, immune-mediated injury of the vessel wall, and hemorheological dysfunction [133-139].

Many so-called autoimmune diseases may eventually turn out to represent immunopathologies induced by one or several yet unknown or unrecognized viral infections, as stated by Rolf Zinkernagel [145]. It is currently assumed that autoimmunity is a breakdown in self-tolerance. However, autoimmunity associated with primary immunodeficiency diseases is not simply defective self-tolerance; rather, an inability of an inherently defective immune system to eradicate persisting microbial pathogens that results in chronic inflammatory responses by less effective alternative immune pathways [146]. Herpesviruses are the archetypal persisting infectious agents that, even in individuals with essentially normal immunity, intermittently escape from normal immune control and produce symptomatic disease. Among these viruses, HCMV has developed a myriad of strategies to evade the host

immunity. Concomitantly, virus replication induces immune modulation and triggers inflammation. Incomplete eradication that leads to chronic replication of HCMV in subjects with potential hidden defects in immune genes may therefore contribute to potentially trigger or perpetuate long-lasting autoimmune disorders.

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Chapter 9

Behçet's Disease: Symptoms, Diagnosis and Treatment

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Abstract

Behçet's disease (BD) is a chronic, relapsing, systemic vasculitis of unknown etiology with the clinical features of mucocutaneous lesions, ocular, vascular, articular, gastrointestinal, urogenital, pulmonary, and neurologic involvement. It is believed to be due to an auto-immune process triggered by an infectious or environmental agent in a genetically predisposed individual. HLA-B51 is the most strongly associated risk factor. The disease usually starts around the third decade of life. Mucocutaneous lesions figure prominently in the presentation and diagnosis, and may be considered the hallmarks of BD. Therefore, their recognition may permit earlier diagnosis and treatment. Although, the treatment has become much more effective in recent years, BD is still associated with severe morbidity and considerable mortality. The main aim of the treatment should be the prevention of irreversible organ damage. Therefore, close monitoring, early and appropriate treatment is mandatory to reduce morbidity and mortality. We will review symptoms, diagnosis and the current state of knowledge regarding the therapeutic approaches for BD.

Keywords: Behçet's disease, symptoms, diagnosis, treatment.

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Introduction

Behçet's disease (BD) is a systemic inflammatory vasculitis of unknown etiology, characterized by relapsing episodes of oral aphthous ulcers, genital ulcers, skin lesions, ocular lesions and other manifestations, including vascular, gastrointestinal, neurological involvement (1, 2). First description of BD, also known as the Old Silk Route disease, has been attributed to Hippocrates in the 5th century BC, in the "Third book of endemic diseases" (2). In 1937, Behçet, a Turkish dermatologist, identified the 3 major signs (recurrent oral aphtae, genital ulcerations, recurrent hypopyon uveitis) and grouped them on a clinical entity, named "triple symptom complex".

The onset of BD is heterogeneous, has variable organ involvement and results in considerable morbidity and increased mortality (3, 4). Recently in Turkey, country with the highest prevalence rate, a cost-analysis of BD was made, proving that it is a considerable economic burden both in direct as in indirect costs (5).

The cause of BD is unknown. It is believed to be due to an autoimmune process triggered by an infectious or environmental agent (possibly local to a geographic region) in a genetically predisposed individual (6, 7). The most probable hypothesis is that of an inflammatory reaction set off by infectious agents such as herpes simplex virus 1 or streptococcus species or by an autoantigen such as heat shock proteins in genetically predisposed individuals (8).

In this review we will describe symptoms, main clinical manifestations, diagnosis and the current state of knowledge regarding the therapeutic approaches for BD.

Epidemiology

BD is a worldwide disease but occurs most frequently between in countries that stretch along the Old Silk Route, an ancient commercial route that stretched between the Mediterranean, Middle East and Far East (2, 9). The estimated prevalence in these countries is between 1/1000 and 1/10,000, being the highest prevalence is in Turkey (80-370 cases per 100,000) (2), followed by Japan (7-8.5/10,000). Reported prevalence in the Asian countries is between 13.5 and 20 cases/100,000; in Western countries (Northern Europe and United States of America) it is lower, ranging from 0.12 to 0.64/100,000 (2, 9, 10).

BD is slightly more frequent in men (2, 9). Men also have a worse clinical course because of a higher risk of eye, cardiovascular and neurological involvement and a younger age of disease onset (2). In addition, men tend to have a worse evolution of clinical features (11, 12).

Disease onset is typically between 30-40 years, rarely appearing before puberty and after the age of 50 (2, 13-16). Younger patients tend to have a more severe disease, however, recent studies, suggest that late-onset BD (age of onset at or after 40 years) may not be as benign as previously thought (2, 9, 16-18).

Familial occurrence has been reported in 1-18% of the patients, occurring especially in juvenile BD(2, 12).

Etiology and Pathogenesis

Like other autoimmune diseases, the precise cause of Behcet's disease is still unknown (2, 9). The human leukocyte antigen (HLA)-B51 allele appears to confer an increased risk to the development of the disease, as it is found in higher prevalence in populations endemic to countries around the Silk Road (2, 6, 9, 19). However this association is less clear in Western countries (2). Therefore, BD is believed to be due to an autoimmune process triggered by an infectious or environmental agent in a genetically predisposed individual (2, 6, 19).

Genetics and human leukocyte antigen (HLA) typing: Most cases of BD are sporadic and the parents of patients are unaffected, but a familial aggregation has been reported previously, especially in juvenile BD (2, 9, 20-32). However an increased prevalence of isolated manifestations of the disease , such as recurrent oral ulcers, genital ulcers, or a positive skin pathergy test, has been observed among first degree relatives (27, 30-31). Mendelian inheritance was not observed in analysis of multicase families (31), but an earlier disease onset in the children of affected parents (26) was found in some studies (26).

HLA-B51 allele located on chromosome 6p, in the major histocompatibility complex (MHC) locus, has been the most strongly associated risk factor for BD in some eastern countries (2, 9). Other genes present in the MHC locus have been studied, including MICA (MHC class I related gene) and TNF genes; however, their participation is considered to be due to linkage disequilibrium with HLA-B51 gene (2). In a study of tumor necrosis factor (TNF) polymorphisms, HLA-B5701 was associated with disease susceptibility in Caucasians from the United Kingdom (7).

Several other genes, located outside the MHC region have been proposed to be involved in BD pathogenesis, namely genes of interleukin-1 (IL-1), coagulation factor V, intercellular adhesion molecule-1 (ICAM-1) and endothelial nitric oxide synthetase (eNOS) (2, 34-38), with conflicting results Mediterranean Fever gene (MEFV), on the other hand, appears to confer susceptibility to BD (39-41).

Environment (infectious agents, heat shock proteins) and self-antigens: It has been shown that individuals from endemic areas who have immigrated to areas with low prevalence of BD have an intermediate risk for developing BD, suggesting that environmental factors play an important role in the etiology of BD (2). Reports have studied herpes simplex virus-1 and *Streptococcus sanguis* (2, 9, 42, 43), however up to date no single infectious agents has been solely implicated as the specific etiologic agent (2). The most accepted theory for the role of infectious agents is the cross-reaction leading to immune response, since various microorganism antigens have high homology with human proteins (like heat shock protein (HSP) 65,) (2, 43,44).

After an eye damage due to uveitis, retinal S antigen, located in the retina, is exposed and due to its homology to HLA-B51 and HLA-B27, and may elicit further immune mediated response (2, 43).

Cytokines and cells of the immune system: The levels of pro-inflammatory cytokines are elevated in serum and cerebral spinal fluid in BD patients, and in many cases correlate with disease activity (2, 6, 46). Th1-type polarization of immune response has also been shown to

occur in BD, the degree of which correlates directly with disease activity (2, 19, 47). The Th1-type polarization of immune response is associated with an increased production of IL-12, and IL-18, which in turn increases neutrophil functions (19). Studies have demonstrated that neutrophils are hyperactive in BD, with increased chemotaxis, phagocytosis, superoxide production, and myeloperoxidase expression (2, 49, 50).

The precise mechanism of neutrophils hyperactivity is not known, however, T cells are fundamental in their activation (2, 918). It is currently believed that complex interactions between T cells, neutrophils and APC are involved in the immune pathogenesis of BD (2, 19).

Clinical Features

BD can affect nearly every system of the body, and have a tendency to recur (2, 9). Presentation and evolution may vary due to ethnic, geographical and individual differences (1, 2, 9). Oral ulcers (OU), genital ulcers (GU), and cutaneous lesions together with ocular lesions and arthropathy are the most frequent features of the disease (1).

Mucocutaneous lesions: Aphtous ulcerations are usually the first and most frequent recurrent manifestation of BD (1, 2, 9). Their recognition may permit earlier diagnosis and treatment, with satisfactory results (51). OU and GU of BD are characterized by recurrent and painful ulcerations and a relapsing course are strongly suggestive of BD. They are identical to aphthae in appearance, but they tend to be more frequent and occur in crops. Most of OU heal without scarring, however bigger ulcers, especially in the oropharynx may leave scars. They can be classified as (51):

- Minor (most common) – 1–5 in number, diameter < 1 cm, shallow, surrounded by an erythematous halo, moderately painful, healing without scarring in 4–14 days
- Major (less frequent) – 1–10 in number, morphologically alike, although larger (>1 cm), more painful, more persistent and may heal with scarring in 2–6 weeks
- Herpetiform (the least common) – recurrent crops of numerous small (2–3 mm) and painful ulcers which may become coalescent.

Genital ulcerations in men typically develop on the scrotum and heal with scarring. In women, the labia are commonly affected although vaginal and cervical ulcers can also occur.

The cutaneous lesions are varied in presentation and include papulopustular lesions (PPL), superficial thrombophlebitis, extragenital ulcerations, reactivity of the skin to needle prick or injection (pathergy test), and other cutaneous vasculitic lesions. erythema nodosum (EN) and pyoderma gangrenosum have been observed in 1/3 of the patients, especially women (1, 2, 9, 52, 53).

Erythema nodosum like-lesions are confined to the lower legs and heal with residual pigmentation. Histologically a greater amount of vasculitis is observed when compared to idiopathic (EN). Superficial thrombophlebitis, may present as nodular skin lesions, and differential diagnosis with EN is mandatory, since the former have an important association with deep vein thrombosis.

The pathergy reaction represents hyperactivity of the skin to simple trauma and a positive pathergy test is considered highly specific for BD (1, 2, 9). A 20-gauge needle is inserted in the skin of the volar surface of the forearm to a depth of 0.5 cm and is then rotated and withdrawn. The appearance of a pustule 42mm at the puncture site 48 hours later constitutes a positive pathergy test. Pathergy is distinctly less common in North American and Northern European patients (10).

Ocular lesions: Ocular involvement is a serious complication of BD. Chronic, relapsing bilateral uveitis is a significant cause of morbidity among these patients. (1, 2, 9, 54). About 25% of patients with ocular disease may become blind despite treatment, although prognosis is improving with the use of modern immunosuppressant therapy and of a more aggressive treatment strategy (1-3, 11, 55-58).

Anterior uveitis with hypopyon, is a characteristic sign of ocular BD, but is observed only in about 20% of patients (59). It is frequently associated with retinal disease. Isolated anterior uveitis and conjunctivitis is rarely observed. Posterior uveal inflammation with involvement of the retina may be severe and lead to blindness. Retinal lesions observed include exudates, hemorrhages, papilledema and macular disease. Other ocular manifestations include iridocyclitis, keratitis, episcleritis, scleritis, vitritis, vitreous haemorrhage, retinal vasculitis, retinal vein occlusion, retinal neovascularization and optic neuritis (60, 61).

Clinical symptoms and signs include blurred vision, photophobia, lacrimation, floaters, hyperemia, periorbital or global pain (4). Recurrent attacks of inflammation lead to secondary complications namely, posterior and/or peripheral anterior synechia, iris atrophy, cataract due to inflammation and/or medication, secondary glaucoma (sometimes neovascular), atrophic retina, optic atrophy, macular oedema, macular degeneration, retinal veins occlusion, sheated vessels, chorioretinal scars and/or proliferative vitreoretinopathy, phthisis bulbi (1, 2, 9, 49, 56, 62).

Musculoskeletal involvement: Non-erosive, non-deforming oligoarthritis, affecting especially the lower extremities that usually last few weeks and is associated with systemic disease activity may be observed in approximately 50% of the patients. (2, 63). In most of the cases treating systemic disease improves the joints, no specific treatment for articular involvement is required in most cases. More rarely a chronic arthritis or osteonecrosis may be seen. Patients with arthritis tend to have more acneiform lesions, sacroiliac joint involvement is, however, not frequent in BD. Localized myositis may occur in BD, a more generalized form is rarely observed.

Vascular disease: BD is a systemic vasculitis affecting both arteries and veins of various sizes. (1, 64). Thrombophlebitis of superficial or deep veins is more often observed in lower extremities (1, 2, 9). Thromboses of the superior and inferior vena cava (occur 0.2–9% of cases), of dural sinuses and of supra-hepatic veins (Budd-Chiari syndrome – in 2–3.2% of patients) may occur, but is rare (1, 2, 9, 66-68).

Arterial involvement is less frequent, pulmonary arterial aneurysm however, has been described and is an important cause of mortality (65).

Cardiovascular involvement: The incidence and nature of cardiac involvement in BD are not yet clearly documented (69). Cardiac involvement includes pericarditis, myocarditis,

endocarditis, mitral valve prolapse, valve lesions, intracardiac thrombosis, endomyocardial fibrosis, myocardopathy, coronary artery lesions (69-71).

Gastrointestinal involvement: Ulceration can occur in any part of the gastrointestinal tract but is most frequently seen in the terminal ileum and cecum. Perforation and bowel infarction from mesenteric vasculitis have also been reported in BD. The ulcerations can be longitudinal, fissured, or aphthoid. Symptoms include abdominal pain, melena, or hematochezia. The clinical and endoscopic appearance may be indistinguishable from that of Crohn's disease, creating difficulty in differentiating these disorders (10). The frequency is variable in different populations (3–26%), being much more frequent in Japan than in the Middle East and Mediterranean (1,72,73).

Neurological involvement: Neurological involvement occurs in 5–10% of patients, usually during the first 5 years of the disease and is associated with high morbidity (1, 74). Central nervous system (CNS) is more frequently involved than the peripheral nervous system (2, 74). Clinical symptoms include bilateral pyramidal signs, extrapyramidal signs, hemiparesis, behavioural changes, sphincter disturbance, cranial nerve palsies, and headache (1, 2, 9, 74, 75). CNS involvement can be divided in parenchymal and non-parenchymal involvement. Parenchymal brain disease is more common (approximately 80%), particularly affecting the brainstem and/or basal ganglia, and is associated with worse prognosis. Non-parenchymal disease includes dural sinus thrombosis, arterial vasculitis and aseptic meningitis (74). CSF is often unspecific, however a high protein or cell count is associated with worse prognosis (1, 2, 9, 74).

Neuro-Behçet's Disease

In neuro-BD (NBD), the CNS can be involved in one or both of two ways: first, and most commonly, through the development of an immune-mediated meningoencephalitis, which predominantly involves the brainstem, but can also involve the basal ganglia, thalamus, cortex and white matter, spinal cord, or cranial nerves; and second, as a consequence of thrombosis within the dural venous sinuses. Headache is a common symptom in BD and does not necessarily indicate CNS involvement. Peripheral nervous system involvement is rare (76).

NBD was reported 2·8 times more often in men than in women (77). This high ratio of men to women was seen in nearly all the reports, although these findings have not been shown in three reports on western European patients (78-80) and one report from Korea (81). Men with BD were at greater risk for morbidity and mortality than were women in a long-term outcome study (82).

The age of onset of NBD is usually 20–40 years. Special caution needs to be applied in diagnosing NBD above the age of 50 years; exclusion of more common neurological disorders, particularly stroke and non-specific changes in white matter on cranial magnetic resonance imaging (MRI), is very important. Neurological complications can occur in children; however, the prevalence of such complications is difficult to ascertain from the

available reports, mainly because of the small numbers of patients and the lack of clear details and definition of the neurological findings (76).

Neurological complications can occur in children; however, the prevalence of such complications is difficult to ascertain from the available reports, mainly because of the small numbers of patients and the lack of clear details and definition of the neurological findings (75).

Classification: The classification of NBD is based in two categories of CNS involvement: parenchymal and non-parenchymal involvement (table 2) (75). Patients with BD who have headache without other neurological symptoms and signs or abnormalities on neuroimaging or in the cerebrospinal fluid (CSF) are not defined as having NBD.

Neuropathology: The neuropathology of parenchymal NBD in the acute phase involves meningoencephalitis with an intense inflammatory infiltration including polymorphs, eosinophils, lymphocytes, and macrophages, with areas of necrosis and apoptotic neuronal loss (82). Intense inflammatory infiltration of small vessels can occur, but fibrinoid necrosis is not seen. NBD is therefore not a cerebral vasculitis rather; it is an inflammatory perivasculitis (82-85).

The structures within the brainstem, thalamus, basal ganglia, and white matter are all seen to be affected. In the progressive phase, inflammatory infiltration remains, although immunohistochemical staining for lymphocytes and cytokines is less prominent (82).

Concentrations of IL-6 are persistently raised in the (cerebrospinal fluid) CSF, and axonal loss and gliosis are also seen at this stage (82-85). These findings correlate with the striking atrophy seen on MRI, particularly in brainstem structures, in advanced stages (86, 87).

Diagnosis: The diagnosis of neurological involvement in BD is done mainly by clinical means; the ancillary investigations noted below help to suggest alternatives, and especially infective complications of treatment, but there is no diagnostic test for NBD. The signs of systemic disease in patients who present with neurological disorders compatible with BD are important. No validated criteria for the diagnosis of NBD exist. Diagnostic criteria are usually helpful to determine standards for research or treatment trials (88).

Blood tests can help diagnose. Erythrocyte sedimentation rate has been found to be associated with disease activity (89). Blood count and biochemical screening is used to identify the nature and severity of the systemic disorder and to identify signs of an infective complication. In the case of cerebral venous thrombosis (CVT), a thrombophilia screen should be undertaken. Early studies suggested a higher prevalence of antiphospholipid antibodies and factor V Leiden mutations, but these data have not been confirmed (90).

CSF constituents are altered in around 70–80% of patients with parenchymal complications (73,91, 92). CSF protein is modestly raised in most cases (73,91). The CSF cell count is often prominently raised and there is usually a CSF neutrophilia in the early stages, replaced later by a lymphocytosis. Patients with CVT or intracranial hypertension have normal CSF constituents. However, in some series, up to 20% of patients present with coexisting parenchymal and vascular complications (79, 93).

Table 1. Criteria for diagnosis of Behçet disease (99)

Finding	Description
Recurrent oral ulceration	Minor aphtous, major aphtous, or herpetiform ulcers observed by the physician or reliably described by the patient, which recurred at least three times over a 12-month period.
Recurrent genital ulceration	Aphtous ulceration or scarring observed by the physician or reliably described by the patient.
Eye lesions	Anterior or posterior uveitis or cells in the vitreous body on slit-lamp examination; or retinal vasculitis detected by an ophthalmologist.
Skin lesions	Erythema nodosum, pseudofolliculitis, papulopustular lesions or acneiform nodules not related to glucocorticoid treatment or adolescence.
Positive pathergy test	Test interpreted as positive by the physician at 24–48 hours.

For a clinical definite diagnosis of BD patient must have recurrent oral ulceration plus at least two of the other findings in the absence of any other clinical explanations.

MRI shows the characteristic lesion in parenchymal involvement that is an upper brainstem lesion that extends into the thalamus and basal ganglia on one side (80, 87). Bilateral lesions are less common, but do occur. Such lesions can be seen as hyperintense T2 lesions with enhancement and are often associated with edema. These lesions reduce in size after treatment and can even disappear altogether on conventional low-strength MRI (94).

Diagnosis and Differentiation from other Conditions

As there are no pathognomonic clinical or laboratorial findings of BD, several diagnostic criteria have been developed during the years, all having in common the 3 major features of oral ulceration, genital ulceration and eye lesions.

Mason and Barnes in 1969 considered Behçet's diagnosis when 3 major criteria or 2 major and 2 minor criteria were present. The minor criteria included gastrointestinal lesions, thrombophlebitis, cardiovascular lesions, arthritis, CNS lesions and family history (95). Behçet's Disease Research Committee of Japan also defined diagnostic criteria in 1972, which were revised in 1987 and 2003 (96). O'Duffy (1974) considered diagnostic oral or genital ulceration and 2 other features and vasculitis on biopsy specimen supported the diagnosis (97). In 1980, Zhang considered more criteria (98). Dilsen et al (1986) also defined a set of criteria emphasizing the pathergy test (95). In 1985 during the Fourth International Conference on Behçet's Disease, in London, an International Study Group (ISG) for Behçet's Disease was created, in order to create a complete set of criteria for the diagnosis of Behçet's disease that could be used in the future. These ISG criteria were published in 1990 (Table 1) (99,100).

Table 2. Classification of NBD (adapted from 75)

	Parenchymal	Non-parenchymal
CNS	Brainstem Diffuse ("brainstem plus") Spinal cord Cerebral Asymptomatic ("silent")	Cerebral venous thrombosis: intracranial hypertension Intracranial aneurysm Extracranial aneurysm/dissection
Peripheral nervous system	Peripheral neuropathy and mononeuritis multiplex <ul style="list-style-type: none"> • Myopathy and myositis 	
Other uncommon but recognised syndromes	Acute meningeal syndrome <ul style="list-style-type: none"> • Tumour-like neuro-Behçet's disease • Psychiatric symptoms • Optic neuropathy 	

Differential Diagnosis

The diagnosis of BD should not be assigned unless other clinical features are present. Other disorders characterized by recurrent mucocutaneous lesions include Crohn's disease, human immunodeficiency virus (HIV) infection, recurrent orogenital aphthosis, herpes simplex infection, and cyclic neutropenia. Differentiation from Crohn's disease may be particularly challenging because many other clinical features are also shared, i.e., anterior uveitis, terminal ileal ulcers, and arthritis. Posterior uveitis is rare in Crohn's disease and bowel histology is often distinctly different (9).

Laboratory Findings

There are no specific laboratory findings for BD. There may be an increase in inflammatory parameters, such as C-reactive protein, erythrocyte sedimentation rate, peripheral leukocytes and platelet counts during the active phase of the disease. Serum levels of several pro-inflammatory cytokines, including TNF- α , IFN- γ , IL-1 β , IL-6 and IL-8, can also be elevated. Mild anemia of chronic disease can be present. Autoantibodies, such as the antinuclear antibodies and rheumatoid factor, are usually absent (3, 101).

Treatment

Treatment of BD has become much more effective in recent years because of advances in

understanding the pathogenesis of the disease, and availability of a wide spectrum of therapeutic agents. The choice of treatment is generally based on the organ(s) is (are) affected and the extension and severity of the involvement. However, the aim of the treatment should be the prevention of irreversible organ damage, especially, during the early, active phase of the disease. Close monitoring helps to control and change the course of the disease.

It is wise to remember that especially male patients and those early onset disease are associated with more severe presentations including major vessel disease, ocular, gastrointestinal, and neurological involvement, and, therefore, require more aggressive treatment (102).

Topical Treatment

The majority of experience in the treatment of OU comes from the studies performed in patients with recurrent aphthous stomatitis (RAS). Controlled studies are still lacking, but the efficacy of topical *corticosteroids* is indisputable based on their favorable and widespread use. Topical corticosteroids suppress the inflammation associated with the formation of aphthae, and they are effective on both OU and GU especially when they are used in the early stage of these lesions. They reduce the pain severity and healing duration. Potent corticosteroid creams alone or in conjunction with antiseptics are also effective in GU (102, 103).

Antimicrobial agents including antiseptic agents and antibiotics are used to control microbial contamination and secondary infection (102). *Anti-inflammatory* agents (benzydamine, diclofenac), *anesthetics* (lidocaine 2–5%, mepivacaine 1.5%, tetracaine 0.5–1% gels, or mucosal ointments) and *silver nitrate*, in general, reduce the pain severity of aphthous lesions (50, 104, 105).

Systemic Treatment (Table 3)

Corticosteroids have been widely used. The compound is an effective choice especially in mucocutaneous lesions, acute uveitis, and neurologic disease. They can be given as monotherapy or in combination with other drugs such as colchicine, IFN- α , cyclosporin, or azathioprine. It is important to remember the effects of long term use; and more corticosteroids do not improve the long term outcome (103).

Colchicine is an anti-inflammatory plant alkaloid that inhibits neutrophil migration by interfering with microtubule formation (3). It is effective in controlling cutaneous and articular involvement and is usually well tolerated in the dose of 1.0–2.0 mg/day (106). Most common side effects are gastrointestinal (nausea, vomiting, diarrhea, abdominal pain). It can cause alopecia and bone marrow suppression, so blood count should be monitored in patients taking colchicine (106).

Table 3. EULAR (European League Against Rheumatism) recommendations for the management of BD 2008 (126)

Eye disease	<ul style="list-style-type: none"> • Azathioprine and local and systemic corticosteroids
Refractory eye involvement	<ul style="list-style-type: none"> • Cyclosporine A or infliximab in combination with azathioprine and corticosteroids • IFN-α alone or with corticosteroids
Major vessel disease	<ul style="list-style-type: none"> • Acute deep vein thrombosis: corticosteroids, azathioprine, cyclophosphamide or cyclosporine A • Thrombosis of the vena cava and Budd–Chiari syndrome: cyclophosphamide • Pulmonary and peripheral arterial aneurysms: cyclophosphamide and corticosteroids; surgery • Anticoagulants, antiplatelet and antifibrinolytic agents are not recommended (pulmonary embolism is rare and there is the risk of major bleeding in case there are concomitant pulmonary aneurysms)
Gastrointestinal involvement	<ul style="list-style-type: none"> • Sulfasalazine, corticosteroids, azathioprine, TNF-α antagonists or thalidomide; Surgery
Articular involvement	<ul style="list-style-type: none"> • Colchicine; IFN-α, azathioprine, TNF-α antagonists in resistant cases
Neurological involvement	<ul style="list-style-type: none"> • Parenchymal disease: corticosteroids, IFN-α, azathioprine, cyclophosphamide, methotrexate, TNF-α antagonists • Dural sinus thrombosis: corticosteroids • Cyclosporine should be avoided in case of neurological involvement due to neurotoxicity
Mucocutaneous involvement (oral, genital and skin lesions)	<ul style="list-style-type: none"> • Topical measures: corticosteroids preparations, lidocaine gel, chlorhexidine, sucralfate suspension • Erythema nodosum: colchicine • In resistant cases: azathioprine, IFN-α, TNF-α antagonists

Dapsone is an anti-infective drug. It inhibits the enhanced chemotactic activity of neutrophils and can be used as an alternative compound to colchicine. This compound also showed a significant decrease on the frequency of EN and PPL. Arthritis and epididymitis were also significantly suppressed by dapsone, but the effect of the compound on arthralgia failed (107). Hemolytic anemia and methemoglobinemia, which can be severe in patients with glucose-6-phosphate dehydrogenase deficiency, are the main side effects, and may significantly limit their use.

Thalidomide selectively inhibits TNF- α synthesis. Thalidomide therapy, however, was associated with exacerbation of EN (108). In addition, the effect of the drug is temporary, and discontinuation of the treatment results in recurrence of the OU and GU. The effectiveness of the thalidomide is lost about 20 days after discontinuation of the drug. Neurological side effects and teratogenic risk of thalidomide limits the clinical application.

Sulfasalazine has anti-inflammatory and immunosuppressive properties including inhibition of prostaglandin and leukotriene synthesis, free radical scavenging, immunosuppressive activity, impairment of white cell adhesion and function and inhibition of cytokine synthesis. It has been used inpatientswith gastrointestinal involvement (58).

Methotrexate. Several open studies of methotrexate has been reported to induce an improvement of a severe mucocutaneous involvement (109) as well as neurological (110, 111) and ocular involvement (112). It is not recommended in pregnancy and lactation. Severe bone marrow depression, liver dysfunction, acute infections, renal insufficiency, and mucositis are important side effects of this drug.

Azathioprine, an important disease-modifying compound, shows an anti-inflammatory effect by suppressing both cellular and humoral immune responses. Some studies concluded that the drug can be used prophylactically to prevent the eye involvement in young, male patients presenting with severe mucocutaneous lesions. Myelotoxicity, gastrointestinal complaints, immunosuppression, opportunistic infections, and hepatotoxicity are the main side effects.Side effects also include gastrointestinal intolerance with anorexia, nausea and vomiting, bone marrow suppression, and infection (113).

Cyclophosphamide has been found a beneficial therapeutic agent for eye disease and systemic vasculitis (neurologic involvement and arterial aneurysms). Myelosuppression, pulmonary fibrosis, renal toxicity, hemorrhagic cystitis, infertility, malignancy, and alopecia are the major adverse effects of cyclophosphamide. Due to the severe toxicity, cyclophosphamide is used in patients who are refractory to other agents (114).

Cyclosporin A interferes with the activation and recruitment of T lymphocytes. The drug is capable of markedly ameliorating uveitis as well as mucocutaneous lesions. It is still one of the most effective agents for the treatment of uveitis which reduces the frequency of ocular exacerbations and improves visual acuity. Cyclosporin is an effective drug; however, it should be reserved for the most severe cases because of its significant long term adverse effects such as renal failure, hypertension, neurologic toxicity and hirsutism. It is wise to remember that neurological manifestations occur more frequently in BD patients under Cyclosporin treatment (115).

Mycophenolate mofetil (MMF), an inhibitor of inosine monophosphate dehydrogenase, preferentially inhibits B cell and T cell function, thereby suppresses both cell mediated and humoral immune responses. MMF is generally well tolerated; the most common side effects include gastrointestinal and genitourinary symptoms. Other less frequent adverse events are neurologic, cutaneous, cardiorespiratory, and metabolic reactions. Rarely, severe leucopenia has also been reported (116).

Interferon- α is a naturally occurring cytokine that has immunomodulatory properties. The mode of action of IFN- α in BD is still unknown. However, their anti-viral and immunomodulatory effects appear to be the possible mechanisms. Recent studies demonstrated that there might be differences in therapeutic efficacy and side effect profile of IFN- α in different patient populations. The primary side effects of IFN- α therapy are flu-like

symptoms (fever, chills, headache, fatigue, myalgia, etc.) that start a few hours after the initiation of the therapy, and continue less than a day. Nausea, vomiting, anorexia, diarrhea, loss of weight, hematologic changes, and transient rising of hepatic transaminases are seen less frequently. Autoantibodies production can also occur. Psychiatric side effects and depression are limiting factors for use of IFN (103).

Anti-TNF- α therapies: TNF- α is a fundamental cytokine in the establishment and maintenance of the inflammatory response. It is considered to be involved in BD pathogenesis based on several findings: higher levels of TNF- α have been found in the aqueous humour and serum of BD patients with uveitis; the number of TNF- α producing cell is increased in the active phases of the disease (117, 118). At present, there are 3 TNF- α inhibitors available: infliximab, a recombinant chimeric monoclonal antibody; adalimumab, a humanized monoclonal antibody; and the fusion protein human p75 TNF- α receptor IgG1 etanercept (118).

Infliximab reduces the frequency of uveitis attacks, is successful treating refractory macular edema and improving visual acuity, especially in cases resistant to combination therapy with azathioprine, cyclosporine and corticosteroids and has a corticosteroid-sparing effect (119-121). There are reports of its efficacy in refractory cases of mucocutaneous, gastrointestinal and CNS involvement, arthritis and one case of pulmonary aneurisms with life-threatening haemoptysis (72, 122, 123). Etanercept has revealed a rapid improvement of mucocutaneous lesions and arthritis. Adalimumab has maintained disease remission in patients with uveitis with no recurrence and stable visual acuities during the follow-up after being switched from infliximab to adalimumab (124). The most common side effects are upper respiratory tract infection and headache. Autoantibody production, infusion reaction, rash, eczema, contact dermatitis and pruritus can also occur (125).

Prognosis

BD has a variable course characterized by relapses and remissions. Prognosis depends on the clinical involvement. Loss of visual acuity and neurological disease are major causes of morbidity and disability. Prognosis of BD improved in the last decade due to the use of modern immunosuppressant therapy and of a more aggressive treatment strategy (55,57).

The disease usually is more severe in males and in the Eastern and Mediterranean regions. The mortality rate in adult cases varies with series; the highest was reported in Turkey (9.8%) and is related to large vessel vasculitis causing sudden-death by aneurysm rupture or thrombosis (64).

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Chapter 10

Immunomodulators as Therapeutic Strategies for Managing Multiple Sclerosis

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Abstract

Immunomodulation and immunosuppression are important strategies for monitoring autoimmune disorders. As imbalances in immune function affect other physiological processes, immunomodulators may have an important role in restoring and maintaining regular neuroimmune activities. In recent years these agents have demonstrated important benefits in controlling the mechanisms associated with deteriorating central nervous system pathologies such as Multiple Sclerosis (MS), where central and peripheral nervous system immune regulation is impaired. MS is characterized by severe compromises to neuroimmune processes involving changes in immune cell function, soluble proteins and modulation of inflammatory processes. The introduction of therapeutic agents in the form of immunomodulators; interferon, phosphodiesterase inhibitors and Glatiramer acetate have proven to be useful to some extent in reducing the severity of MS. Herein the implications and effects of these molecules on the immune system in MS are reviewed. Additionally, the available evidence on the mode of action of neuropeptides in MS, their effectiveness on clinical measures, and current knowledge are also reviewed.

I. Introduction

Multiple Sclerosis (MS) is a heterogeneous and multifactorial neuroimmune disorder characterised by formation of numerous scar tissues in various brain regions, causing impairments in neuroimmune physiological activities and characterised by demyelination and axonal degeneration [1]. The symptoms of MS due to white matter lesions include, numbness, weakness in motor skills, optic neuritis, lack of coordination, just to mention a few [2]. MS can be sub-grouped into three main groups as relapsing-remitting MS (RRMS), primary-progressive (PPMS) and secondary progressive (SPMS) [3,4]. About 85% of all MS cases denote a RRMS pattern while the remainder 10-15% have PPMS [4-6]. RRMS is mainly characterised by acute attacks of new or recurrent neurological symptoms where patients recover partially [3,4]. SPMS and PPMS however entail a progressive deterioration in neurological activities [7].

The pathogenesis of this disorder may denote an autoimmune profile where compromises to various neuroimmune modalities are important components of the disease mechanism. These immune compromises include heightened pro-inflammatory lymphocyte function, impairments in myelin integrity, oligodendrocytes function and alteration in HLA-haplotypes resulting in neurodegeneration and demyelination in the central nervous system (CNS) [8-13]. Although a causative agent for MS is yet to be determined, genetic, environmental, immunological and idiopathic factors may collectively have a role in initiating and promoting the development of this disorder. Various therapeutical strategies have been developed for the management of MS and this paper aims at reviewing some of these therapies along with their potential role in MS.

II. MS and Immunological System

Interactions between innate and adaptive immune mechanisms assist in maintaining physiological homeostasis. Perturbations in either one or both systems have severe consequences on health and wellbeing. Most autoimmune diseases involve atypical bi-directional communication between both systems, with subsequent alterations in cellular activity, proteins and gene expression. Initial resistance towards pathogenic antigenic infiltration is usually mediated by agents of the innate immune system including dendritic cells, macrophages, natural killer cells (NK), monocytes and soluble proteins. Pathogenic or toxic interference triggers a sequence of events involving recognition by toll-like receptors (TLRs), activation of soluble proteins, induction of phagocytosis, cytotoxic responses, recruitment of additional cells and antigen peptide presentation to the adaptive T and B lymphocytes. Memory cells and antibodies against target antigens are also generated following T and B cell in the adaptive immunity.

In MS, several theories have been proposed to explain the observed deficiencies or deviation in the immune function. The mechanism of altered immune response in both human and animal experimental autoimmune encephalomyelitis (EAE) models of MS may involve a plethora of inflammatory detrimental effects. Increase in dendritic cell (DC) accumulation within the CNS has a direct effect in activating autoreactive T cells in EAE [14-17]. A mechanism that is both dependent on the activation state of DCs and the mechanism of

antigen recognition [18]. Overly activated DCs, usually have increased expression of CD40 and CD80, uncharacteristic secretion of pro-inflammatory cytokines and decreased expression of programmed death ligand-1 (PDL1) all to which enhance survival and prevalence [19]. It has been demonstrated that DC overactivity is more prominent in SPMS patients compared to RRMS patients implying a more severe implication for DCs [19]. In addition, monocyte-derived DCs are found with heightened levels of pro-inflammatory cytokine secretions. IFN- γ , TNF- α , IL-6 and IL-23 over secretion demonstrates a pro-inflammatory cytokine spectrum in disease occurrence [20-23]. Increased expression of osteopontin in brain lesions and DCs has been shown to be related to high levels of Th1 and Th17 cells in both human MS and EAE mice [24,25]. Hence, DCs are important in converting the immune response towards pro-inflammatory response in MS.

The role of microglial cells in MS is still misunderstood with both negative and positive effects on CNS activities. CNS microglial activation is responsible for damaging neuronal synapses, inducing white matter inflammation and increasing levels of TLRs, IL-6 and IL-17 in MS [26]. Furthermore, microglial cells are responsible for demyelination, with active phagocytosis, a process associated with a surge production of reactive oxygen species that affects neuronal signalling [27-30]. Conversely, the same microglial cells enhance neurogenesis and anti-inflammatory immune response with the release of neurotrophic factors, IL-10 and TGF- β attenuating CNS inflammation [30]. Duality of function of microglial cells remains a mystery especially with more pro-inflammatory hallmarks taking place in progressive forms of MS. Developing effective therapies for MS patients that are aimed at targeting detrimental overactive microglial cells or conferring high neuroprotection could prove to be highly beneficial.

An extensive prevalence of pro-inflammatory cytokines and chemokines are observed during disease progression in MS, this is due to perturbation or deficiencies in both aspects of the immune system. Lymphocytes especially CD4+T cells are important contributors to MS progression and pathophysiology. Substantial amounts of IFN- γ enhance pro-inflammatory related immune responses in MS and most likely encourage a predominant Th1 immune profile. Overall, an increase amount of CCL-2-8 (induced by TNF- α) and CXCL-10 (induced by IFN- γ) chemotactic peptides is observed. This activates autoreactive T cells, Th17 cells and promotes further cytokine release at sites of CNS inflammation [31,32] leading to subsequent BBB and neural breakdown within the CNS [33].

Particular therapeutic strategies have been suggested to be potentially effective in reducing the symptoms of this disorder and these may be useful in curbing and possibly averting the neurological damage in MS. Current drugs with immunomodulatory and immunosuppressant properties include interferon beta (IFN- β), glatiramer acetate (GA), phosphodiesterase inhibitors (PDEIs) and neuropeptides. The efficacy of these drugs may not be generalised to the entire MS affected population though they may be of considerable benefit in reducing either the severity or the onset of illness. Although the exact mechanisms for these therapies have not yet been entirely expounded, they have been shown to dampen pro-inflammatory effects of DC and Th1 cells. Drug trials in EAE have illustrated plausible means to maintain CNS integrity while reducing heightened inflammation [34]. Manufactured MS drugs, immunomodulators have anti-inflammatory roles in suppressing of autoreactive T cells.

The following section is a discussion on drug mechanisms that are related to PDEIs, GA, IFN- β s and neuropeptides, molecules used in the treatment of MS.

A. Phosphodiesterase Inhibitors (Pdei)

Phosphodiesterases are enzymes that regulate intracellular levels of cAMP by catalyzing the hydrolysis of 3'5' cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) into 5'-monophosphates AMP and GMP [35]. Regulation of these enzymes is important in maintaining immune homeostasis. PDEs are coded as different types of isoforms with particular PDEs noted to be increased in expression in MS individuals. Heightened levels of PDEs are usually associated with decreased levels of cAMP, a process reversed through the use of specific PDEIs [36]. With their ability to cross the BBB and to migrate to areas of CNS damage, PDEIs have effects on Th1 and pro-inflammatory modulators in the CNS [37,38].

Common PDEIs used in MS therapy include ibudilast, rolipram (PDE4 inhibitor), pentoxifylline, vinpocetin, propentophylline and theophylline [38-40]. Depending on the type of PDEIs used, a dose dependant effect is prescribed and administered. Administration of 60mg/day of ibudilast, a non-selective PDEI is responsible for an increase in NKT cells and triggers a Th1 to Th2 shift in MS [41]. In contrast, higher doses of pentoxifylline 1600 or 2400mg/day, may be necessary to achieve such a Th1 to Th2 shift and is reducing the levels of TNF- α , IL-12 as well as it is enhancing Th2 cytokines IL-4 and IL-10 release [39,40]. Anti-inflammatory ibudilast shows benefits both in MS and EAE animals [42,43]. Importantly ibudilast inhibits PDE3, PDE4, PDE10 and PDE11 [44] conferring both improvement in cerebral blood flow and prevention of gliosis formation. In patients with relapsing MS, ibudilast acts as a neuroprotector although its effectiveness in reducing MS cases may not necessarily be beneficial in the long run [45]. Selective PDE-4 inhibitors such as rolipram elevate cAMP levels in autoreactive T cells leading to drastic reduction levels of TNF- α , lymphotxin- α and IFN- γ [46-48].

PDEIs can be used either as mono- or combination therapy in MS to effectively reduce neurodegeneration and sustaining normal inflammatory processes [38]. Incidentally, when rolipram is administered in conjunction with lovastatin, reductions in Th1 and Th17 activities as well as an immune response skewing towards Th2 related reactions are observed [49,50]. Combination therapies in MS may therefore prevent axonal loss, reduce CNS infiltration of inflammatory cells, increase CNS neurorepair and remyelination. Such benefits are most effectively as combined therapy when compared to single drug administration [49,50].

B. Glatiramer Acetate (Copaxone)

Glatiramer acetate (GA), a tetrapeptide immunomodulatory drug, is composed of L-glutamic acid, L-lysine, L-alanine and L-tyrosine [51] and has shown positive therapeutical results following administration to either murine animals or MS patients. GA lessens the frequency of relapses and progression in EAE and RRMS respectively [52-56]. It is thought that GA acts by binding with high affinity to specific HLA-DR molecules present on the surfaces of antigen presenting cells (APC). GA facilitates recognition of GA-restricted CD4 $^{+}$ T cells, that while in the CNS stimulates the release of anti-inflammatory cytokines [57,58]. The anti-inflammatory reactions of GA are important in both innate and adaptive immunity. In the innate immune system, GA-stimulated monocytes release high levels of

anti-inflammatory cytokines including IL-10 and TGF- β , while limiting the secretion of pro-inflammatory markers such as TNF- α and IL-22 [59]. Monocytes primed by GA direct the maturation of T cells towards an anti-inflammatory profile [60]. An increase of IL-1Ra is also observed, a molecule that antagonizes the effects of IL-1 β and playing such role through the binding effect of GA to IL-1 receptors [61,62].

Furthermore the effects of GA in the adaptive immune system, is attributed to the ability of GA to promote proliferation of the anti-inflammatory immune cells CD4 $^{+}$ and CD8 $^{+}$ cells [63,64]. GA induced T cells in the cerebrospinal fluid may indirectly or directly produces abundant anti-inflammatory IL-10 and TGF- β [65]. The secretion of cytokines from astrocytes and microglia cells might be directed by GA induced T cell activation [66]. Combined production of IL-10 from both innate and adaptive immunity may be the underlying mechanism necessary to sustain remyelination and reduce inflammation in the CNS. Additionally homeostasis, is maintained when GA stimulates CD8 $^{+}$ T and CD4 $^{+}$ CD25 $^{+}$ T cells by inducing suppressive effects on inflammatory CD4 $^{+}$ T cells [67,68]. GA treated T cells also produce neurotrophic factors within the CNS [69,70], which may recruit and help in differentiating oligodendrocyte precursor cells to promote both neuronal remyelination [71] and neuronal survival [72,73]. Importantly, GA treatment significantly reduces the chemokine receptor CXCR3 which is noted to be elevated in MS patients [74]. Decreases in CXCR3 and CXCL10 ligand binding reduce autoreactive T cell activities in the CNS. Similar to PDEIs, GA induces an immune Th2 shift and downregulates pro-inflammatory damages to myelin of the CNS.

Additionally, GA induces novel naive T cells proliferation from the thymus to neutralize the effects of pre-existing impaired memory T cells ensuring therefore a more efficient and stringent immune response that can reverse MS inflammatory pathogenesis [75]. This promotes the generation of specific memory and activated anti-inflammatory antibodies that prevent the ongoing impaired anti-inflammatory environment in the periphery and CNS [76]. GA induces changes in the expression of genes associated with neuroimmune dysfunction in MS, in particular genes that regulate antigen presentation, apoptosis, inflammation and endothelium adhesion [77]. The ability of GA to evoke anti-inflammatory reactions on diverse immune cell types of both the innate and adaptive immune system is a very effective method for regulating the perturbed inflammatory profile in MS.

C. Interferons Beta

Another group of molecules is effective in the treatment of MS and consists of interferons (IFNs), in particular IFN- β , glycosylated IFN β -1a and unglycosylated IFN β -1b. The effectiveness of these molecules is more potent where treatment is administered at early stages of the disease [78,79]. IFN- β acts to ensure complete clearance of autoreactive T cells by hindering the antigen recognition activities following MHCII and T cell interactions [80]. It also ameliorates the activation of myelin reactive T cells through suppression of B7/CD28 and CD40/CD40L [81,82]. Importantly inhibitory effects of IFN- β may limit dendritic cell migration and activation of both naive and autoreactive T cells in lymph nodes and CNS respectively. This mechanism occurs via the reduction of CCR7, matrix metalloproteinase (MMP)-9 and IFN- γ production [83-85]. Additionally another important contributor to the

pro-inflammatory profile in MS are Th17 cells producing IL-17 molecules, a cytokine that is lowered in the presence of IFN- β [86]. Similar to glatiramer acetate, IFN- β inhibits the production of IL-12 and CXCL10 [87,88] and increases the levels of the anti-inflammatory IL-10 cytokines [88,89] re-directing as such the immune response towards an anti-inflammatory profile. Increasing levels of cytokine inflammatory suppressors such as soluble TNF-receptor II and IL-1 receptor antagonists may be a potential mechanism for IFN- β to control cytokine secretion and consequently preventing CNS damage [90]. In relation to B cells, IFN- β stimulates the release of the B-cell-activating factor causing B cells to thrive in MS patients [91]. In addition, blood brain barrier integrity in the pathogenesis of MS can be restored through the application of IFN- β . IFN- β preserves BBB integrity by limiting the expression of adhesion molecules, VCAM/VLA-4, on both activated and memory T cells thus preventing autoreactive T cells infiltration across the BBB [92-94]. IFN- β also increases both the production of ectoenzyme CD73 (stimulating adenosine release) and several anti-inflammatory mediators consequently averting autoreactive T cells away from the CNS and preventing further neuronal dysfunction [95].

Similarly to PDEIs, IFN- β can be combined with other drugs to enhance the effectiveness of MS treatment. Administration of IFN β -1a with Natalizumab is shown to dramatically reduce the rate and number of lesions observed in MS patients [96]. Long-term use of IFN β -1a in RRMS patients show a better prognosis, better quality of life with little or absence of side effects. Such beneficial effect of IFN β -1a is possible if administered at appropriate dose and shows high treatment efficiency in patients with low disability [97,98].

D. Neuropeptides

Neuropeptides such as vasoactive neuropeptides (VN) are a class of peptides with diverse physiological regulatory roles. The two most important VNs that are associated with neuro-immune disorders are vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating peptide (PACAP). These VNs are ubiquitously expressed in the mammalian body and importantly are present in the CNS and lymphoid organs. Receptors are found on immune cells such as mast cells, T lymphocytes and granulocytes [99,100]. VIP and PACAP are highly expressed in the CNS and lymphoid tissues are specifically found in the cortex, thymus, spleen, lymph nodes, hypothalamus, colon, pituitary gland, neurosecretory fibres, gonads, adrenal, endocrine, germ cells, gastrointestinal tract, ganglia, neurons and muscle fibres [99,101-109]. These peptides participate in the regulation of important physiological processes such as maintaining the BBB/BSB [110], cerebellum development [111], cholinergic and overall neurotransmission [112], inflammatory modulation [113], nitric oxide [NO] regulation [114], hypoxia [115] and apoptosis inhibition [116].

VIP and PACAP act through G-protein coupled receptors (GPCRs), named respectively *Vasoactive Intestinal Peptide Receptor* (VPAC)-1 and VPAC-2 and pituitary adenylate cyclase activating peptide receptor (PAC)-1. VIP binds with high affinity to receptors VPAC1 and VPAC2 but partially to PAC1, whilst PACAP binds to all three receptors [117]. VIP and PACAP following receptor binding activate intracellular stimulatory subunit of the GPCR protein (Gs). Gs cause adenylate cyclase (AC) to catalyse the production of cyclic AMP (cAMP) from ATP. cAMP acts as the regulatory protein of protein kinase A which results in

the phosphorylation of various intracellular signalling pathways [100,118]. VIP and PACAP intracellular signalling cascade can inhibit production of pro-inflammatory cytokines IL-6, IL-12, TNF- α and nitric oxide (NO) in macrophages and T lymphocytes [119-121]. Both ligands also control immune processes related to chemokine release of CCL2, CCL5, CCL9, CXCL1, CXCL2, CXCL3, CXCL8, and CXCL10. Such control on chemokine release has impacts on chemotaxis and diapedesis of monocyte and neutrophils to sites of infections [122]. In addition, they activate anti-inflammatory mechanisms, suppress macrophage related functions (phagocytosis), respiratory burst, and chemotaxis. All to which leads to limitation of lymphocyte recruitment and prevention of pro-inflammatory factors secretion [100,123,124].

In human MS condition, a limited amount of data on the potential efficacy of these VNs compounds is available. However a number of studies in animal models of MS have demonstrated significant benefits [125]. Evidence for decreased VIP concentration in the cerebrospinal fluid is reported in MS patients [126,127]. In EAE mice, VIP promotes expansion of Foxp3 Tregs, deterring the frequency and movement of autoreactive T cells in the CNS [128]. The presence of VIP-Tregs prevents CNS inflammatory infiltration of CD4 $^{+}$ T cells and macrophages, potentially activating improvement in recovery [129], lessening both CNS infiltration and release of a plethora of inflammatory molecules. These pro-inflammatory reductions alter inflammation within the brain and spinal cord, encouraging anti-inflammatory reactions to restore the altered neuroimmune mechanism within the CNS [130,131]. Hence, VIP and PACAP also promote a Th2 anti-inflammatory immune profile, indicating the ability of these VNs to induce Th2 type cytokines and chemokines thereby regulating strongly inflammation [119,132,133]. This preferential selection enhances the capacity of Th2 cytokines demonstrating the ability the protective mechanism in preventing autoimmune related episodes.

As CD4 $^{+}$ Th2 cells express predominantly VPAC1 and VPAC2 [134] receptors, these cells are important in sustaining Th2 mechanism in MS. VPAC1 inhibits excessive production of pro-inflammatory markers produced by macrophages and microglia cells while VPAC2 sustains Th2 survival and endorses anti-inflammatory effectors [135]. VIP and PACAP therefore act through their receptors to prevent persistent inflammation in an attempt to maintain homeostasis.

III. Conclusion

In summary, immunomodulators may have potential benefits in the current therapeutical strategies undertaken in MS. The ability of these agents to dampen the heightened pro-inflammatory status in MS patients is important in ensuring immunological homeostasis in both the periphery and the CNS. These drug interventions are important in sustaining the BBB integrity by modulating and reducing cytokine/chemokines responsible for BBB breakdown. These drugs reduce the production of mRNA and protein molecules of IL-23, IL-17, IFN- γ , TNF- α and IL-1 β cytokines both in the periphery and in the CNS. The outcome of these effects is also showing an increase in Tregs, foxp3, IL-10 and IL-4 signifying positive shift towards a Th2 immune response.

Despite these suggested benefits, the heterogeneous and complex nature of MS may affect the effectiveness of various drugs. Hence in certain subtypes of MS, little or no improvement may be observed after administration of the above mentioned therapies. However, combination therapy may be an alternative in some instances as they have shown to be useful. It may be necessary to determine whether combinations of the above mentioned drugs may adequately target the BBB and CNS dysfunction observed in these individuals.

Majority of the studies on therapeutic strategies in MS have been performed using animal models in particular EAE. Although the presentation of EAE mimics highly the MS pathology, the effectiveness of drugs in EAE previously documented in several reports may vary from animal to human. Consequently, further drug trial studies are needed to evaluate the efficacy of these drugs in human MS patients. Additionally, the time point at which these drugs are applied may play an important role in reversing the ongoing damage observed in MS patients. It may be important to administer these drugs during early diagnosis or onset of the disease, or perhaps prior to onset where a patient is noted to be more susceptible or likely to have MS.

These drugs may also be applied via various means, perhaps the pathway to effective drug treatment needs to be accurately established to promote better prognosis (although this may not necessarily be important given that all routes lead to the CNS). Age and disease severity may also need to be considered when addressing the effectiveness of these drugs in MS patients. It may be that patients in the later stages of life may be less likely to benefit from some of these drugs while younger adults with less progressive symptoms may have higher beneficial outcomes. Disease severity and progression are important determinants of the effectiveness of medication. Most of these drugs may not show immediate improvement in the short term, however, over time in the long run they may generate positive outcomes provided they are taken consistently with regular compliance monitoring.

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Chapter 11

Premenstrual Syndrome: A Disease with an Autoimmune Component?

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Abstract

Premenstrual syndrome (PMS) is a disorder characterized by depressed mood, anxiety, affective lability, irritability, decreased interest in usual activities, difficulty concentrating, low energy, changes in appetite, sleep disturbances, a sense of being overwhelmed or out of control, headaches, joint or muscle pain, breast tenderness, and abdominal bloating.[1] Women with fewer than five of these symptoms are typically diagnosed with premenstrual syndrome. Women who experience more of the symptoms, or fewer symptoms at a debilitating level, meet the diagnostic criteria for premenstrual dysphoric disorder (PMDD). To meet the PMDD criteria, symptoms should be specific to the premenstrual period and at least one of the symptoms should directly relate to mood disturbances.[1] PMS is a phenomenon which impacts the majority of adult women on some level, with millions of women affected severely enough to disrupt daily life. Nevertheless, it is an under-investigated disorder that is still without a definitive etiology. The pronounced gender discrepancy in the prevalence of autoimmune diseases strongly implicates estrogen and/or progesterone in their development; it is known that the hormonal fluctuations of the menstrual cycle cause exacerbation of the symptoms of autoimmune diseases, particularly those with cutaneous manifestations. The demonstration of a dramatic comorbidity of premenstrual exacerbations of cutaneous allergic and autoimmune disorders with PMS, the presence of hypersensitivity reactions to estrogen and progesterone in PMS patients but not in normal controls, and the ability

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of desensitization therapy to improve symptoms in PMS patients suggests that autoimmunity may play a role in the origin of PMS symptoms.

Keywords: premenstrual syndrome, atopic dermatitis, autoimmune progesterone dermatitis, estrogen dermatitis

Introduction

Premenstrual syndrome (PMS), including Premenstrual Dysphoric Disorder (PMDD) —a designation reserved for those with more severe symptoms, is characterized by an eclectic collection of both psychological and somatic signs and symptoms. Psychological symptoms associated with the disorder include depression, hopelessness, low self-esteem, feeling edgy or wired, mood swings, anger, irritability, lethargy, apathy, food cravings, and sleep disturbances, while common physical complaints include breast tenderness and/or swelling, bloating, headaches, joint pain, and muscle pain.[1] Psychological symptoms are often emphasized, with physical ones lumped together in one category by the standard diagnostic guidelines,[1,2] despite the fact that physical symptoms are more often reported as severe than psychological ones.[3] Although less than 10% of all women have symptoms severe enough to disrupt personal relationships and daily productivity, more than 85% of women are reported to suffer cyclically from various PMS symptoms.[2,4] Virtually every woman in the western world experiences some PMS symptoms at some time.[4] Despite the prevalence of PMS in adult women and the often substantial burden that the disease represents, PMS has been under investigated with regard to other mental disorders with comparable prevalence and dysfunction levels and it is still a disorder without a definite etiology.[5]

Symptoms of PMS tend to increase in severity as menses approaches but disappear during pregnancy and menopause, leading to the inevitable hypothesis that the cause of the disorder lies in monthly fluctuation of female sex hormones.[2] It is known that estrogen initiates or mediates an impressive array of biological functions, with receptors in a multitude of tissues and cell types. In fact, fluctuating estrogen levels have been shown to have physiological effects on virtually every organ system in the body, while the effects of progesterone are less studied.[6]

Symptomatology of the disorders, however, has not evidenced a strong correlation with serum levels of suspected hormones.[7] To date, progesterone, estrogen, prolactin, norepinephrine, thyroid hormones, growth hormones, prostaglandin, insulin, cortisol, follicle stimulating hormone, and antidiuretic hormone have all been investigated as potential causative factors, but with a similar lack of significant association with existing symptomatology.[2]

Measurements of serum levels, however, may not predict available levels of active hormone. Estrogen synthesized in the peripheral tissues is largely active in the same tissues, making serum levels of estrogen an unreliable indicator of its potential for tissue activity.[8] In addition, it is known that sex -steroid sensitivity varies across individuals as well as individual tissues[9] and that tissue-specificity of steroid effects exists.[10] Sex steroids are active in many peripheral tissues (including breast, gastrointestinal, and uterine tissues).[10] It

is possible that susceptible individuals experience hypersensitivity to fluctuations of hormone levels across multiple tissues and systems.[10]

Although PMS symptoms were initially hypothesized to be the result of overproduction of sex steroids—particularly progesterone and estrogen, it is now believed that PMS patients produce normal hormone levels but have a particular hypersensitivity to the hormones produced.[2] Exaggerated response to normal hormones is postulated to produce abnormal biochemical events in the central nervous system (CNS), possibly resulting in an imbalance between sex hormones and neurotransmitters.[2] Of particular focus is the neurotransmitter serotonin. Use of selective serotonin reuptake inhibitors (SSRIs), currently the first line of therapy in PMS, is based on the belief that serotonin is integrally involved in the development of PMS in reproductive women.[11] It has been postulated alternatively that PMS may represent a set of infectious illnesses exacerbated by a cyclic immunosuppression induced by changing hormone levels.[12] In contrast, a substantial number of physicians consider PMS to be of purely psychological origin.[1]

Cutaneous Disease and the Menstrual Cycle

The week preceding onset of menses is characterized by an exacerbation of symptoms in many systemic diseases including asthma, porphyria, hereditary angioedema, aphthous ulcers, Behcet syndrome, epilepsy, migraines, myasthenia gravis, and allergic rhinitis,[13] and many autoimmune diseases like Crohn's Disease,[14] systemic lupus,[15] and multiple sclerosis.[16]

Many skin diseases are negatively affected by the hormonal changes associated with the approach of the onset of menstrual flow. Estrogen (secreted by the ovaries) and progesterone (produced by the corpus luteum that develops at the site of a ruptured ovarian follicle) stimulate development of the endometrium and modulate pituitary production of other gonadotrophic hormones.[17] Estrogen levels peak at about day 12 (shortly before ovulation), progesterone levels peak in the mid-luteal phase at about day 21.[17] The skin contains numerous receptors for estrogen and progesterone and is highly sensitive to the effects of these two steroid sex hormones.[18] Women commonly report a worsening of acne vulgaris[19] during the premenstrual period, as well as other chronic skin diseases such as urticaria, eczema, folliculitis, angioedema,[19] dermatitis, herpetiformis, erythema multiforme, lichen planus, pompholyx, and psoriasis.[20] Exacerbation of skin disease typically begins several days before menstruation and substantially improves within 2 days after menstruation.[21,22]

Several disorders of the skin recognized as atopic also show a definite cycle of exacerbation and then improvement of skin eruptions in concert with the menstrual cycle. Atopic dermatitis was found, in two early large-scale studies, to worsen during the premenstrual period in about 50% of atopic-dermatitis patients.[23,24] In a more recent survey which evaluated 286 women diagnosed with atopic dermatitis, 47% were observed to have a monthly premenstrual worsening of skin symptoms.[21] In 129 out of the 134 subjects who experienced skin deterioration, an increase in skin lesions began during the week before menstruation and improved within a couple of days after onset.[21]

In general, atopic dermatitis is increasingly recognized as a disease that may well be autoimmune in origin, as many patients produce specific immunoglobulin E (IgE) against a broad variety of human proteins, including those of epidermal origin.[25;26]

Autoimmune Diseases of the Skin and the Premenstrual Period

Some women who experience premenstrual skin eruptions have been shown to exhibit autoimmunity to sex steroids themselves. A cutaneous manifestation of an autoimmune reaction to estrogen known as estrogen dermatitis has been described.[27] The most commonly identified autoimmunity to sex hormones, however, is hypersensitivity to endogenous progesterone.[28] Now termed autoimmune progesterone dermatitis (APD), the disorder currently envelops a widely diverse set of possible presentations (see **Table 1**). Shared features of APD are eruptions that appear during the luteal phase of the menstrual cycle (presumably in response to increasing serum progesterone levels) and menarche consistently occurring before onset of disease.[29] Eruptions are more common on the face, neck, upper trunk, and arms, and closely associated with receptor density which is highest in these areas.[27] Lesions typically appear during the week before the onset of menses and resolve quickly once menses ensues.[30,31]

Beyond these common parameters, there is little agreement in reported findings. Eruptions may be localized or generalized,[31] both mucosal and perineal involvement has been described,[32] and both immediate and delayed hypersensitivity reactions have been observed.[31] Although many develop the disorder (or see symptoms worsen) during pregnancy or after use of exogenous progesterone,[13] other patients do not fit this profile.[20]

Diagnosis has relied on positive response to intradermal progesterone or intramuscular or oral progesterone challenge[31] or occasionally demonstration of antiprogestrone circulating antibodies.[20] Other lines of evidence have offered support for progesterone as the causative factor in APD, including in vitro degranulation of rabbit basophils and morphologic alteration of rat mast cells in the presence of patient serum (suspected antibody) and progesterone (suspected antigen),[30] immediate induction of both urticaria and erythema multiforme by progesterone injection,[33] suppression of symptomatology by antiovulatory agents or oophorectomy,[27,30,33] and demonstration of serum antibodies to progesterone by indirect immunofluorescence localized around cell membrane of luteinizing cells of the corpus luteum (although this is not consistently observable).[30]

However, positive response to intradermal and/or intramuscular testing is not always achieved, and APD has occasionally been diagnosed on the basis of a history of cyclic, recurrent luteal phase skin and oral lesions in the absence of positive progesterone test results.[30] In addition, intradermal testing and intramuscular testing results do not always agree. In evaluation of patients with estrogen dermatitis, two out of seven cases reacted only to intradermal estrogens, while the other five responded only in the tuberculin-type skin test.[27] The variety of responses to different testing approaches imply the potential for several different etiologies for APD.[13]

The diagnosis, APD, may in fact be a collection of disorders with premenstrual cutaneous eruptions. It has been postulated that a subset of those with APD may be patients with a premenstrual flaring of atopic dermatitis or chronic urticaria,[34] both also autoimmune disorders. There is also comorbidity among the disorders associated with perimenstrual flares, for example, a recognized association between Crohn's and Behçet's disease,[35] eczema and asthma,[36] migraines and mood disorders,[37] and even monthly urticarial episodes in one patient associated with eosinophilia.[19] The genetic bases for these comorbidities are just beginning to be unraveled. [25,37,38]

Table 1. Autoimmune Progesterone Dermatitis.

Presentation	Reference
Eczematous lesions (particularly the pompholyx type)	32
Eczematous, fixed drug-like eruption, with reactivation of specific lesions or during future menstrual cycles	31
Erythema annulare centrifugum	53
Eosinophilia	38
Folliculitis	54
Maculopapular lesions	32
Papulovesicular lesions	20
Pruritus ani (estrogen)	27
Pruritus vulvae (estrogen)	27
Ulcerations of oral mucosa	30
Urticaria	32
Vesiculobullous eruptions which may deteriorate into anaphylaxis	13
Vesiculopustular lesions mimicking dermatitis herpetiformis	55

Table 2. Gender Dimorphism in the Prevalence of Autoimmune Diseases

Autoimmune Diseases	Female:Male Ratio	Reference
Hashimoto thyroiditis	50:1	56
Graves-Basedow disease	8:1	56
Scleroderma	5:1	56
Myasthenia gravis	2:1	57
Multiple sclerosis	5:1	58
Autoimmune diabetes mellitus	5:1	59
Sjögren's syndrome	9:1	60
Rheumatoid arthritis	3:1	61
Autoimmune hepatitis	9:1	62
Chronic urticaria	2:1	34

Estrogen and Autoimmunity

Estrogens are known to have a substantial effect on the immune system,[39] particularly in autoimmunity. Estrogens appear to enhance humoral immunity, including the production of both cytokines and antibodies.[40] Estrogen is also implicated as the main effector in the significant gender discrepancy that exists in many autoimmune diseases.[39] (See **Table 2**) The incidence of systemic lupus erythematosus (SLE) in women, for example, is estimated to be twenty times as high as that in men.[40] Severity of SLE dermatitis was shown to fluctuate in concert with estrogen levels.[41] It is postulated that estrogens may effectively hyperexpress existing intrinsic autoantigens, creating the huge preponderance of female autoimmune patients.[40] It is known that intensity of patch-test reactions varies over the menstrual cycle,[42] with skin reactivity to both irritants and antigenic substances increasing during the premenstrual phase.[21] For more information on that topic, see the paper discussing sex hormones and both irritants and allergic responses in relation to skin testing.[18, 63]

PMS, Autoimmune Diseases, and the Skin

Interestingly, two research groups have demonstrated a strong association between premenstrual flares of atopic dermatitis and concurrent PMS. The first evaluated 204 atopic dermatitis patients of reproductive age from one dermatology clinic, surveyed by a postal questionnaire.[42] Patients reported the time points at which exacerbation of their atopic dermatitis was experienced, as well as recording additional negative symptoms experienced over the menstrual cycle and their severity. Surprisingly, the authors found a significant association of premenstrual flaring of atopic dermatitis with the presence of PMS symptoms ($p < 0.002$).[42]

A second study evaluated 286 women of reproductive age with atopic dermatitis through personal interview in which patient history of both atopic dermatitis and PMS symptoms were recorded, and then skin conditions of the patients were verified by study personnel during the premenstrual period. The investigators were able to demonstrate a virtually complete association of premenstrual exacerbation of atopic dermatitis with PMS.[21] Every patient (129/129) with deterioration of skin conditions in the premenstrual period also reported symptoms of PMS, while only one of the 152 patients whose dermatitis was not observed to deteriorate during the premenstrual cycle reported PMS symptoms.[21] Although the mechanisms which produce PMS symptoms specific to those who also have premenstrual exacerbations of atopic dermatitis are as yet unclear, it is hard to escape the conclusion that an immunological influence of estrogen and/or progesterone plays a role in the pathogenesis of both disorders.[43]

A more recent investigation enrolled 30 women diagnosed with PMS and compared PMS patients with concomitant skin disease (pruritus vulvae, papular eruption, acne vulgaris, and hyperpigmentation) to both normal controls and women with PMS free of associated skin disease.[44] Women completed a questionnaire related to PMS symptoms as well as gynecologic, dermatologic, and laboratory examinations. Intradermal testing with estradiol valerate, progesterone, and placebo also took place. Fifteen patients (all patients with PMS

only and half of those with PMS as well as skin disease) underwent desensitization therapy to progesterone and/or estrogen (depending on results of intradermal testing).[44]

All of the patients with concomitant skin disease were experiencing a premenstrual skin flare at the time of enrollment. PMS symptoms evaluated included mood swings, tension, irritability, breast fullness, headaches, and cravings for sweet and salt.[44]

Intradermal testing in patients with PMS and concomitant skin disease demonstrated that all patients with PMS as well as a concomitant skin disease had a positive response to either progesterone or estrogen, while no women in the control group had any positive reaction to these hormones. Other results included: 8/10 tested positive to estrogen, 8/10 responded positively to progesterone, and 6/10 were positive to both. Intriguingly, similar results were observed in patients with PMS free of associated skin disease as follows: 8/10 tested positive to estradiol and 9/10 to progesterone.[44] Similarly to patients with PMS and skin disease, all patients with PMS alone demonstrated a positive response to either progesterone or estrogen.

Women who exhibited immediate hypersensitivity reactions to estradiol had similar rates of positive reaction as follows: 9/10 women with both PMS and concomitant skin disease had a positive response to estradiol and 8/10 tested positively to progesterone. No patient tested positively to the placebo.[44]

Evaluation of delayed type hypersensitivity reactions showed slightly fewer positive responses to estradiol. Six out of ten patients with both PMS and concomitant skin disease were responsive to estradiol, although 9/10 remained positive for progesterone. Again, no patient responded to the placebo.[44]

Findings were confirmed by the histopathologic findings of perivascular lymphocytic infiltrates in biopsies from positive intradermal test sites.

The authors' final analysis evaluated the effects of desensitization to the endogenous sex steroids estrogen and progesterone on patient symptoms. Desensitization either improved or normalized skin symptoms in patients with skin manifestations.[44] Surprisingly, desensitization also improved PMS symptoms in patients with PMS alone as well as PMS with concomitant skin disease.[44]

The authors suggest that the presence of a delayed hypersensitivity reaction to one's own female sex hormones may be a significant pathogenetic mechanism of PMS itself, suggesting that PMS may have an autoimmune component. Findings may be related to changes in the balance of the autonomic nervous system response, wherein delayed hypersensitivity to endogenous sex hormones in females initiates a cascade of immunologic and neuroendocrine events, resulting in the collection of signs and symptoms associated with premenstrual syndrome.[44] This hypothesis is provocatively supported by the observation that exacerbation of psychological symptoms of PMS shows a temporal pattern of premenstrual flares virtually identical to that reported for autoimmune progesterone dermatitis and estrogen dermatitis. In a bipolar patient, the menstrual cycle regularly induced a manic state 2 to 3 days before menstrual flow which resolved 2 days after menstruation ensued.[45] Similar patterns were observed in two patients who experienced premenstrual auditory hallucinations.[45]

The finding that all PMS patients in this study reacted positively to intradermal testing with either estradiol and progesterone (while none of the patients not diagnosed with PMS responded positively to challenge) strongly supports the use of intradermal challenge as a diagnostic method for patients with suspected PMS and in those with PMS and related dermatologic conditions, as long as it is performed in the luteal phase of the menstrual cycle.[44]

The finding that virtually all of the patients with skin exacerbations also reported PMS symptoms also suggests that skin diseases that exacerbate premenstrually, particularly APD and estrogen dermatitis, should be considered part of the PMS syndrome.[44]

Potential Mechanisms for an Autoimmune Etiology of PMS

The role of estrogen autoimmune disorders and its ability to produce such a striking differential in the prevalence of autoimmune disorders in men and women is one of the most provocative questions in women's medicine.[46] Estrogen and progesterone levels in reproductive women, influence virtually every tissue and organ system in the female body.[39] An understanding of the role of those hormones in producing PMS and its associated variety of cutaneous eruptions (including the autoimmune dermatitises) will involve elucidating the interdependent effects of these two hormones (and possibly others implicated in PMS and autoimmune disorders) across the endocrine, nervous, and immune systems.

It is known that the acquired immune system of women differs in function from that of males. Estrogens stimulate immunological processes driven by CD4+T_H2 cells and B cells, while androgens stimulate the immunologic function of CD4+ T_H1 and CD8+ cells.[46] With regard to autoimmune progesterone dermatitis and estrogen dermatitis, exogenous (and therefore foreign) forms of the hormone may be taken up by antigen presenting cells and then presented to T_H2 thymocytes, resulting in an increase in IgE synthesis.[19] Only a subset of patients, however, has a history of prior exposure to exogenous hormones. It has been postulated that there may be cross-sensitivity of antibodies to similar molecules like hydrocortisone or 17- α -OH-progesterone, but at least one study has found no such reactivity.[28] It has also been proposed that an aberrant and allergenic form of progesterone may be produced in some patients that induces an immunologic response.[33]

The neurological, endocrine, and immune systems have multiple levels of interactions and are integrated through a network of signal molecules—cytokines, hormones, and neurotransmitters that act on a common set of receptors.[47] Interaction between the peripheral nerves and the immune system is conducted through cutaneous nerve fibers that release neuromediators that activate receptors on target cells in the skin, including keratinocytes, mast cells, Langerhans cells, fibroblasts and infiltrating immune cells.[48] It has been observed that neuropeptides and neuropeptide-positive nerve fibers are significantly increased in the lesions of atopic dermatitis. [48] In addition, neurokinins from autonomic and sensory nerves in the skin have recently been demonstrated to interact with antigen presentation in dermal Langerhans cells and other processes in the development of atopic skin disease.[49]

The neuro-endocrine-immune network (NEI) is known to play a role in mediating atopic dermatitis as well as other allergic diseases through an overproduction of neuropeptides and cytokines.[50] Cutaneous allergic and atopic processes are influenced by neuronal mediators released from neural cells, which induce the production of both IgE antibodies (which produce type I, immediate or anaphylactic hypersensitivity) and other mediators produced by T-cells (which produce type IV, cell mediated or delayed type hypersensitivity).[51]

Although *in vivo* support for its role is still lacking, the hypothalamic-pituitary-gonadal axis may be integrally involved in the pathogenesis of autoimmunity as related to sex hormones.[52]

It could be postulated that steroids in peripheral tissues produce metabolites that may enter the vasculature and eventually be captured by brain tissue with the result of modulating mood. Increased production of steroids in very active tissues could result in high concentrations of metabolites. Excessive levels of metabolites which act on brain tissue to modulate mood, in turn, could produce the extreme mood swings and other psychophysiological effects characteristic of PMS.[10]

Conclusions

It is known that sex hormones influence irritant and allergic response, with high estrogen levels stimulating antibody production and increasing response to skin testing with both irritant and allergenic compounds. It is arguably possible that the widely observed increase in activity of autoimmune disease in the premenstrual period may represent non-specific upregulation of all antibody-mediated immune processes. However, the strong association between PMS and cutaneous diseases known to be of autoimmune origin, as well as the ability of intradermal challenge with progesterone or estrogen to identify PMS patients, suggests a possible role of autoimmunity for PMS—a disease whose etiology is still a profound mystery.

Existing data should be supplemented by larger studies, characterized by rigorous study design and statistical analysis, in which association with other autoimmune diseases that show strong gender bias is explored. Further research needs include efforts to unravel the mechanisms and factors controlling neuromediators and their receptors, with the objective of finally understanding the receptor-mediated and intracellular signal pathways involved in neuroendocrine modulation of allergy and autoimmunity. For millions of women across the globe who suffer from autoimmune diseases (including cutaneous autoimmune disorders) and PMS, the ultimate research goal would be an understanding of the extremely complex interactions between the neurological, endocrine, and immune systems in order to define therapies with the potential to abort disease pathogenesis, rather than simply palliate its sequelae.[46] PMS may represent the ideal model for these endeavors. As a disease that profoundly impacts millions of women, its etiology begs to be solved and the potential autoimmune component unraveled to facilitate appropriate and timely diagnosis as well as treatment.

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Chapter 12

The Primary Target Antigenic Site in Heymann Nephritis is the Renal Proximal Convolved Tubules' Brush Border Associated Zone of Cells

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Abstract

Following the induction of Heymann nephritis or slowly progressive Heymann nephritis immunopathological events are primarily directed against the renal proximal tubules' brush border associated zone of cells by the developing pathogenic immunoglobulin G autoantibodies. Pathogenic immunoglobulin G autoantibodies making their way from the circulation through the glomeruli into the renal tubular environment get absorbed and attack the target nephritogenic autoantigens which reside in the brush border region of the renal proximal tubules.

Due to damage by the pathogenic immunoglobulin G autoantibody directed against the nephritogenic autoantigen the release of autoantigens into the urine and circulation will occur. If the immunopathological attack is continuous then the absorption/retention of vitally important components of the glomerular filtrate will be disturbed resulting in electrolyte imbalance.

If the immunological attack continues into a chronic progressive phase then additional secondary/tertiary injuries will take place to the kidney's glomeruli and renal

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proximal tubules. Nephritogenic autoantigen, pathogenic immunoglobulin G autoantibody against the nephritogenic antigen and complement components settle in the form of immune complexes on the epithelial side of the glomerular basement membrane. Immune complex glomerular nephritis further complicates the functional integrity of the kidney by leaky glomeruli causing non-selective proteinuria.

Since the primary target is the nephritogenic autoantigen of the renal proximal tubules' brush border zone of cells, the elimination of modified and native nephritogenic autoantigens from the circulation would halt the production of autoimmune disease causing pathogenic immunoglobulin G autoantibodies against the nephritogenic autoantigens. It has been demonstrated that prevention and/or termination of Heymann nephritis can be achieved by the implementation of a new vaccination technique that we have developed and call modified vaccination technique.

Keywords: Heymann nephritis, immune complex glomerulonephritis, modified vaccination technique, nephritogenic antigen.

Introduction

When kidney lesions of Heymann nephritis (HN) are described in the medical literature, the emphasis is on a detailed account of the characteristic glomerular lesion that can be observed by histology, fluorescence antibody (ab) test and electronmicroscopy [1, 2, 16, 23, 25, 27]. Information gathered from these investigations vividly describes the obvious renal lesion by deposition of immune complexes (ICs) on the epithelial side of the glomerular basement membrane (GBM). E.g. it is known that the IC on the GBM is composed of renal tubular nephritogenic autoantigen (aag)-like antigen (ag) that is produced by the epithelial cells of the GBM [29, 32] (according to some investigators), pathogenic immunoglobulin G (IgG) autoantibody (aab) directed against the nephritogenic aag and complement components (C3, C5b-9). The tubular lesions are occasionally mentioned and most often do not receive a prominent description. However, a few researchers have provided a detailed account of the lesions observed in the renal proximal convoluted tubules [14, 30].

To bring attention to the significant role the brush border (BB) associated aag plays:

- in normal rats following its release into the circulation;
- in the induction/maintenance of the autoimmune kidney disease HN and slowly progressive Heymann nephritis (SPHN);
- in renal tubular and glomerular lesion development;
- in the downregulation/termination of SPHN;

we provide the following explanation below that the chemically or otherwise modified native aag initiated/maintained disorders can be terminated by specific high titred immunoglobulin M (IgM) aabs directed against the native nephritogenic aag. Such a treatment modality can be achieved through the implementation of a new vaccination technique that we have developed and call modified vaccination technique (MVT) [3, 4, 7, 12, 13, 17].

Since most autoimmune diseases are manifested in predisposed individuals (with genetic, gender, geographic backgrounds) exposed to modified self ags or molecules similar to self ags (molecular mimicry), the MVT – by being able to remove from the circulation the disease

causing modified and disease contributing aags – should be able to terminate in most cases the tissue damaging autoimmune response in both cell mediated and aab mediated conditions.

In SPHN and in other experimental autoimmune diseases and in human autoimmune diseases as well, we should not concentrate only on the pathogenic autoimmune response caused lesions to find solutions for treatments e.g. by immunosuppressive agents, but find ways to institute immunological interventions for the termination of autoimmune disease causing events specifically and without side effects.

Fate of Released Nephritogenic aag from the Renal Proximal Convolute Tubules during Normal Healthy State

In most living beings, autoimmunity has predominantly beneficial aspects by keeping the internal environment of the host free of unwanted endogenous aags [34, 37].

Like any high filtration/absorption/reabsorption tubular structure (like the small intestine's villi) that is regenerated at regular intervals, the BB zone of cells of the renal proximal convoluted tubules, likewise, are shed and replaced. The shed cellular components find their ways into the urine (excreted) or circulation where the fate of the native nephritogenic ag is as follows:

- specific nonpathogenic IgM aabs in the circulation directed against the nephritogenic ag [rat kidney fraction 3] (rKF3) assist in their removal prior to being degraded into small molecular weight peptides in mononuclear cells [12, 20, 24, 31, 33];
- released nephritogenic aags continuously stimulate the IgM producing cell lines (secondary immune response); in a physiological sense we are not tolerant to our own intracytoplasmic components [35, 37]; Note: specific IgM aabs directed against various intracytoplasmic components – following cell death due to various reasons – keep the internal environment free of released intracytoplasmic aags [33, 37], thereby preventing toxic accumulation and possible chemical alterations (by drugs, infectious agents, toxins etc.) of aags that could lead to autoimmune diseases. In a significant way, circulating specific IgM aabs throughout life assist in the removal of native and modified self aags from the circulation, thereby maintaining tolerance to self [36].
- released nephritogenic aags combining with circulating IgM aabs directed against the nephritogenic aag form ICs. These ICs further stimulate the production of IgM aabs against the nephritogenic aag keeping a constant level of circulating IgM aabs in the circulation throughout life.

Notable Observations Made During the Course of SPHN

- the true autoimmune insult – culminating in the autoimmune kidney disease: SPHN – is initiated/maintained by the developing pathogenic IgG aabs directed against the renal tubular BB zone associated aag;
- the immunological insult – manifesting in IC depositions in the glomeruli – does not represent the primary or most important immunopathological process responsible for

the maintenance of the autoimmune kidney disease. It represents a secondary injury to the kidney;

- as longs as the modified self ag is present in the system, it evokes pathogenic IgG aab production and injuries to the kidney's tubular BB and glomeruli [10, 27, 30];
- as far as the pathogenic IgG aab mediated autoimmune disease is concerned, the titre of the pathogenic IgG aab in the circulation must be zero to achieve termination of the disease [9, 11, 15];
- specific IgM aabs against the target renal tubular BB ag during the autoimmune disease are elevated aiming to downregulate/terminate the pathogenic immune response [18, 19, 26, 28].

Induction and Maintenance of SPHN in Rats

Some autoimmune diseases are cell mediated and some are pathogenic IgG aab initiated and mediated disorders.

SPHN is a relevant example of a pathogenic IgG aab initiated/maintained autoimmune disease [8]. The developing pathogenic IgG aabs' primary target resides in the renal proximal tubular BB associated zone of cells [14] where according to our findings 100% of the native nephritogenic ag is located [6].

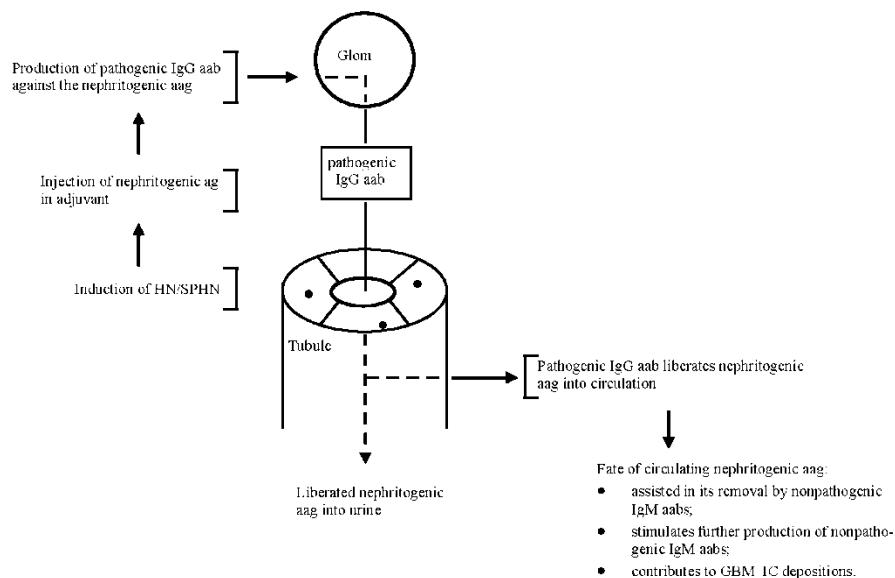


Figure 1. The primary injury – by the developing pathogenic IgG aab – is directed against the nephritogenic aag in the renal proximal convoluted tubules' BB cells

The nephritogenic aag is 100% produced/resides in the renal proximal convoluted tubules' BB associated zone of cells. It is the primary target for pathogenic IgG aab damage causing a chronic progressive autoimmune kidney disease.

Abbreviations: aab, autoantibody; aag, autoantigen; ag, antigen; BB, brush border; GBM, glomerular basement membrane; Glom, glomerulus; HN, Heymann nephritis; IC, immune complex; IgG, immunoglobulin G; IgM, immunoglobulin M; SPHN, slowly progressive Heymann nephritis

SPHN in the rat does not represent all autoimmune diseases but it represents autoimmune diseases which are initiated by:

- modified aags → capable of evoking pathogenic IgG aabs against the native aag that resides in the target organ; in our case, in the BB region of the renal proximal convoluted tubules;
- pathogenic IgG aabs produced as a result of modified self aags e.g. native homologous rKF3 ag injected in adjuvants, i.e., in Freund's complete adjuvant [27]; or as a native ag chemically modified into a haptene-protein conjugate [10]. Developing pathogenic IgG aabs will damage the primary aag bearing site, the BB zone of cells of the renal proximal convoluted tubules [8]. As a result of damage, nephritogenic aags will be liberated into the urine and circulation (Figure 1). Note: The nephritogenic aag is also present in the form of ICs around the GBM and in the mesangium of normal rat kidneys as: rKF3 x rat anti-rat kidney fraction 3 (rarKF3) IgM aab [6]. These ICs are localized in the glomeruli likely due to charge related properties of the IC.

The development and maintenance of ICs in the glomeruli by pathogenic IgG aabs, nephritogenic ag and complement components during the course of the autoimmune kidney disease is a secondary immunological event and will not necessarily be observed in many other autoimmune diseases (Figure 2).

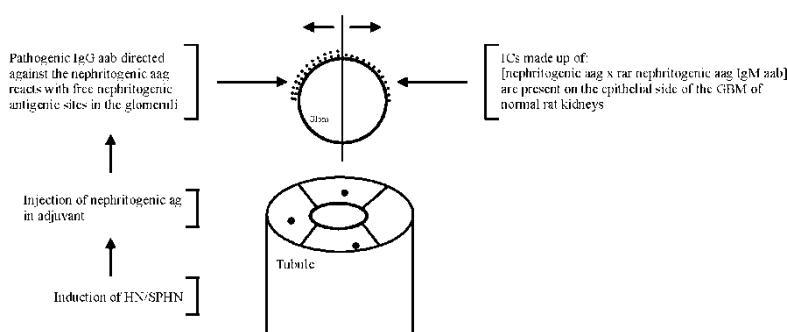


Figure 2. The secondary injury – by the developing pathogenic IgG aab – is directed against the GBM localized IC's nephritogenic aag around the glomerular capillary loops

In normal rats small beaded depositions of nephritogenic aags around the glomerular capillary loops can be detected [8, 14]. According to some investigators the nephritogenic aag is produced by the epithelial cells of the glomeruli [12]. This is not the case. We have conclusively shown that the glomeruli of pre- and post-natal rats which are not yet open to the circulation [20] do not have the nephritogenic aag. We have also shown that the glomerular localized nephritogenic aag in the adult rat kidney is present in the form of IC (and not produced locally), i.e., [nephritogenic aag x rar nephritogenic aag IgM aab] [24]. These localized ICs in the glomeruli or in its related structures cause no detectable morphological/functional changes.

During the initial stages of SPHN the developing pathogenic IgG aabs react with the glomeruli deposited ICs' native renal proximal tubular aags, causing gradual build-up of ICs resulting in ICGN. Abbreviations: aab, autoantibody; aag, autoantigen; ag, antigen; GBM, glomerular basement membrane; Glom, glomerulus; HN, Heymann nephritis; IC, immune complex; ICGN, immune complex glomerulonephritis; IgG, immunoglobulin G; IgM, immunoglobulin M; rar, rat anti-rat; SPHN, slowly progressive Heymann nephritis

The continuous damage to the kidney (i.e., tertiary renal injury) will be as follows:

- circulating IgG aabs – directed against the BB localized nephritogenic aag of the renal proximal convoluted tubules – will damage and release nephritogenic aags (i.e., rKF3) [8];
- the fate of the released nephritogenic aag during the chronic progressive phase of the autoimmune disease:
 - released nephritogenic ags from the renal tubules are assisted in their catabolism by specific IgM aabs;
 - released nephritogenic ags stimulate continuous production of specific IgM aabs (IgM appears in the circulation);
 - released nephritogenic ags will contribute to layered depositions of ICs in the glomeruli i.e., pathogenic IgG aab directed against the nephritogenic aag will react with free nephritogenic aag sites in the glomeruli to which complement components and circulating nephritogenic aags will join; the event continues as long as circulating pathogenic IgG aabs are present to damage the renal BB associated cells (Figure 3).

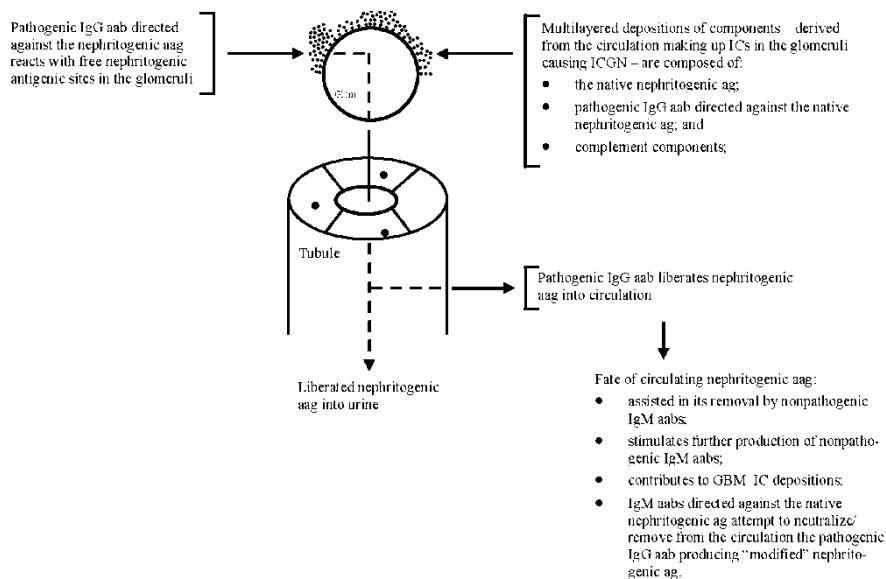


Figure 3. The tertiary injury – by the developing pathogenic IgG aab – is directed against the nephritogenic aag in the renal proximal convoluted tubules' BB cells and the GBM localized ICs. Ultrastructural changes in the renal proximal convoluted tubules' BB cells and the GBM result in a chronic progressive ICGN manifesting in:

- disturbed retention/reabsorption/elimination of small and large MW molecules;
- multilayered depositions of components: of free nephritogenic aag (released into the circulation) joining the glomerular localized pathogenic IgG aab and complement components on the epithelial side of the GBM in the form of ICs.

Morphological changes in the kidney can lead to severe proteinuria and non-compensatory renal failure.

Abbreviations: aab, autoantibody; aag, autoantigen; ag, antigen; BB, brush border; GBM, glomerular basement membrane; Glom, glomerulus; IC, immune complex; ICGN, immune complex glomerulonephritis; IgG, immunoglobulin G; IgM, immunoglobulin M; MW, molecular weight

Termination of Autoimmune Disease Causing Processes in SPHN Utilizing the MVT

In order to terminate the autoimmune disease causing process:

- one must remove the modified native aag from the system that initiated/maintained the continuous production of pathogenic IgG aabs; or
- one must remove the agent (if known; it can be a drug, toxin, smoking, infectious agent etc.) that is able to chemically or otherwise modify the aag.

Knowing the etiology and pathogenesis of the autoimmune kidney disease SPHN allowed us to develop a new vaccination method called MVT [5, 9, 12, 13, 15]. To implement the MVT – to downregulate the pathogenic aab mediated kidney disease (i.e., SPHN) – the aim was to specifically increase the level of naturally occurring nonpathogenic IgM aabs in the circulation which were directed against the BB derived nephritogenic aag. Specific nonpathogenic IgM aabs (well documented in the medical literature) assist in the catabolism of cell debris (derived from cells after cell death) [37].

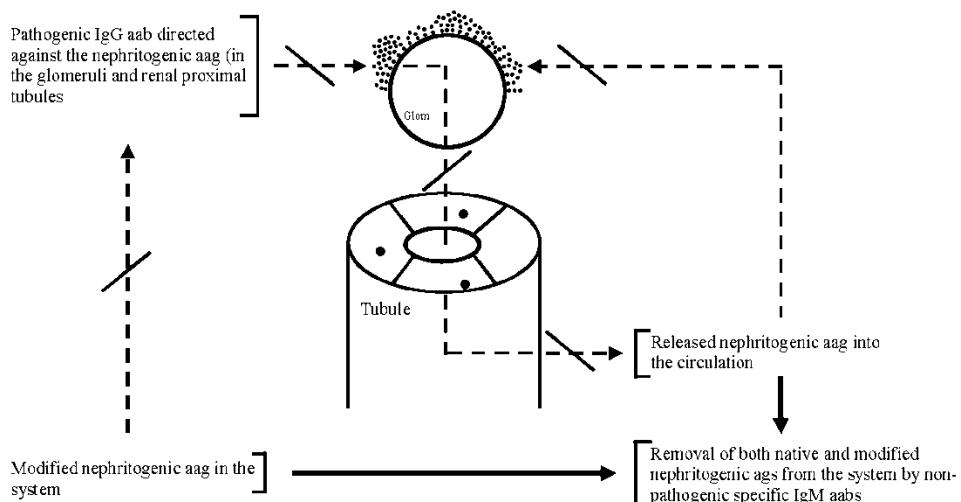


Figure 4. Termination of autoimmune disease causing immunopathological events by the implementation of the MVT

Using ICs for immunization composed of [nephritogenic ag x rar nephritogenic ag IgM ab] at ag excess produced in recipient rats the same ab with the same specificity against the target ag as was present in the IC, namely, elevated levels of rar nephritogenic aag IgM aab. The increased level of rar nephritogenic aag IgM aab in the circulation by taking out both the native and modified nephritogenic aags from the system halted the production of disease causing pathogenic IgG aabs and possible contribution of released nephritogenic aag from the renal proximal convoluted tubules, to glomerular deposits. The cycle of pathogenic autoimmune events was halted and tolerance to self was regained. Abbreviations: aab, autoantibody; aag, autoantigen; ag, antigen; ab, antibody; Glom, glomerulus; IC, immune complex; IgG, immunoglobulin G; IgM, immunoglobulin M; MVT, modified vaccination technique; rar, rat anti-rat

—→ termination of pathogenic immune responses and lesion contributing aag

—→ activated immune response for the removal of modified and native nephritogenic aags

We produced ICs made up of the homologous rKF3 ag x rarKF3 IgM ab at slight ag excess. The injected ICs produced the same ab with the same specificity against the target ag that resided in the IC, namely rarKF3 IgM aab. The developing nonpathogenic IgM aabs directed against the renal tubular nephritogenic ag reacted with:

- the chemically modified aag in the system and neutralized/eliminated it. No more chemically modified aag in the system meant no more stimulation of the pathogenic IgG aab producing cell lines and no more deposition of IgG aabs in the glomeruli;
- the liberated native aag in the system and neutralized/eliminated it. No more native aag (i.e., rKF3) in the circulation meant no contributing aag to glomerular deposits (Figure 4).

Conclusion

The MVT will not only work for the prevention and treatment of an IC glomerulonephritis called SPHN in rats, but also has the potential for preventing and treating all modified aag initiated and maintained autoimmune diseases.

The glomerular lesion (by the depositing aggregated ICs in the kidney's glomeruli leading to proteinuria) is due to a secondary immune event. The primary immune event – as in all autoimmune diseases – is initiated/maintained by the developing pathogenic IgG aabs (produced by altered/modified aags) against the native aag bearing organ (in our case the kidney's renal tubular associated BB aag). The pathogenic IgG aabs were initially and subsequently produced by the modified native-like rKF3 ag. These pathogenic IgG aabs attacked (since the developing pathogenic IgG aabs are cross reactive, being able to react with the modified and native aags) the BB related zone of cells where the nephritogenic aag resides, i.e., the rKF3 aag. The immunological insult/reaction with the nephritogenic ag resulted in the damage and release of the BB associated nephritogenic aag. If no other immune event would have taken place it still would have been enough to incapacitate the normal renal function by interfering with retention/reabsorption of vitally important small and large molecular weight substances which were present in the circulation.

It is well documented that:

- modified aags (or molecules similar to self ags [10] [molecular mimicry] [21, 22, 38] and not native aags) initiate/maintain an autoimmune disease process, therefore, their removal/neutralization from the system is paramount;
- native aags are targets [8] which principally reside in normal rat kidneys' renal proximal convoluted tubules' BB region and are also sparsely present (in the form of ICs; specific IgM aabs combined with the nephritogenic aag) and targeted in the glomeruli.

Therefore, the elimination/neutralization of the modified nephritogenic ag – that initiated and maintained the production of pathogenic IgG aabs, kidney lesions in the proximal convoluted tubules and in the glomeruli – would specifically and without side effects

terminate the immunopathological processes that caused/maintained the autoimmune kidney disease.

The prevention and when present, the specific termination of the kidney disease SPHN (resulting in tolerance to self) can be achieved by the implementation of the third vaccination technique that we have developed and call MVT [9, 11, 15].

We assert:

- when molecules with identical molecular sequences/structures to normal self ags are produced;
- when against normal self ags specific IgM abs are produced; and
- when ICs made up of the native self ags and specific IgM abs at slight ag excess are created and injected into patients to prevent/terminate certain autoimmune diseases;

the developing high titred nonpathogenic IgM aabs which are directed against the target ag will neutralize the modified aag, thereby terminating the autoimmune disease causing processes.

The MVT promises not only to downregulate/terminate immunopathological events in SPHN (against a target nephritogenic aag that caused the pathogenic IgG aab induced kidney disease in rats; primarily against the renal proximal tubules) but also to terminate autoimmune diseases which are initiated by altered or modified self ags.

When ICs made up of native aags (or native aag equivalent molecules) and specific homologous IgM abs against the native aags and injected into patients at slight ag excess, they will have the potential of producing the same abs with the same specificities against the native target ags that resides in the inoculum, namely, human anti-human native ag IgM aabs. This heightened nonpathogenic IgM aab response against the native target aag (the aabs being cross reactive) could assist in the removal of the pathogenic IgG aab producing modified native aag from the system, thereby terminating the autoimmune disease causing events specifically and without side effects.

List of Abbreviations

aab, autoantibody; aag, autoantigen; ab, antibody; ag, antigen; BB, brush border; GBM, glomerular basement membrane; HN, Heymann nephritis; IC, immune complex; IgG, immunoglobulin G; IgM, immunoglobulin M; MVT, modified vaccination technique; rKF3, rat anti-rKF3; rKF3, rat kidney fraction 3; SPHN, slowly progressive Heymann nephritis

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Chapter 13

Dysregulation of Autoimmunity Caused by Silica Exposure: Fas-Mediated Apoptosis in T Lymphocytes Derived from Silicosis Patients

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Abstract

Silicosis patients suffer not only from respiratory disorders, but also from autoimmune diseases. To clarify the mechanisms involved in the dysregulation of autoimmunity found in silicosis patients, we have been focusing on Fas and Fas-related

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molecules in the Fas-mediated apoptotic pathway because Fas is one of the most important molecules regulating autoimmunity in T cells. Our findings have shown that in comparison to healthy donors, silicosis patients exhibit elevated serum soluble Fas levels, an increased relative expression of the soluble fas and dcr3 genes in peripheral blood mononuclear cells, other highly detectable variant messages of the fas transcript, a relatively decreased expression of several physiological inhibitors (survivin and tso), and a dominancy of lower membrane Fas expressers in lymphocytes, which transcribe soluble fas dominantly. These findings are consistent with immunological factors such as serum immunoglobulin G levels and the titer of anti-nuclear autoantibodies. In addition, anti-caspase 8 autoantibody and anti-Fas autoantibody were detected in serum from silicosis patients, and a functional assay showed that anti-Fas antibody stimulated Fas-mediated apoptosis. We hypothesize that there are two subpopulations of silicosis lymphocytes. One is a long-term survival fraction including a self-recognizing fraction showing lower levels of membrane Fas and inhibition of Fas/Fas ligand binding in the extracellular spaces. The other is a fraction exhibiting apoptosis caused by silica/silicates, recruitment from bone marrow, higher levels of membrane Fas, and sensitivity to anti-Fas autoantibody. Further investigations should be performed to confirm the effects of silica/silicates on the human immune system.

In addition, results concerning whether serum soluble interleukin-2 receptor and soluble CD40 ligand levels should be considered immunological markers in silicosis patients are discussed.

Furthermore, based on our previous reports showing *in vitro* activation of peripheral T cells by silica and reduced function of the CD4+CD25⁺ fraction in which FoxP3⁺ regulatory T cells (Treg) are located, the reconstitution of the CD4+CD25⁺ fraction in silicosis patients (SILs) was analyzed. Since T cells in peripheral CD4+CD25⁺ as well as CD4+CD25⁻ fractions from SILs showed higher expression of pd-1 (marker gene for T-cell activation) compared to that of healthy donors (HDs), chronic T-cell activation is thought to have occurred in SILs. In addition, surface Fas expression of Treg was higher in SILs than HDs. The *ex vivo* experiments using freshly isolated peripheral blood mononuclear cells (PBMCs) from SILs and HDs cultured with or without silica showed loss of Treg by Fas-mediated apoptosis and an increase of activated CD25⁺ T cells in PBMCs from SILs as well as HDs. Although T cells in SILs are thought to have been exposed to low-dose silica for a long time, their effector T cells (Teff) and Treg still possess the capacity to be activated by silica. These activations of both Teff and Treg cause re-constitution of the peripheral Treg fraction, loss of Treg and contamination of activated Teff, resulting in a reduction of the size and function of Treg. These results might contribute to an elucidation of the development of autoimmune diseases found in silicosis patients.

Silicosis and Dysregulation of Autoimmunity

Patients with silicosis are known to not only have respiratory disorders such as progressive pulmonary fibrosis, but also immunological complications such as rheumatic arthritis (known as Caplan syndrome), systemic sclerosis (SSc), and systemic lupus erythematosus (SLE) [1-6]. These immunological abnormalities have been found not only in patients with silicosis who were exposed to and inhaled natural crystalline silica (SiO_2), but also in patients who have received plastic surgery with implants containing silicone ($[\text{SiO}_2\text{-O}]_n$) [7-12]. These epidemiological findings indicate that crystalline silica causes dysregulation of the human immune system, particularly autoimmunity. Silica-induced disorder of

autoimmunity is considered due to the adjuvant-type effects of silica. However, more precise analyses are needed, especially given recent immunomolecular biological findings.

In this chapter, we describe 1) alteration of Fas/CD95 and its related molecules in silicosis patients who have not yet manifested symptoms of autoimmune diseases, since the Fas molecule is known to be important for inducing apoptosis of lymphocytes and the mouse model of mutated Fas shows sensitivity to autoimmune diseases such as SLE. We thought patients with silicosis and without any symptomatic autoimmune diseases have already shown subclinical abnormalities related to Fas and its related molecules.

In addition, the CD4+25+FoxP3+ regulatory T cell (Treg) has recently been shown to be a main key cell type regarding the regulation of stimulation/activation of the effector T cell (Teff) to foreign and/or self antigens[13-18]. Several reports indicated that reduction of the size and/or function of Treg may cause continuous maintenance of activated Teff to induce dysregulation of autoimmunity. Thus, even when we consider silicosis patients as representing a pre-status condition of autoimmune diseases, there might be dysfunction concerning a size reduction of the Treg population.

All patients with silicosis reported here were employees of the brickyards in Bizem, Okayama Prefecture, Japan. The percentage of free silica inhaled by these patients has been estimated to be 40–60%. Each patient received diagnosis of pneumoconiosis, according to records of the International Labor Office (ILO) published in 2000 [19]. They showed no clinical symptoms of autoimmune disease, including sclerotic skin, Raynaud's phenomenon, facial erythema or arthralgia, and had no malignancies. Specimens were obtained only in cases in which informed consent had been obtained.

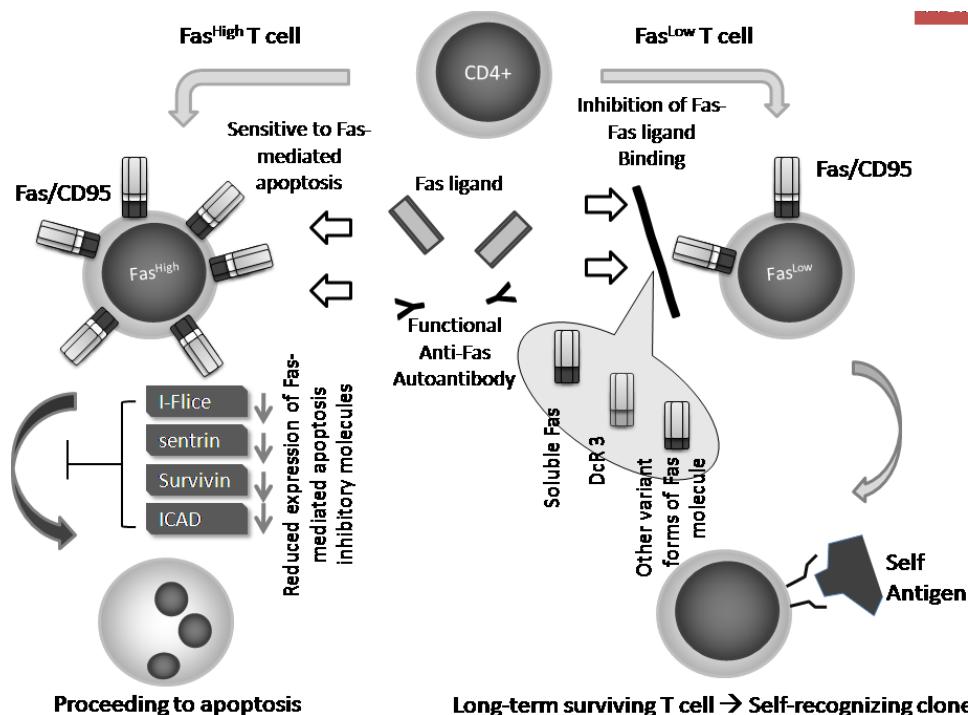


Figure 1. Schematic model of dysregulation of the Fas and Fas-related molecules, and Fas-mediated apoptosis found in silicosis patients

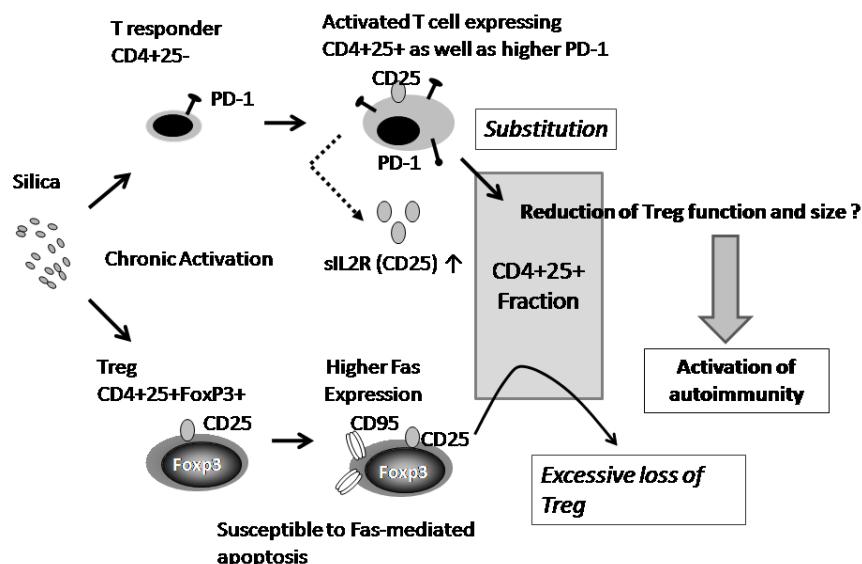


Figure 2. Schematic models of alteration of the peripheral CD4+25+ fraction in silicosis patients, caused by chronic activation of not only Teff but also Treg, due to chronic and recurrent exposure to silica

Fas and Fas-related Molecules Found in Silicosis Patients

Fas/CD95 is mainly expressed on the cell surface of lymphocytes as a membrane type and forms a trimer after binding with the Fas ligand [20-29]. After binding, the cell death signal is transduced using the death domain located in the intracellular domain of Fas and recruits FADD and procaspase 8/10 to form the active death-inducing signaling complex (DISC). Thereafter, activated caspase 8/10 triggers a caspase-cascade involving CAD/CPAN/DFF40 (by removing its inhibitor, ICAD/DFF45), DNA fragmentation, and finally apoptotic cell death. The soluble Fas, the most typical alternative spliced variant of the wild-type *fas* gene transcript, lacks 63 bp of the transmembrane domain and can be secreted from cells to inhibit binding of membrane Fas and the fas ligand. If high levels of soluble Fas exist in the extra-cellular region, lymphocytes may be protected from Fas-mediated apoptosis and survive longer. Higher serum levels of soluble Fas have been reported in several autoimmune diseases.

A comparison of the cellular and molecular changes of fas and Fas-related molecules between silicosis patients (SILs) and healthy donors (HD) yielded the following findings:

- 1). Serum soluble Fas was elevated in SILs compared to HD [30].
- 2). The soluble Fas message was dominantly expressed in peripheral blood mononuclear cells (PBMCs) from SILs compared to those from HD [31].
- 3). Gene expression of decoy receptor 3 (DcR3), which functions in a manner similar to soluble Fas, was higher in PBMCs from SILs than HD [32].

- 4). Various alternative transcript variants of the *fas* gene, which were supposed to function in a manner similar to the soluble Fas from their molecular components, were detected frequently in PBMCs from SILs [33].
- 5). Lymphocytes presenting the lower surface Fas mainly expressed the soluble *fas* message, and the higher surface Fas mainly expressed the wild-type *fas* [34].
- 6). The expressions of various genes inhibitory to fas-mediated apoptosis, such as *I-Flice*, *sentrin*, *survivin* and *ICAD*, decreased in PBMCs from SILS relative to HD [35, 36].
- 7). Functional Fas-stimulus anti-Fas autoantibody was detected in the serum of SILs [37].

Taken together, these results led us to suppose that there might be two populations of T lymphocytes in silicosis patients (Fig. 1). One is a population which survives longer and includes auto-recognizing T cell clones. Characteristics of this population may include a lower surface Fas expression, production of soluble Fas, DcR3 and other variant messages, with Fas-mediated apoptosis being inhibited by these products. The other population is one which proceeds to undergo Fas-mediated apoptosis. This population is characterized as possessing enough surface *fas* expression, a reduced expression of inhibitory molecules against fas-mediated apoptosis, and is sensitive to the *fas* ligand and autoantibody of the Fas found in silicosis patients.

Treg Status in Silicosis

Treg have been well investigated and possess special characteristics such as suppression of T-cell stimulation *in vivo*, anergy *in vitro*, production of interleukin (IL)010/transforming growth factor (TGF)- β , CTLA-4 expression, glucocorticoid-induced tumor necrosis factor receptor family-related protein (GITR) expression, and forkhead box P3 (FoxP3) expression. Treg are thought to contribute to T-cell homeostasis, the prevention of autoimmune diseases, tolerance after transplant, and the prevention of graft-versus-host disease, allergy and hypersensitivity. Detrimental effects of Treg may be a down-regulation of tumor immunity and immune-defense to various pathogens [13-18].

We previously reported that the CD4+25+ fraction in PBMCs derived from silicosis patients showed reduced Treg function to suppress auto-Teff proliferation against allo-antigen in comparison with HD, and even these silicosis patients did not show any autoimmune symptoms except higher serum anti-nuclear antigen titers [38]. We also reported that silica can slowly and gradually stimulate peripheral blood T cells *in vitro* as monitored by expression of CD69, an early expressing antigen of T-cell activation [39]. If chronic and recurrent exposure to silica causes continuous activation of T cells, activated Teff will present CD25 as an activation marker. Thus, if PBMCs circulating in silicosis patients include chronically and continuously activated Teff, the CD4+25+ fraction may consist of Treg and activated Teff. This may be one of the reasons why the CD4+25+ fraction from silicosis patients showed reduced function as Treg. However, if this hypothesis is correct, and although there may be an increase of the CD4+25+ fraction in PBMCs of silicosis patients

relative to HD, the Treg function of the CD4+25+ fraction may not be disturbed since there is no way of reducing Treg cells.

We then compared surface CD95/Fas expression in Treg between SILs and HD and found that Treg in SILs exhibited a high expression of Fas on their surface. This is reasonable since it has been reported that activated Treg may express higher Fas and that this may be the homeostasis process to close the Treg function. These findings lead to the consideration that silica exposure activates not only Teff, but also Treg cells. Activated Treg expressing higher Fas may proceed to apoptosis by the fas ligand produced by other cells such as macrophage and Teff, which are also activated by silica exposure, and Treg may decline from the peripheral CD4+25+ fraction in PBMCs. In addition, activated Teff will enter the CD4+25+ fraction instead of pure Treg. These considerations may explain why the Cd4+25+ fraction in PBMCs from silicosis patients showed reduced function as Treg. Analyses to confirm this hypothesis are ongoing and we have demonstrated that the peripheral regulatory T cell from silicosis patients is susceptible to CD95-mediated apoptosis [39].

Conclusion

Chronic silica exposure causes dysregulation of autoimmunity. This has been confirmed by many epidemiological analyses. However, little is known about the mechanisms concerning how inhaled silica can disturb autoimmunity and induce a hyper-reaction to the self-antigen. Our investigations may provide insights into the effects of silica on the human immune system and the pathophysiology of autoimmune diseases since we can regard silicosis patients as representing a pre-status condition of autoimmune disease. Further investigations may further validate these points and contribute to recognition of the occurrence of factors and environmental effects that induce dysregulation of autoimmunity.

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Chapter 14

Unusual Renal Localization of Vasculitis in Sjögren's Syndrome: A Clinical Case Report

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Abstract

A rare case of a patient with Sjögren's syndrome is described associated with proteinuria, microscopic hematuria, and gradually deteriorating kidney function. Renal biopsy has shown interstitial nephritis and vasculitis. The patient also had recurrent chronic genital herpes and was positive to human papilloma virus. The patient was treated with high dose corticosteroids. Due to the worsening renal function caused by the progressing vasculitis, cyclophosphamide therapy was inevitable even though the viral infection was a contraindicating factor. Therefore, along with antiviral therapy (acyclovir), we initiated cyclophosphamide therapy whereby the patient's kidney functions improved without progression of the infection. In patients with Sjögren's syndrome the deterioration of the renal function may raise the possibility of ongoing renal vasculitis, therefore thorough nephrological investigations are necessary and if the diagnosis is proven adequate immunosuppressive therapy needs to be initiated immediately, despite the risk of activating any associated infectious diseases.

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Keywords: Sjögren's syndrome, interstitial nephritis, vasculitis, HPV.

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune disease that prototypically affects exocrine glands and characterized by a wide variety and combination of symptoms. The major symptoms of pSS are keratoconjunctivitis sicca and xerostomia, as a result of lymphocytic infiltration and destruction of the salivary and lacrymal glands. In about 60-70 % of the cases, the first sign is the glandular involvement alone [1, 2], while in some instances the disease begins with systemic/extraglandular manifestations.

Renal involvement has been described in about 15-25% of the cases; both tubular and glomerular damage have been shown in pSS, although glomerular manifestations are rare [1, 3]. The renal pathology shows prominent tubulointerstitial nephritis, without specific symptoms. Histopathology indicates vascular signs more frequently (25-40%), involving the small and medium-sized arteries, clinically appearing as purpura in approximately 10% of the cases [4-6]. Since vasculitis rarely affects renal vessels and the association of different forms of renal manifestations in pSS is quite unique, hereby we depict a case of renal localization of vasculitis in pSS.

Case Report

In 2007, a 27-year-old Caucasian woman had significant weight loss and increased erythrocyte sedimentation rate (ESR), presenting with positive anti-nuclear antibody (ANA), anti-extractable nuclear antigen antibody (aENA), aSS-A and rheumatoid factor (RF) titers, splenomegaly, exanthema, leukopenia, lymphocytopenia at the beginning of her disease. Proteinuria (1g/day) and microscopic hematuria were observed along with a progressive decline in the kidney function; the estimated glomerular filtration rate (eGFR) was 50ml/min (normal range for GFR is 100-120 ml/min). The obligatory glandular symptoms were confirmed by appropriate investigations, including salivary gland biopsy, and indicated pSS. Based on the clinical and laboratory assessment, the patient fulfilled the classification criteria for pSS [5]. No clinical, or immunoserological markers were indicative of systemic lupus erythematosus. Renal biopsy was also performed and showed interstitial nephritis together with vasculitis (Figures 1, 2 & 3). Along with these symptoms, the patient suffered from recurrent and chronic persistent genital herpes and human papilloma virus (HPV) infection, associated with condyloma. Consequently, at this time she was treated only with high dose glucocorticosteroids, although only mild to moderate therapeutic effect could be detected. The progressive deterioration of the renal function, probably due to renal vasculitis required cyclophosphamide therapy. Despite of the risk of activation, or progression of viral infections, in agreement with infectologists, we started cyclophosphamide infusions, according to the National Institute of Health (NIH) recommendations, combined with antiviral (acyclovir) prophylaxis. Up till now, she received 6 cycles of cyclophosphamide (0.75g/m²), without any side effect. Cyclophosphamide proved to be effective, indicated by the normalization of the GFR, and the reduction of proteinuria. Regular gynecological controls have not indicated

progression, or malignant transformation of HPV infection; the condyloma could be well controlled by local therapy. No relapse of genital herpes has occurred.

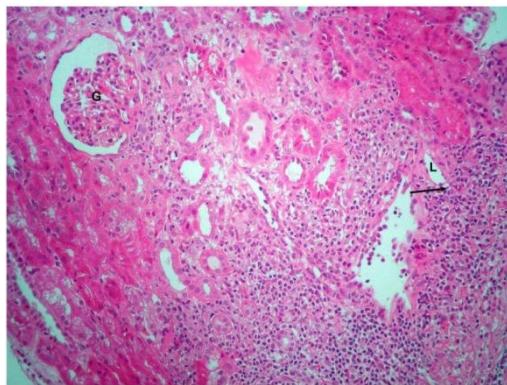


Figure 1. Vasculitis of the kidney

Extensive cellular infiltration destructs the vessel wall (arrow). (L = vascular lumen, G = glomerulus). HE staining. Magnification: 200x

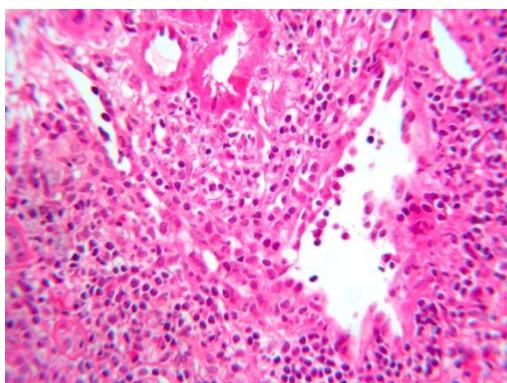


Figure 2. Vasculitis of the kidney

Extensive mononuclear cell infiltration around a renal vessel. Magnification: 400x

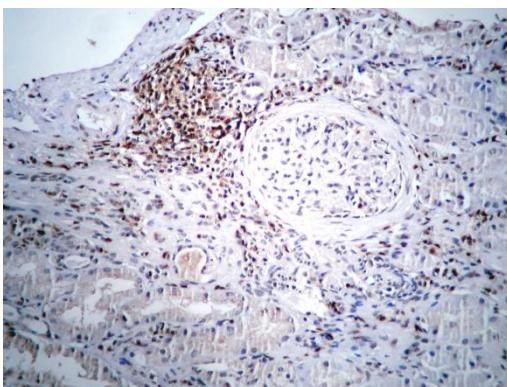


Figure 3. Prominent T-cell dominance within the interstitial cellular infiltration

Brown color marks T-cell positivity. Immunohistochemistry using anti-CD3 antibody (DAKO), detection using EnVision and diaminobenzidine. Magnification: 200x

Conclusion

Renal manifestations, as part of the extraglandular manifestations of the disease can be detected in about 15-20 % of patients with pSS. Histologically, it appears as tubulo-interstitial nephritis and may lead to renal tubular acidosis, hyposthenuria, rarely proteinuria, and aminoaciduria [10]. Vasculitis is another relatively common manifestation of pSS; however it localizes mostly to skin vessels. In pSS, the systemic or internal manifestation of vasculitis is less frequent, although may affect any of the small and medium size vessels. Skin vasculitis, manifested as purpura shows a strong association with tubulopathy. Among the renal manifestations tubulointerstitial nephritis is the most common, however glomerulonephritis also may occur [11]. Up till now, this is the first case on interstitial nephritis, associated with renal vessel vasculitis described in a patient with pSS.

Vasculitis is considered to be a severe complication and renal localization may affect vital organ functions. Therefore, in such situations cyclophosphamide is an adequate therapy for the induction of remission [12], while more aggressive immune suppression may increase the risk for different infections.

The etiology of pSS is unknown, although genetic and environmental factors including hormonal imbalance (female dominancy) are strongly suspected. One of the most important pathogenic steps is the disproportional B cell activation and pathogenic autoantibody secretion [7]. Otherwise, the central role for an infectious agent in the development of pSS has been advocated and indeed a number of viruses have been suspected to launch such a disease. These include herpes virus 6, cytomegalovirus(CMV), Epstein-Barr virus(EBV), hepatitis C virus(HCV), human T lymphotropic virus type 1(HTLV), human immunodeficiency viruses(HIV), human intracisternal A-type retroviral particle, and human retrovirus 5 [8]. There are some studies regarding to direct detection viruses e.g. double stranded DNA viruses (CMV, EBV) and four retroviruses (HTLV-1, HIV, Intracisternal A type particles, and human retroviruses 5) [8, 9]. The role of viruses is suspected in the development of the pSS. Sicca syndrome affects exocrine organs, leading to keratoconjunctivitis sicca, xerostomia and genito-urinary dryness, as well. The epithelial barrier damage (e.g. of the genitourinary system) can contribute to the development of bacterial and viral sexually transmitted diseases (STD), such as HPV, gonococcus, HIV. In general, many viruses can be responsible for systemic vasculitis, the most frequent being hepatitis B virus-related polyarteritis nodosa (HBV-PAN), HIV, erythrovirus B19, CMV, varicella-zoster virus and HTLV-1. On the other hand, some bacteria, fungi or parasites can also cause vasculitis [14].

We report the case of pSS with associating tubulo-interstitial nephritis and renal vasculitis leading to progressive deterioration of kidney functions. We believe that in patients with pSS the worsening renal function may raise the possibility of ongoing renal vasculitis, therefore thorough nephrological investigations are necessary and if the diagnosis is proven adequate immunosuppressive therapy should be initiated immediately, despite the risk of activating any associated infectious diseases.

List of Abbreviations

aENA, anti-extractable nuclear antigen antibody; ANA, anti-nuclear antibody; aSS-A, Anti-Sjögren's Syndrome antibody A; CMV, cytomegalovirus; EBV, Epstein-Barr virus; ESR, erythrocyte sedimentation rate; GFR, glomerular filtration rate; HBV-PAN, hepatitis B virus-related polyarteritis nodosa; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HPV, human papilloma virus; HTLV, human T lymphotropic virus type 1; NIH, National Institute of Health; pSS, Primary Sjögren's syndrome; RF, rheumatoid factor; STD, sexually transmitted disease

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