



Pathophysiologic Mechanisms in Acquired Aplastic Anemia

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Aplastic anemia, an unusual hematologic disease, is the paradigm of the human bone marrow failure syndromes. Absence of hematopoietic cells has been recognized from the characteristic morphology for a century; an immune pathophysiology has been inferred from improvement in blood counts with immunosuppressive therapy in the majority of patients. Molecular mechanisms underlying both T cell effector cells and the target marrow stem and progenitor cells are now being identified. Activated type 1 cytotoxic T cells and type 1 cytokines have been

implicated in cell culture experiments; clues to the molecular basis of the aberrant immune response include cytokine gene polymorphisms and abnormalities in the regulatory pathways for γ -interferon. For stem cell depletion, mutations in genes of the telomere repair complex are present in some patients with apparently acquired aplastic anemia. Telomerase deficiency is associated with short telomeres and a quantitative reduction in marrow progenitors and likely also a qualitative deficiency in the repair capacity of hematopoietic tissue.

Historically, patients with severe forms of marrow failure have faced a dismal prognosis, and there are formidable practical difficulties of experimentation in a rare disorder in which the cells of interest have disappeared. **Early observers of the disease emphasized putative etiologies and the relationship of aplastic anemia to environmental factors, such as chemicals (especially benzene) and idiosyncratically to specific medical drug exposures (most notably chloramphenicol).** Over several decades, and following on the observations of improvement of blood counts in the majority of patients who were treated with immunosuppressive therapies, understanding of aplastic anemia has cohered around a unified immune mechanism of hematopoietic cell destruction. Technical advances in cell biology, flow cytometry, molecular biology, and immunology provided methods to measure numbers and function of very limited numbers of cells. Most recently, acquired aplastic anemia has been linked to inherited genetic mutations, and the molecular biology of the disease has been elucidated by inferences from the function of the affected genes.

This review is extracted from a contemporary publication in *Blood*¹ and developed more extensively in the author's textbook²; the reader is referred to other monographs, textbook chapters, and reviews.^{3,4}

Etiologies

Clinical associations

Since Ehrlich's description of the first case of aplastic anemia in a pregnant woman, precipitating factors have been

sought from the individual patient's history. An enormous literature, dating from the beginning of the 20th century, described chemical- and drug-induced disease, stimulated by observations of the effects of benzene on blood counts, of dipyrone's association with agranulocytosis, and a seeming epidemic of aplastic anemia after the introduction of chloramphenicol. **It is noteworthy that neither chemicals nor drugs appear to account for a large proportion of cases of acquired aplastic anemia in more modern and formal series.**

Clinical associations are worth reassessment in the context of the immune hypothesis of marrow failure. **Pregnancy appears a real association, as deduced more from the documented improvement of blood counts with its termination than from formal epidemiologic study.⁵ The unusual syndrome of eosinophilic fasciitis also is strongly linked to aplastic anemia. Five to ten percent of cases of aplastic anemia follow an episode of seronegative hepatitis, in which immune activation is inferred from the pattern of T cell activation, cytokine production, and HLA association; despite intensive and sophisticated efforts, an infectious agent has yet to be identified.⁶ Benzene, or more correctly its metabolites, is a marrow toxin in animals and man,** but antique case reports leave doubt as to whether marrow failure in benzene workers was not often myelodysplasia rather than aplastic anemia. Unpredictable marrow failure in the setting of routine medical drug use is devastating to the patient and physician and has serious legal ramifications in pharmaceutical development. The study of idiosyncratic drug reactions, by definition so extremely rare, is difficult. That genetic differences in drug metabolism, especially in detoxification of reactive intermediate compounds, likely underlie susceptibility is thus poorly supported by experimental data. Overrepresentation of deletions in the drug metabolizing glutathione-S-transferase genes (*GSTM1*, *GSTT1*, which would increase concentrations of toxic drug intermediates) have been observed in some series.^{7,8} Nevertheless, no satisfactory mechanisms have been developed

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for the most notorious pharmaceutical, chloramphenicol, or for other heavily inculpated agents such as penicillamine or gold. Many drugs on "black lists" also more commonly cause mild marrow suppression. Regular but only modest destruction of marrow cells may sometimes be a prerequisite for a much more infrequent immune response to an exposed neoantigen. There is little demographic or clinical difference between patients with idiopathic aplastic anemia and those with an assumed drug etiology. Claims of permanent aplastic anemia after idiosyncratic exposure to minuscule quantities of chloramphenicol, as in ophthalmic solutions, may reflect observation and reporting biases rather than extreme sensitivity to a hidden metabolite. Conversely, very few chemotherapeutic agents, despite being designed as cell poisons and administered in milligram or gram quantities, directly result in irreversible marrow destruction without obvious effects on other organs.

Epidemiology

The broad study of large populations and intensive investigation of clinically well-characterized patients are two complementary approaches to identifying an etiologic agent. Unfortunately, neither has yielded conclusive results. The two largest controlled epidemiologic studies that have been conducted are the International Aplastic Anemia and Agranulocytosis Study in Europe and Israel in the 1980s and the recently completed Thai NHLBI Aplastic Anemia Study performed in Bangkok and a northeast rural region in the 1990s.⁹ The incidence of aplastic anemia in the West is 2 per million and is about 2- to 3-fold higher in Asia. Benzene and pesticides, while significantly associated, accounted for only a small number of cases in both studies, and medical drugs have a negligible role in Asia. In rural Thailand, exposure to nonbottled water as well as to certain animals (ducks and geese), to animal fertilizer, and also to pesticides suggested an infectious etiology.

Autoantigens

A few putative antigens have been teased from screening antibodies in patients' sera against a peptide library (by expression of genes expressed in fetal liver or leukemic cell lines). Kinectin, a widely expressed protein, bound to antibodies from about 40% of aplastic patients.¹⁰ Another antigen that bound to antibodies, in a smaller minority of marrow failure patients, was diazepam-binding related protein-1, an enzyme essential in the oxidation of unsaturated fatty acids and broadly distributed in tissues.¹¹ The relevance of these autoantibodies to a cellular pathophysiology of aplastic anemia is unclear. For kinectin, reactive cytotoxic T cells could be generated *in vitro* and inhibited human hematopoietic colony formation, but anti-kinectin T cells were not found in patients.¹⁰ For diazepam-binding related protein-1, a putative T cell epitope derived from this protein could stimulate cytotoxic T cells obtained from one patient, and T cell precursors with peptide-binding activity were present in two cases.

Pathophysiology

In most cases, aplastic anemia behaves as an immune-mediated disease. Cellular and molecular pathways have been mapped in some detail for both effector (T lymphocyte) and target (hematopoietic stem and progenitor) cells. The combination of exposure to specific environmental precipitants, diverse host genetic risk factors, and individual differences in the characteristics of the immune response likely account for the disease's infrequency, variations in its clinical manifestations, and patterns of responsiveness to treatment.

Immune-mediated T cell destruction of marrow

An immune mechanism was inferred from the recovery of hematopoiesis in patients who failed to engraft after stem cell transplant, when renewal of autologous blood cell production was credited to the conditioning regimen. The majority of syngeneic transplants in which bone marrow was infused without conditioning failed, indicating that the pathophysiology in these cases must be more complex than simple stem cell absence. The responsiveness of aplastic anemia to immunosuppression remains the best evidence of an underlying immune pathophysiology: the majority of patients show hematologic improvement after only transient T cell depletion by antithymocyte globulins (ATG); relapse also usually responds to repeated immunosuppression; and dependence of adequate blood counts on administration of very low doses of cyclosporine is not infrequent. As immunosuppressive regimens have intensified, from early attempts with corticosteroids to aggressive strategies such as high-dose cyclophosphamide, and the proportion of responders has risen, the willingness to ascribe an immunological mechanism has also increased. Indeed, little distinguishes responders to immunological therapy from refractory patients (other than age, because children show higher rates of both hematologic recovery and survival). Failure to respond to immunosuppression has been interpreted as indicating an alternative pathophysiology but is also consistent with either severe stem cell depletion or immunological mechanisms not susceptible to current therapies.

In early laboratory experiments, removal of lymphocytes from aplastic bone marrows improved colony numbers in tissue culture, and their addition to normal marrow-inhibited hematopoiesis *in vitro*. The effector cells were identified by immunophenotyping as activated cytotoxic T cells expressing Th1 cytokines, especially γ -interferon. CD8 cells containing intracellular interferon may now be measured directly in the circulation¹² and oligoclonal expansion of CD8⁺ CD28⁻ cells, defined by i) flow cytometric analysis for T cell receptor (TCR) V β subfamilies; ii) spectratyping to detect skewing of CDR3 length; and iii) sequencing of the CDR3 region to establish a molecular clonotype.¹³ Patients at the time of clinical presentation have oligoclonal expansions of a few V β subfamilies, which diminish or disappear with successful therapy; original

clones reemerge with relapse, sometimes accompanied by new clones, consistent with epitope spreading of the immune response. Occasionally, a new clone persists in remission, perhaps evidence of T cell tolerance.

The impact of T cell attack on marrow can be modeled *in vitro* and *in vivo*. γ -Interferon (and tumor necrosis factor- α) reduces the numbers of human hematopoietic progenitor-derived colonies *in vitro*; the cytokines efficiently induce apoptosis in CD34 target cells, at least partially through the Fas-dependent pathway of cell death. Also *in vitro*, sustained exposure to low concentrations of γ -interferon markedly reduces the output of long-term culture-initiating cells (LTIC-IC), consistent with local amplification of toxicity in the marrow milieu. *In vivo*, the infusion of parental lymph nodes cells into F1 hybrid donors causes pancytopenia, profound marrow aplasia, and death. Murine ATG, cyclosporine, and also monoclonal antibodies to interferon and tumor necrosis factor abrogate hematologic disease and rescue animals; there is a powerful “innocent bystander” effect, in which activated cytotoxic T cells kill genetically identical targets.¹⁴

Why T cells are activated in aplastic anemia is unclear. HLA-DR2 is over-represented among patients, suggesting a role for antigen recognition, and its presence is predictive of a better response to cyclosporine.¹⁵ Polymorphisms in cytokine genes, associated with an increased immune response, also are more prevalent: a nucleotide polymorphism in the tumor necrosis factor- α (*TNF2*) promoter at -308^{16,17} and homozygosity for a variable number of dinucleotide repeats in the gene encoding γ -interferon.¹⁸ Constitutive expression of Tbet, a transcriptional regulator that is critical to Th1 polarization, occurs in a majority of aplastic anemia patients.¹⁹ Mutations in *PRF1*, the gene for perforin, are responsible for some cases of familial hemophagocytosis; mutations in *SAP*, a gene encoding for a small modulator protein that inhibits γ -interferon production, underlie X-linked lymphoproliferation, a fatal illness associated with an aberrant immune response to herpesviruses and aplastic anemia. Perforin is overexpressed in aplastic marrow.²⁰ Perforin and *SAP* protein levels are markedly diminished in a majority of acquired aplastic anemia cases (Solomou E, unpublished data). Mesenchymal cells from aplastic bone marrow also may inadequately suppress T cell activation.²¹ Genome-wide transcriptional analysis of T cells from aplastic anemia patients has implicated components of innate immunity in aplastic anemia, including Toll-like receptors and natural killer cells,²² for which there is some preliminary experimental support.^{23,24}

Hematopoiesis

Immune attack leads to marrow failure. The pallor of the modern biopsy core or empty spicules of an aspirate, few or no CD34 cells on flow cytometry, and minimal numbers of colonies derived from committed progenitors in semisolid media all reflect the severe reduction in hematopoietic cells that defines the disease. Stem cell “surrogate” assays, LTC-

IC or cobblestone-forming cells, which measure a primitive infrequent and quiescent multipotential progenitor cell, also show marked deficiency, and from the product of the low percentage of marrow cellularity and the scant numbers of LTC-IC per mononuclear cell, suggest that only a few percent of residual early hematopoietic cells remain in severely affected patients by the time of clinical presentation. Qualitative features of these few cells, as measured, for example, by poor colony formation per CD34 cell or inadequate response to hematopoietic growth factors, are harder to interpret, but recent genetic studies have suggested explanatory mechanisms (see below). The reduced number and function of the marrow is secondary to cell destruction, and apoptosis is prevalent among the few remaining elements.²⁵ Microarray of the scant CD34 cells from marrow failure patients revealed a transcriptome in which genes involved in apoptosis, cell death, and immune regulation were upregulated²⁶; this transcriptional signature can be reproduced in normal CD34 cells exposed to γ -interferon.²⁷

One peculiar feature of white blood cells in aplastic anemia is short telomeres. Telomere shortening was initially most easily blamed on stem cell exhaustion. However, the discovery, first by linkage analysis in large pedigrees, that the X-linked form of dyskeratosis congenita was due to mutations in *DKC1* and subsequently purposeful identification of mutations in *TERC* in some autosomal dominant patients with this constitutional marrow failure syndrome indicated a genetic basis for telomere deficiency. Central to the repair machinery is an RNA template, encoded by *TERC*, upon which telomerase, a reverse transcriptase encoded by *TERT*, elongates the nucleotide repeat structure; other proteins, including the *DKC1* gene product dyskerin, are associated with the telomere repair complex. Systematic surveys of DNA disclosed first *TERC*²⁸ and later *TERT* mutations²⁹ in some patients with apparently acquired aplastic anemia, including older adults. Family members who share the mutation, despite normal or near normal blood counts, have hypocellular marrows, reduced CD34 cell counts and poor hematopoietic colony formation, increased hematopoietic growth factor levels, and of course short telomeres. The clinical presentation of most adult patients with *TERC* and *TERT* mutations is much later than in typical dyskeratosis congenita, and they lack typical physical anomalies.^{28,29} Chromosomes are also protected by several proteins that bind directly to telomeres, and polymorphisms in their genes (*TERF1*, *TERF2*) are also more or less prevalent in aplastic anemia compared to healthy controls.³⁰ A few of our patients also have heterozygous mutations in the Shwachman-Bodian-Diamond syndrome (*SBDS*) gene. Almost all children with this form of constitutional aplastic anemia are compound heterozygotes for mutations in *SBDS*, and their white cells have extremely short telomeres; however, the *SBDS* gene product has not been directly linked to the telomere repair complex or to telomere binding. A parsimonious inference from all these

data is that inherited mutations in genes that repair or protect telomeres are genetic risk factors in acquired aplastic anemia, probably because they confer a quantitatively reduced hematopoietic stem cell compartment that may also be qualitatively inadequate to sustain immune mediated damage. Telomeres are short in one-third of aplastic anemia patients, but mutations have only been identified in less than 10% of cases. The most interesting explanation is involvement of other genes, including for other members of the large repair complex, telomere-binding proteins, still obscure components of the alternative repair system, and some DNA helicases. Alternatively, telomere shortening may be secondary to stem cell replication.

Clonal Evolution

Clinically, aplastic anemia may coexist or appear to evolve to other hematologic diseases that are characterized by proliferation of distinctive cell clones, as in paroxysmal nocturnal hemoglobinuria (PNH) or myelodysplasia (MDS). The mechanisms linking immune-mediated and premalignant pathophysiologies are not well elucidated in marrow failure or in parallel circumstances (chronic hepatitis and hepatocellular carcinoma, ulcerative colitis and colon cancer, and many others). The presence of tiny clones at the time of diagnosis of aplastic anemia, detected using extremely sensitive assays—phenotypic (flow cytometry for PNH) or cytogenetic (fluorescent *in situ* hybridization for MDS)—also creates problems of disease classification and patient diagnosis.

PNH

Fifty percent or more of patients at presentation with pancytopenia have expanded populations of PNH cells, easily detected by flow cytometry due to the absence of glycosylphosphatidylinositol-linked membrane proteins, the result of somatic *PIG-A* gene mutations.³¹ Most clones are small and do not lead to clinical manifestations of hemolysis or thrombosis, but classic PNH can be dominated by marrow failure (the “aplastic anemia/PNH syndrome”), and all PNH patients show evidence of underlying hematopoietic deficiency. The global absence of a large number of cell surface proteins in PNH has been hypothesized to allow “escape” and survival of a pre-existing mutant clone. Association of an expanded PNH clone with HLA-DR2¹⁵ and with autoantibodies,¹¹ and as a predictor of responsiveness to immunosuppressive therapies,³² all suggest that the escape is from immune attack. However, there is little concrete experimental evidence of reproducible differences in either immune responsiveness or susceptibility of PNH clones compared to phenotypically normal target cell populations.^{33,34} In contrast to cells of normal phenotype, the marrow PNH clone retains its proliferative capacity in tissue culture and does not overexpress Fas³⁵; comparison by microarray shows that residual cells of normal phenotype in the PNH bone marrow upregulate the same apoptosis and cell death genes as do CD34 cells in aplastic marrow,

while the *PIG-A* clone appears transcriptionally similar to CD34 cells from normal donors.³⁶ Despite a few provocative studies,^{23,33,37} no satisfying mechanism to explain clonal escape has convincing empiric support (see Ref ³¹ for review).

MDS

Stereotypical patterns of aneuploidy develop in a minority of patients over time: monosomy 7 or trisomy 8 are most characteristic.³⁸ Trisomy 8 MDS shares many immune abnormalities with aplastic anemia. Patients respond to immunosuppressive therapies, and oligoclonal T cell expansions are usually present.³⁹ T cell oligoclonal appear to recognize the aneuploid cells, and specifically WT1 antigen that they express at high levels, but the target cells are not killed due to upregulation of anti-apoptosis genes, including *c-myc*, *survivin*, and *CDK1*.⁴⁰ For trisomy 8, abnormal cells targeted by the immune system appear to be selected for their capacity to survive cytotoxic lymphocyte attack.

Monosomy 7 is also a frequent cytogenetic abnormality in aplastic anemia but has a poorer prognosis, patients usually succumbing to refractory cytopenias or evolving to acute leukemia.³⁸ Emergence of monosomy 7 has been linked to exogenous use of granulocyte colony-stimulating factor (G-CSF) in aplastic anemia,⁴¹ as occurs also in treated severe congenital neutropenia. Laboratory studies suggest that monosomy 7 clones expand in an abnormal cytokine milieu: high G-CSF concentrations lead to selection of cells that bear a short isoform of the G-CSF receptor that signals proliferation but not differentiation.⁴²

Prospects

If residual stem cell numbers indeed limit recovery in a substantial proportion of patients treated with immunosuppression, *ex vivo* expansion of hematopoiesis may be possible, as for example using Hox box proteins. An expanding view of immune activities in aplastic anemia and in other human autoimmune diseases may explain why immunosuppressive therapy is sometimes ineffective or inadequate. Quantitative and practical measurements of oligoclonal T cell activity and of hematopoietic stem cell number and function may allow laboratory testing to guide treatment decisions. Ultimately, definition of genetic risk factors, affecting hematopoietic cell function and the immune response, will clarify how agents in the environment initiate and perpetuate the marrow destruction of aplastic anemia.

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