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Gender-related influences on the development of chronic graft-*versus*-host disease-induced experimental lupus nephritis

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SUMMARY

Autoimmune diseases are far more common in women than in men. In the incidence of systemic lupus erythematosus (SLE), the female-to-male ratio is as high as 10:1. This suggests that sex hormones may play a fundamental role in determining the susceptibility to these diseases. In order to investigate the sex-related differences in the inducibility of chronic graft-*versus*-host disease-related experimental lupus nephritis, lymphocytes from female DBA/2 donor mice were administered to either male or female (C57BL10 × DBA/2)F1 recipients. An additional group of male recipients received lymphocytes from male DBA/2 donors. After four cell transfers, female recipients developed a significantly higher albuminuria than both male groups. Serum concentrations of autoantibodies against glomerular basement membrane (GBM), collagen IV, and laminin were significantly higher in females 2–4 weeks after induction. Levels of circulating autoantibodies against renal tubular epithelial antigens (RTE) and nuclear antigens were not different between the sexes. In transfer studies, the necessity of the presence of anti-GBM and anti-RTE autoantibodies for the development of glomerulonephritis was confirmed. These findings indicate that: (i) in this model of lupus nephritis, susceptibility to glomerulonephritis is strongly influenced by sex-related genes; and (ii) among the variety of autoantibodies occurring in this model of SLE, both anti-GBM and anti-RTE autoantibodies play a key role in the pathogenesis of glomerulonephritis.

Keywords autoimmunity lupus nephritis graft-*versus*-host disease sex systemic lupus erythematosus

INTRODUCTION

Physiological differences between males and females include the immune system. It is well known that autoimmune diseases are far more common amongst women. Females in general have higher immunoglobulin levels [1], increased antibody production after immunization [2], decreased susceptibility to infections [3], and decreased graft rejection time [4]. The pathogenetic mechanisms responsible for these differences are still not fully known.

Experimental murine chronic graft-*versus*-host disease as a model for systemic lupus erythematosus (SLE) has provided us with a source of information on the pathogenetic pathways of autoimmunity in general and lupus nephritis in particular [5,6]. We used this model to investigate sex-related differences in experimental lupus nephritis. For the induction of chronic graft-*versus*-host disease, 8–10-week old (C57BL10 × DBA/2)F1 hybrids were used as recipients of DBA/2 donor lymphocytes. The chronic graft-*versus*-host reaction is associated with the absence of allocytotoxic $CD8^+$ anti-F1 T cells in the DBA/2-derived inoculate. Recipient B cells are activated by anti-allo-MHC class II $CD4^+$ donor T cells, which in turn leads to a lupus-like autoimmune syndrome. The recipient mice develop a variety of pathological alterations associated with the formation of autoantibodies directed against nuclear antigens (ANA), against components of the glomerular basement membrane (GBM), and against renal tubular epithelial antigens (RTE), and a positive Coomb's test. Renal histopathological alterations are reminiscent of lupus nephritis, as classified by the WHO, including the development of albuminuria, glomerulosclerosis and end-stage renal failure [7–10].

We studied gender-related differences in the development of immune complex glomerulonephritis in this model by examination of albuminuria, autoantibody levels, light microscopy, and by immunofluorescence studies.

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MATERIALS AND METHODS

Animals

Male and female C57BL10 ($H\text{-}2^b$) and DBA/2 ($H\text{-}2^d$) mice were purchased from Olac Ltd. (Bicester, UK). (C57BL10 × DBA/

2)F1 hybrids were bred in our animal facilities. Female and male DBA/2 mice 7–8 weeks old were used as donors. Female and male F1 hybrids, aged 8–10 weeks, were used as recipients of lymphocytes.

*Induction of chronic graft-*versus*-host disease*

Spleens, mesenteric, cervical and inguinal lymph nodes, and thymi were removed from DBA/2 donors under aseptic conditions. Single-cell suspensions were prepared by mincing the tissues in sterile RPMI 1640 medium and gently pressing the fragments through a steel sieve (150 μm pore diameter). The suspensions were then filtered through a sterile Pasteur pipette loosely packed with cotton wool. After two washes in RPMI 1640 medium, each followed by centrifugation twice for 10 min at 250 ***g***, the pellets of spleen, lymph node cells, and thymocytes were resuspended separately in RPMI 1640. The proportion of viable DBA/2 cells was invariably over 90%, as determined by trypan blue exclusion and phase contrast microscopy. The total number of cells in each suspension was determined with a flow cytometer (Cellcounter 134, Analyse Instruments, Stockholm, Sweden). The suspensions were then washed again, the pellets resuspended in Dulbecco's PBS solution, and the suspensions were pooled. On days 0, 3, 7 and 10 the F1 recipients were injected intravenously with 50×10^6 viable DBA/2 cells in 0·25 ml of Dulbecco's PBS, in a constant ratio between thymocytes, lymph node cells and spleen cells, each dose being composed of approximately 60% spleen cells, 30% thymocytes, and 10% lymph node cells in constant ratios. The percentages of T cell populations in inoculates derived from male and female donor mice were analysed using fluorescence-activated cell sorting (FACS). For these studies, we used rat anti-mouse Lyt-2(CD8) FITC-labelled antibody (1:500; Pharmingen, San Diego, CA), and biotin-labelled anti-mouse $\alpha\beta$TCR antibody (1:1000; Pharmingen) with streptavidin-PE (1:200; Southern Biotechnology Assoc. Inc., Birmingham, AL) as a second step.

Experimental design

Twenty-two male and 22 female (C57BL10 × DBA/2)F1 hybrid mice received lymphocytes from female DBA/2 mice, with an additional group of 10 male mice receiving lymphocytes from male DBA/2 mice. The development of albuminuria in diseased animals was followed for 12 weeks after the initial injection. During the course of the disease we collected serum and urine every 2 weeks, and every 4 weeks the kidneys from three randomly chosen animals were collected.

Follow up of F1 mice

One week before and 2 weeks after the first injection of parental cells and then at 2-week intervals, the albumin content of the urine of the diseased F1 mice was determined. Animals were kept in urine collection cages for 18 h with free access to water and food. Urine albumin levels were assessed by rocket electrophoresis against rabbit anti-mouse albumin, with the use of purified mouse serum albumin (Sigma Chemical Co., St Louis, MO) as a standard. After their stay in the urine collection cages all mice were anaesthetized with diethylether and blood was collected from the orbital plexus for the preparation of serum, which was frozen in aliquots at −20°C. Serum samples were tested for the presence of autoantibodies by ELISA performed as mentioned earlier [11]. Results from each sample were corrected for non-specific binding to bovine serum albumin (BSA). For the ELISA studies, RTE and GBM were prepared from fresh (C57BL10 × DBA/2)F1 mouse kidneys, and the purity of these antigens was verified by absorption studies, gel electrophoresis, ELISA, and immunoblotting as described [11,12]. Laminin and type IV collagen were purified as mentioned elsewhere [11].

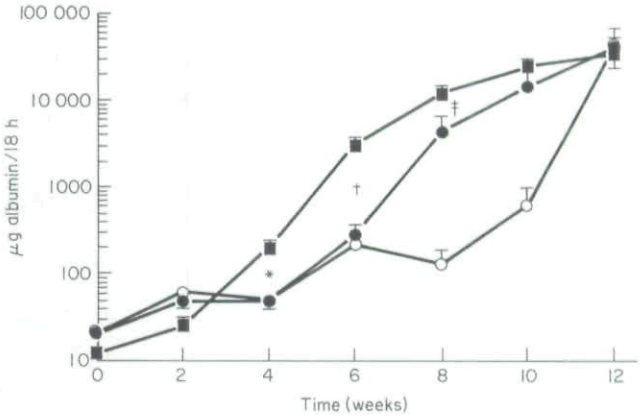

Fig. 1. Albuminuria of male and female (C57BL10 × DBA/2)F1 mice after induction of chronic graft-*versus*-host disease. ○, Male donor lymphocytes into male recipients; ●, female donor lymphocytes into male recipients; ■, female donor lymphocytes into female recipients. Values are means ± s.e.m. *$P = 0{\cdot}0001$ in week 4; †$P = 0{\cdot}0015$ in week 6; ‡$P = 0{\cdot}0257$ in week 8 for female donor lymphocytes into female recipients *versus* female donor lymphocytes into male recipients.

Histological methods

For the morphological studies on the development of renal disease, three mice of each group were killed at weeks 4, 8 and 12. One kidney was snap frozen in CO_2 ice-cooled isopentane and stored at −70°C until use. The other kidney was fixed in phosphate-buffered formalin and embedded in paraffin. Tissue was processed for light microscopy and immunofluorescence studies as described elsewhere [11]. The conjugates used for direct immunofluorescence studies were FITC-labelled rabbit anti-mouse IgM (dilution 1:50), goat anti-mouse IgG (dilution 1:300), and goat anti-mouse C3 antibodies (dilution 1:300) (all from Nordic Immunology, Tilburg, The Netherlands). Direct immunofluorescence studies were performed on cryostat sections of kidneys from diseased mice. Sections from normal mouse kidneys were used as negative controls. Indirect immunofluorescence was used for the detection of autoantibody activity against nuclear antigens [11]. Serum was diluted 1:10 in 2% BSA. As a second step we used rabbit anti-mouse IgG-FITC and anti-mouse IgM-FITC (Nordic).

Statistical analysis

Statistical significance was determined by unpaired Student's *t*-tests. A P value $< 0{\cdot}05$ was considered as statistically significant.

RESULTS

FACS analysis

Percentages of TCR^+ cells were $87{\cdot}6 \pm 0{\cdot}5$ and $88{\cdot}0 \pm 1{\cdot}2$ for female and male donor-derived inoculates, respectively

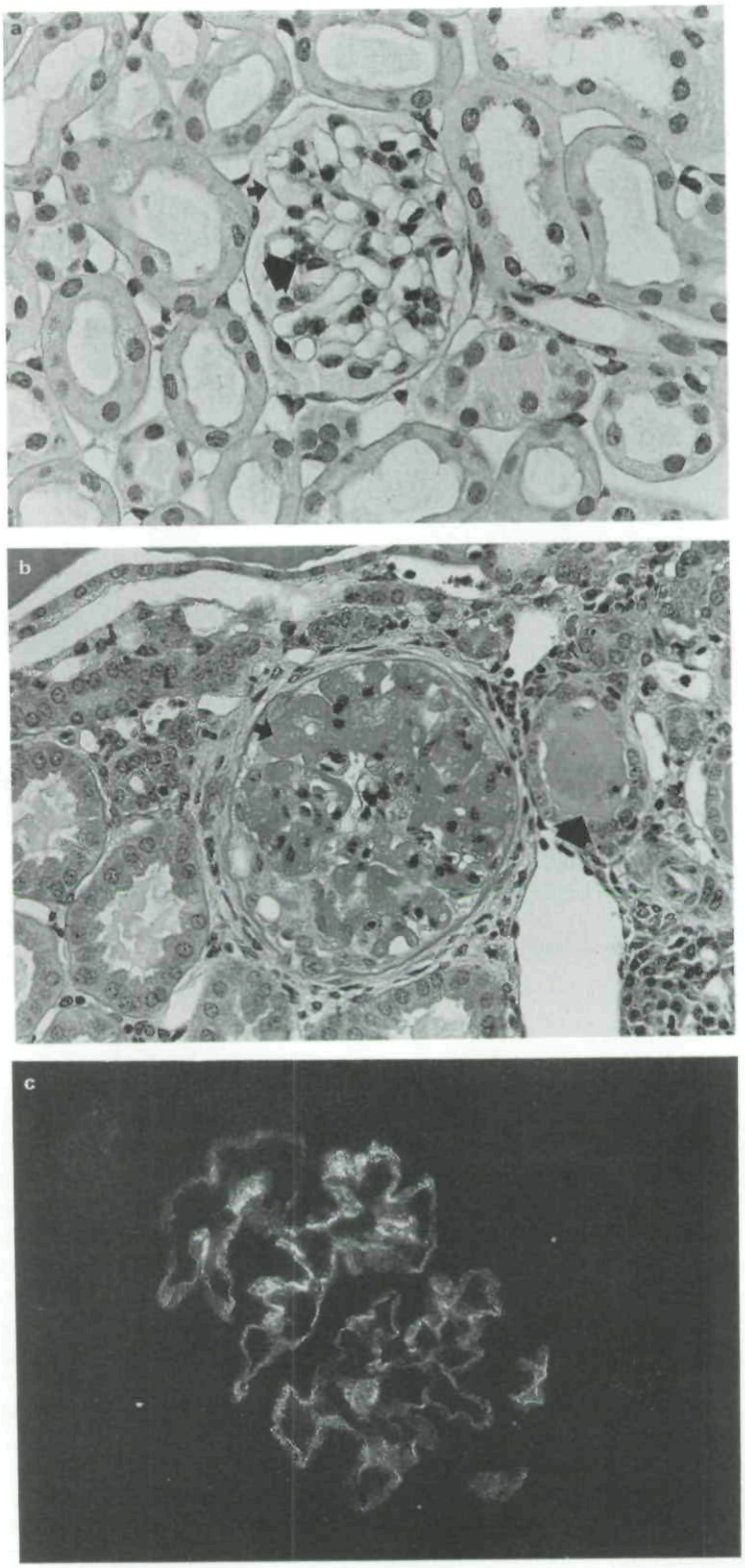

Fig. 2. (a) Light micrograph, showing a normal glomerulus in the kidney of a healthy control mouse. Small arrow, glomerular capillary wall; large arrow, mesangial area (H&E, × 240). (b) Light micrograph, showing focal hyalinosis and glomerulosclerosis (small arrow) in a kidney of a female C57BL10 × DBA/2 F1 mouse 12 weeks after the induction of chronic graft-*versus*-host disease. Note protein casts in tubuli (large arrow) (PAS, × 240). (c) Direct immunofluorescence of the kidney from C57BL10 × DBA/2 F1 female mouse in week 4, showing a mixed granular and linear distribution of IgG along the glomerular basement membrane (GBM) (× 400).

Table 1. Immunofluorescence findings in (C57BL10 × DBA/2)F1 hybrids 4 weeks after the induction of chronic graft-*versus*-host disease

		Anti-IgG		Anti-IgM		Anti-C3	
		GBM	mes	GBM	mes	GBM	mes
m	M	+	+	+/−	+	+/−	++
f	M	+/−	+	+/−	+	+/−	++
f	F	++	++	+/−	++	+	++

−, No staining; +/−, focal staining; +, clear staining; ++, intense staining; GBM, glomerular basement membrane; mes, mesangial area; m, male donors; f, female donors; M, male recipients; F, female recipients of lymphocytes.

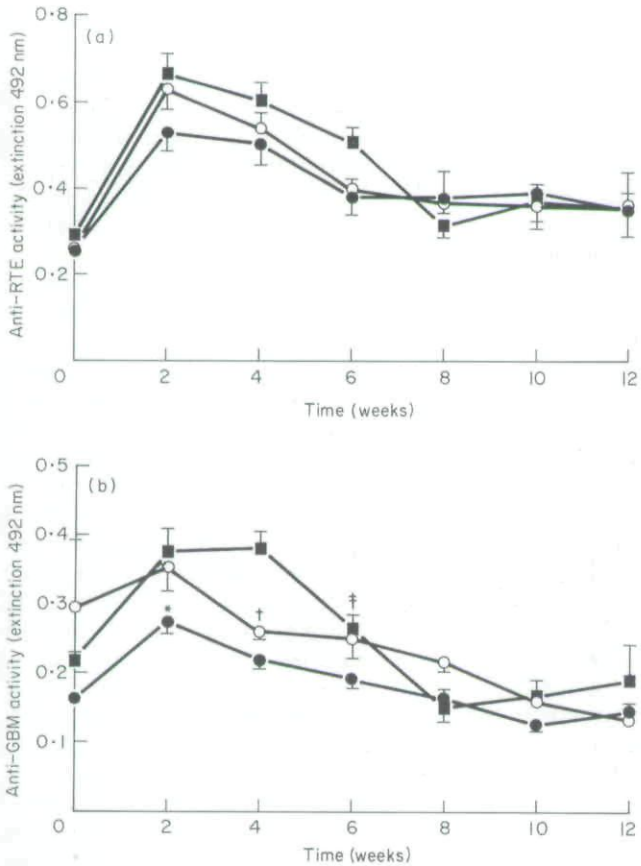

Fig. 3. (a) ELISA results showing the presence of anti-renal tubular epithelial (RTE) antibodies in the sera of mice at different time points after the induction of chronic graft-*versus*-host disease. ○, Male donor lymphocytes into male recipients; ●, female donor lymphocytes into male recipients; ■, female donor lymphocytes into female recipients. Values are mean ± s.e.m. (b) Anti-glomerular basement membrane (GBM) antibodies present in the sera of mice suffering from chronic graft-*versus*-host disease, as determined by ELISA (mean ± s.e.m). ○, Male donor lymphocytes into male recipients; ●, female donor lymphocytes into male recipients; ■, female donor lymphocytes into female recipients. *$P = 0{\cdot}0133$ in week 2; †$P = 0{\cdot}0019$ in week 4; ‡$P = 0{\cdot}0036$ in week 6 for female donor lymphocytes into female recipients *versus* female donor lymphocytes into male recipients.

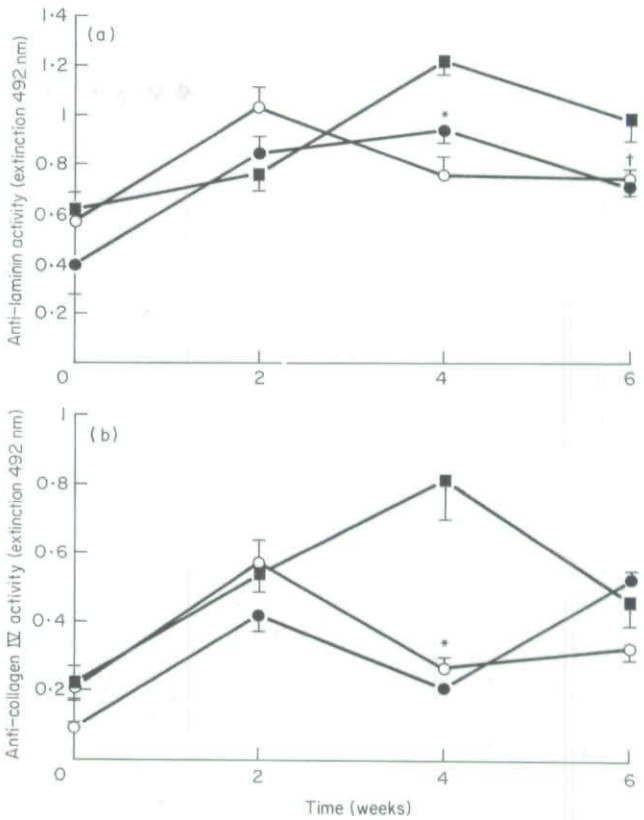

Fig. 4. (a) Anti-laminin antibody activity in sera of diseased mice as determined by ELISA. ○, Male donor lymphocytes into male recipients; ●, female donor lymphocytes into male recipients; ■, female donor lymphocytes into female recipients. Mean ± s.e.m. * $P = 0{\cdot}0095$ in week 4; † $P = 0{\cdot}0310$ in week 6 for female donor lymphocytes into female recipients *versus* female donor lymphocytes into male recipients. (b) ELISA results showing anti-collagen IV antibody activity in the sera of diseased mice. ○, Male donor lymphocytes into male recipients; ●, female donor lymphocytes into male recipients; ■, female donor lymphocytes into female recipients. Mean ± s.e.m. * $P = 0{\cdot}0006$ in week 4 for female donor lymphocytes into female recipients *versus* female donor lymphocytes into male recipients.

($P = 0{\cdot}26$). Percentages of CD8/TCR$^+$ cells were $53{\cdot}7 \pm 2{\cdot}5$ and $48{\cdot}1 \pm 0{\cdot}8$ for female and male donor-derived inoculates, respectively ($P = 0{\cdot}20$). As a control, percentages of CD8$^+$ and Thy-1$^+$ T cells were not significantly different between male and female donor-derived inoculates.

Albuminuria

As shown in Fig. 1, all animals reached the highest level of albuminuria in week 12. Proteinuria of female recipients receiving lymphocytes from female DBA/2 donors started to increase after week 2. The levels remained higher than those of the other groups until week 12. Albuminuria levels of female mice receiving female donor lymphocytes were significantly higher than those of male mice receiving female donor lymphocytes in weeks 4, 6 and 8 ($P = 0{\cdot}0001$, $P = 0{\cdot}0015$, and $P = 0{\cdot}0257$, respectively). The albuminuria of the males receiving male DBA/2 lymphocytes remained at the same level as the albuminuria of the male groups receiving female DBA/2 lymphocytes until week 6. After week 6, albuminuria of the males receiving

male lymphocytes became lower than the albuminuria of the male recipients of female lymphocytes, but this difference was not significant. Reproducibility of these findings and those mentioned below was confirmed in separate experiments (not shown).

Light microscopy

During the course of the disease no significant histological differences were observed between the different groups. In week 4, reabsorption granules in tubular epithelial cells, corresponding to an increased proteinuria, were seen, as well as inflammatory cells infiltrating in glomeruli. These were predominantly LFA-1$^+$ leucocytes, as determined in a separate study (C. J. Kootstra *et al.*, manuscript in preparation). In week 8, lobular hypercellularity, mesangial expansion, thickening of the GBM, and protein casts were observed. In week 12, focal hyalinosis and glomerulosclerosis had developed (Fig. 2).

Immunofluorescence studies

Depositions of IgG, IgM and C3 were studied in the kidneys of all three animal groups 4 weeks after the first injection of parental lymphocytes (Table 1). Female recipients of female DBA/2 lymphocytes showed intense mixed linear and granular staining of IgG along the GBM (Fig. 2). Male recipients of female and male DBA/2 lymphocytes showed IgG mainly in a mesangial distribution. The distribution of IgM and C3 was mainly mesangial in all groups.

Autoantibodies

Serum levels of antinuclear antibodies, as determined by indirect immunofluorescence, corresponded to earlier findings described in detail elsewhere [11], and were not significantly different between the experimental groups ($P=0{\cdot}31$). In Figs 3 and 4 the results of the ELISA studies of autoantibodies in the sera of the animals injected with either female or male DBA/2 lymphocytes are presented. Anti-RTE titres were noted in the sera of all mice, reaching maximum levels 2 weeks after the first immunization. Anti-RTE antibody levels were not significantly different between the experimental groups at any time point of the observation period (Fig. 3) The anti-GBM autoantibody titres in female recipients were at maximum levels 2 and 4 weeks after immunization. Male recipients of female donor cells reached maximum anti-GBM titres in week 2. At weeks 2, 4, and 6 the anti-GBM titres in females receiving female lymphocytes were significantly higher than in males receiving the same type of lymphocytes ($P=0{\cdot}0133$, $P=0{\cdot}0019$, and $P=0{\cdot}0036$, respectively). The titres of antibodies against GBM for the male group, which received male lymphocytes, were higher than those of the males receiving female lymphocytes (Fig. 3). The antibody titres against the extracellular matrix (ECM) component laminin reached maximum levels in week 4 in the female group. The titres in sera of the male groups remained at a lower level. The levels were significantly lower than those of the female group ($P=0{\cdot}0095$, Fig. 4). The anti-collagen IV antibody titres in female recipients increased in week 2, reaching maximum levels in week 4. Both male groups reached maximum levels in week 2. In week 4 the anti-collagen IV antibody level of females receiving female lymphocytes was significantly higher than that of males receiving female lymphocytes ($P=0{\cdot}0006$; Fig. 4).

DISCUSSION

The immune response of males and females is not identical. It has long been recognized that autoimmune diseases are many times more common amongst women [13–15]. The etiology of these differences in susceptibility for autoimmune diseases is not fully known. The influence of sex hormones on the development of lupus disease has been investigated by Carlsten *et al.* [16] in MRL *lpr/lpr* mice. These authors presented evidence that sex steroids have a major impact on the progression of lupus disease in this mouse strain. Roubinian and colleagues showed that the administration of high doses of oestrogen to castrated NZB/W male mice resulted in an increase of both mortality and autoantibody formation [17]. Blank and coworkers studied sex hormone involvement in the induction of experimental SLE by a pathogenic anti-DNA idiotype in naive mice [18].

In a previous report we demonstrated that genes linked to non-H-2 regions contributed by the C57BL10 strain determine the difference in susceptibility for renal involvement in murine chronic graft-*versus*-host disease [19], an inducible model for lupus nephritis [6]. The current study was undertaken to investigate the influence of sex-related genes in this model. Chronic graft-*versus*-host disease was induced in male and female (C57BL10 × DBA/2)F1 hybrid recipients by injection of female DBA/2 lymphocytes. In addition, a group of male mice was included, which received male donor DBA/2 lymphocytes. The transfer of male donor cells into female recipients was omitted because of an anticipated strong anti-H-Y response leading to acute rejection of the graft [20].

Female mice treated with female DBA/2 lymphocytes responded with an earlier and stronger autoimmune response than male recipients of female lymphocytes. We found significant and reproducible differences between males and females in the levels of albuminuria in weeks 4, 6 and 8. Light microscopy showed no significant differences between the groups. Reabsorption granules were observed in week 4, reflecting increased proteinuria in all groups. The immunofluorescence studies showed different patterns of deposition of IgG, IgM and C3 in the three groups. Females showed a clear mixed linear and granular IgG pattern along the glomerular capillary walls. Male recipients showed only a weak granular pattern along the GBM for IgG, IgM and C3. Activities of serum autoantibodies against nuclear antigens (indirect immmunofluorescence) and against RTE (ELISA) were not significantly different between the groups. In contrast, serum anti-GBM antibody titres at weeks 2, 4 and 6 were significantly higher in the female recipients than in the male recipients when both received female DBA/2 lymphocytes. The anti-laminin and anti-collagen antibody titres of the two sexes both receiving female lymphocytes were significantly higher for the female recipients in week 4. These results clearly indicate an influence of the gender of both the donor and the recipient of lymphocytes on the susceptibility for glomerulonephritis in this lupus model. In FACS experiments, we found no differences in the percentages of T cells (T cell receptor) or allocytotoxic Lyt-2$^+$ T cells between the inoculates of male and female donors. This excludes differences in the composition of the donor inoculates as a cause of the differences in autoimmunity.

In concert with our findings, Lahita emphasized the importance of oestrogens in SLE. The association between steroid levels and specific HLA haplotypes implicates sex hormones as

immune modulators and perhaps as predisposing factors for autoimmune disease [21]. Blank *et al.* demonstrated that both androgens and oestrogens can modify the severity of autoimmunity in mice, which develop severe SLE-like disease upon injection with a pathogenic anti-DNA idiotype [18]. A study of Ahmed *et al.* showed that highly enriched splenic $CD5^+$ B cells from normal female mice are more efficient producers of autoantibodies than equal numbers of $CD5^+$ B cells from normal males [22]. The increased autoantibody production in females was shown to be related to the effect of oestrogen on the immune response. Importantly, oestrogen did not increase the numbers of either B or $CD5^+$ B cells, but augmented the ability of B cells to produce antibodies [22]. Blank and coworkers suggested that the improvement noted with androgen and the deterioration observed with oestrogen in their model is mediated via their effects on T suppressor cells [18]. The production of cytokines such as IL-1, IL-2, and interferon-gamma (IFN-γ) by lymphocytes and macrophages is also affected by sex hormones, and this may further influence the development of autoimmune disease [18,23]. Finally, sex hormones direct the expression of Ia on immune as well as non-immune cells, which constitutes a third mechanism by which sex hormones may mediate their effects in autoimmunity [18]. It is feasible that similar mechanisms operate in our experimental model of lupus nephritis, and further studies will be directed at this possibility.

We found sex-related differences not only in proteinuria, but also in autoantibody titres. Titres of antinuclear antibodies and anti-RTE were not significantly different between the experimental groups. The differences we found in the titres of autoantibodies against GBM are significant. We showed earlier that antibodies directed against GBM components can be directly involved in the formation of subepithelial electron-dense deposits. This results in an early linear and a late granular pattern of immunoglobulin deposition along the GBM and the development of proteinuria [11, 24–26]. In passive transfer studies in the Heymann model, we found that the presence of anti-GBM antibodies within the serum used to induce the disease is a *conditio sine qua non* for the induction of immune-complex glomerulonephritis [27]. Our studies indicated that anti-GBM antibodies induce damage to the GBM, allowing for anti-RTE antibodies to travel through the glomerular capillary wall and reach their target antigens located on the surface of glomerular epithelial cells [25,27]. This then led us to speculate on a similar role for anti-GBM antibodies in the graft-*versus*-host model for lupus nephritis, in which we found anti-GBM antibodies in sera and renal eluates already in the early stage of the renal disease [11,25,28]. The current genetic study now provides us with elegant proof of the crucial role for the anti-GBM antibodies in the induction of the renal disorder, in that a significant decrease of anti-GBM but not of anti-RTE titres is related to significantly lower levels of albuminuria. This is the first time that such a role for anti-GBM antibodies is shown in an active model for immune-mediated glomerulonephritis. The further consequences of this notion with regard to the basic concepts of anti-basement membrane glomerulopathy and immune-complex glomerulonephritis have been extensively discussed by us elsewhere [25], and are beyond the scope of this study.

Our results indicate that susceptibility to glomerulonephritis in chronic graft-*versus*-host disease is governed by gender-related factors. To our knowledge, such a role for sex hormones has not been described previously in an experimental model of active autoimmunity induced by allogeneic polyclonal B cell stimulation in inbred animals with no genetic predisposition to autoimmunity. These findings provide us with a unique model for the study of hormonal and genetic influences on autoimmune disease. Furthermore, our results show that among the variety of autoantibodies occurring in this model of lupus nephritis, both anti-RTE and anti-GBM autoantibodies play a key nephritogenic role. The mechanism underlying the gender-dependence of the titres of anti-GBM antibodies but not of anti-RTE antibodies remains an enigma and requires investigation. Further studies are in progress applying hormonal manipulation to determine more specifically the influence of sex-related non-MHC genes on the susceptibility to lupus glomerulonephritis. Because of its therapeutic potential, hormonal modulation of autoimmune disease remains a field for continued study [17,29–31].

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