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Autoimmune disease after neonatal injection of semi-allogeneic spleen cells in mice: involvement of donor B and T cells and characterization of glomerular deposits

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SUMMARY

Balb/c neonates injected with semi-allogeneic (A/J × Balb/c) F_1 hybrid spleen cells develop an autoimmune disease associated with an immune-complex glomerulonephritis. The successful induction and maintenance of B cell chimerism is required for the occurrence of autoimmunity. The percentage of chimeric mice displaying autoimmune features increases in parallel with the number of cells injected at birth. T cell depleted inocula although readily inducing B cell chimerism were found unable to induce hypergammaglobulinaemia, circulating immune complexes and glomerulonephritis. IgG_1 is the most and IgG_3 the least represented IgG isotype among the immunoglobulins deposited in the glomeruli. Immunoglobulins bearing donor (A/J) allotype are detected in the glomeruli of six out of 11 chimeric mice. Rheumatoid factor activity is significantly concentrated within the immunoglobulins eluted from the kidneys, whereas anti-DNA activity is not.

Keywords neonatal allotolerance chimerism autoimmunity glomerulonephritis

INTRODUCTION

We and others have recently described the occurrence of an SLE-like disease characterized by immune complex-mediated glomerular lesions and circulating autoantibodies in mice made tolerant to alloantigens by neonatal injection of semi allogeneic spleen cells (SC) (Goldman *et al.*, 1983; Tateno *et al.*, 1985; Luzuy *et al.*, 1986). In this model, F_1 hybrid donor cells injected at birth persist in the parental host for a long period of time because they induce unresponsiveness to themselves. Lymphoid chimerism is required for the development of autoimmunity, donor B cells being responsible for the production of anti-DNA antibodies (Luzuy *et al.*, 1986). We were interested to determine whether donor B cells are also involved in the formation of nephritogenic antibodies and to investigate the possible relationship between B cell chimerism, autoimmunity and glomerulonephritis in this experimental situation. We also considered a possible role for donor T cells in the development of the syndrome.

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MATERIALS AND METHODS

Mice. The inbred strains A/J (H-2^a) and Balb-c (H-2^d) were purchased respectively from the Jackson Laboratory, Bar Harbor, ME, USA and from the Centre d'Energie Nucléaire, Mol, Belgium while (A/J × Balb/c) F_1 (hereafter referred to as F_1) were bred at our own colony.

Preparation of T cell depleted inocula. T lymphocytes were removed by treating SC with a monoclonal anti-Thy-1.2 antibody for 15 min at room temperature before the addition of guinea-pig complement (Bio-Merieux, Lyon, France) for another 45 min at 37°C. The cells were then pelleted and the same procedure was repeated. The T cell depletion was controlled in each individual experiment either by the abrogation of the *in vitro* responsiveness of the spleen cells to phytohaemagglutinin or concanavalin A, or by the detection of less than 1% of cells stained by a sandwich procedure using the biotinylated anti-thy-1.2 antibody and fluorescent streptavidin.

Neonatal inoculations. Experimental animals were obtained by inoculation of newborn Balb/c mice (less than 24 h after birth) either intraperitoneally or intravenously. The intravenous route (via the anterior facial vein) was used for the injection of $1{\cdot}5 \times 10^7$ or 3×10^7 untreated F_1 SC, or 3×10^7 T cell depleted F_1 SC. The intraperitoneal route was used for the injection of 10×10^7 F_1 SC or 10×10^7 Balb/c SC (controls).

A/J allotype determination. The presence of A/J immunoglobulins in the sera of neonatally injected mice was searched for by using a monoclonal antibody specific for an A/J allotope expressed on the IgG_2b subclass (kind gift of Dr J. Marvel). The serum level of IgG_2b bearing the A/J allotype was evaluated by haemagglutination titre on Sheep Red Blood Cells (SRBC) conjugated with the monoclonal antibody. The sensitivity of the method is 10 ng/ml, which represents 0·05% of the amount of IgG_2b found in a normal F_1 serum. Results were expressed as percentages of the allotype level present in F_1 normal serum.

Anti-IgCH allotype antiserum. Ascites fluid rich in anti-Ig^e allotype antibodies were raised in Balb/c mice by repeated injections of A/J immunoglobulins (Ig), which bear the Ig^e allotype (Lieberman, 1978).

The Ig fraction of the ascites was obtained by salt precipitation and was further affinity purified on immobilized A/J Ig. The affinity purified antiserum was then conjugated to biotin and used in immunofluorescence studies.

Detection of anti-DNA and rheumatoid factor antibodies. These tests were performed by solid-phase enzyme-linked immunosorbent assay (ELISA). Serial dilutions of the serum samples were incubated in polystyrene plates precoated with either DSDNA (Pisetsky & Peters, 1981) (double-stranded DNA) (Calf thymus DNA, type V; Sigma Chemical Co; St Louis, MO), SSDNA (single-stranded DNA, obtained by heat denaturation of calf thymus DNA) or a mixture of heat-aggregated monoclonal IgG_2a, IgG_2b and IgG_3 antibodies (Litton Bionetics, Kensington, USA). Anti-DNA antibodies were revealed by an alkaline-phosphatase conjugated goat anti-mouse IgG antiserum (Sigma Chemical Co, St Louis, MO) while the bound rheumatoid factors were revealed by an alkaline-phosphatase conjugated goat anti-mouse IgG_1 antiserum (Amersham Int plc, Buckinghamshire, England).

Circulating immune complexes (CIC) and serum IgG levels. Both tests were performed by ELISA. Circulating immune complexes were detected in polystyrene plates coated with 1 μg/well of Clq (Hay, Nineham & Roitt, 1976). Thirty microlitres of serum were mixed with 30 μl PBS and precipitated by 60 μl of polyethylene glycol. The precipitate was resuspended in EDTA 0·2 M Tween 0·8% buffer, pH 7·5, and distributed in duplicate wells. The bound immune complexes were revealed by a goat anti-mouse IgG antiserum coupled to alkaline phosphatase.

The amount of CIC in serum was obtained by referring to a standard curve constructed with mouse aggregated gamma-globulins. Serum IgG levels were quantified in plates coated with goat anti-mouse IgG antiserum (Sigma Chemical Co, St Louis, MO). Bound IgG were revealed by the same antiserum conjugated to alkaline phosphatase. A standard curve was constructed with pooled normal Balb/c serum and the individual IgG levels were expressed in percent of the normal serum value.

Immunofluorescence studies of kidneys. At autopsy, kidneys were immediately snap-frozen and stored in liquid nitrogen until used. Frozen sections were cut at 4 μm, washed in PBS and incubated

with one of the following reagents: FITC-conjugated goat anti-mouse IgG, IgG_1, IgG_2a, IgG_2b, IgG_3 (Litton Bionetics, Kensington, USA) or biotinylated anti-A/J allotype antiserum. In this latter case, streptavidin-FITC was added for another 30 min. The slides were then washed, mounted and viewed under a Leitz orthoplan microscope.

Kidney elution studies. Kidneys from 17 diseased mice were pooled and eluted as previously described (Goldman *et al.*, 1983). Briefly, washed and homogenized kidney tissue was incubated in 0·02 M citrate buffer, pH 3·2 at 37°C for 90 min. After centrifugation at 3,500 *g* for 15 min, the supernatant was dialysed against PBS. The globulin fraction was obtained by precipitation in 50% ammonium sulphate. IgG_1, IgG_2b and IgG_3 levels were measured in kidney eluate and in a serum pool of the corresponding mice by ELISA in the following way.

Briefly, polystyrene plates were coated with subclass-specific goat anti-mouse antiserum. After incubation of the samples, bound immunoglobulins were revealed by an alkaline-phosphatase conjugated goat anti-mouse IgG antiserum. The specificity of these tests was ascertained by the fact that non-relevant purified monoclonal antibodies of various classes and subclasses were always at least a 1000-fold less bounded than the relevant ones. The anti-DSDNA, anti-SSDNA and anti-mouse immunoglobulin activities of the immunoglobulins eluted from the kidneys were quantified by the same methods as those used for the sera.

Statistical analysis. Comparisons between groups were done by Student's *t*-test. In the assays for autoantibodies and circulating immune complexes, samples giving values exceeding the mean value of control mice + 3 s.d. were considered positive.

RESULTS

Immunopathology in chimeric mice. We prospectively compared the incidence of autoimmune features in mice injected at birth with three different doses of F_1 SC. Only mice harbouring stable B cell chimerism as indicated by the persistent presence of donor allotype in serum are reported. It can be seen from Table 1 that the proportion of mice with immunopathological changes increased together with the number of semi-allogeneic cells injected. The autoimmune syndrome was most reproducibly induced by the intraperitoneal injection of 10×10^7 F_1 SC, a cell dose which induced long-lasting chimerism in 18 out of 19 mice injected.

Although chimerism was not always associated with immunopathology, no autoantibodies were detected when B cell chimerism failed to be induced at birth (Table 2). A close association between B cell chimerism and autoimmunity was further observed in four mice in which neonatal injection of F_1 SC induced only transient chimerism. In these animals disappearance of chimerism was always associated with vanishing of circulating anti-DNA antibodies (Table 2).

Table 1. Autoimmune disease in mice made neonatally chimeric: relation with cell dose

Neonatal inoculum	Number of mice	Anti-ds DNA*	Circulating immune complexes	Glomerular† deposits of IgG
10×10^7 F_1 SC	18	94‡	78	94
3×10^7 F_1 SC	11	91	64	73
$1{\cdot}5 \times 10^7$ F_1 SC	17	65	27§	47
10×10^7 Balb/c SC‖	10	0	10	0

* Serum analysis were performed at 6 weeks.
† Indirect immunofluorescence studies of kidneys were done between 8 and 25 weeks.
‡ Percent of positive mice.
§ 11 mice tested.
‖ Controls.

Table 2. Association between B cell chimerism and autoimmunity

Pattern of chimerism	No. of mice*	Age (weeks) 6: Donor allotype	6: Anti-SSDNA†	30: Donor allotype	30: Anti-SSDNA
Transient	4	+	+	−	−
Absent	9	−	−	−	−

* Mice were injected as newborns with 3×10^7 F_1 SC.
† Mice were considered positive if exceeding the mean value of controls + 3 s.d.

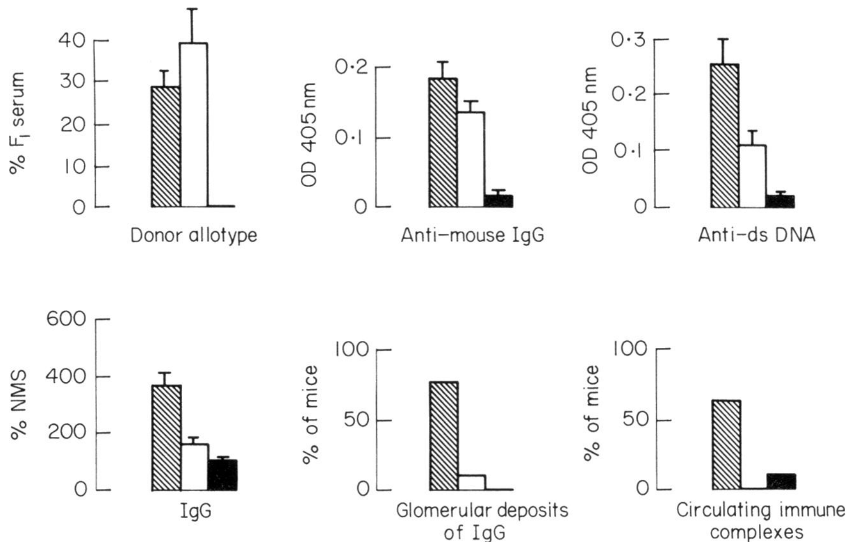

Fig. 1. Serum levels (mean + s.e.) of donor allotype, rheumatoid factor, anti-DSDNA and IgG (at 6 weeks), as well as percentages of mice with glomerular deposits of IgG and circulating immune complexes are shown. Mice (at least ten per group) were injected as newborns with 3×10^7 F_1 SC (hatched column, Group I), 3×10^7 T cell depleted F_1 SC (open column, Group II) or 3×10^7 syngeneic Balb/c SC (controls, closed column, Group III). Levels of statistical significance: donor allotype, not significant (NS) between Group I and Group II; rheumatoid factor, NS for Group I vs Group II, $P < 0{\cdot}02$ for Group I or Group II vs Group III; anti-DSDNA, $P < 0{\cdot}01$ for Group I vs Group II and $P < 0{\cdot}02$ for Group II vs Group III; IgG, $P < 0{\cdot}01$ for Group I vs Group II and NS for Group II vs Group III.

Requirement for donor T cells in the development of immunopathology. T cell depleted F_1 SC were as efficient as untreated F_1 SC in inducing long-lasting chimerism as indicated by the similar levels of serum donor allotype at 6 weeks (Fig. 1). Autoantibodies were detected in mice injected with T cell depleted F_1 SC but anti-DNA antibody levels were significantly lower than in mice injected with untreated SC (Fig. 1). Interestingly, neither hypergammaglobulinemia nor circulating immune complexes were present in these animals. Moreover, granular deposits of IgG were observed in only one out of ten mice.

Specificities of the immunoglobulins deposited in the glomeruli. We first analysed by immunofluorescence with anti-IgG subclass antisera the kidneys of 11 mice displaying mesangial and

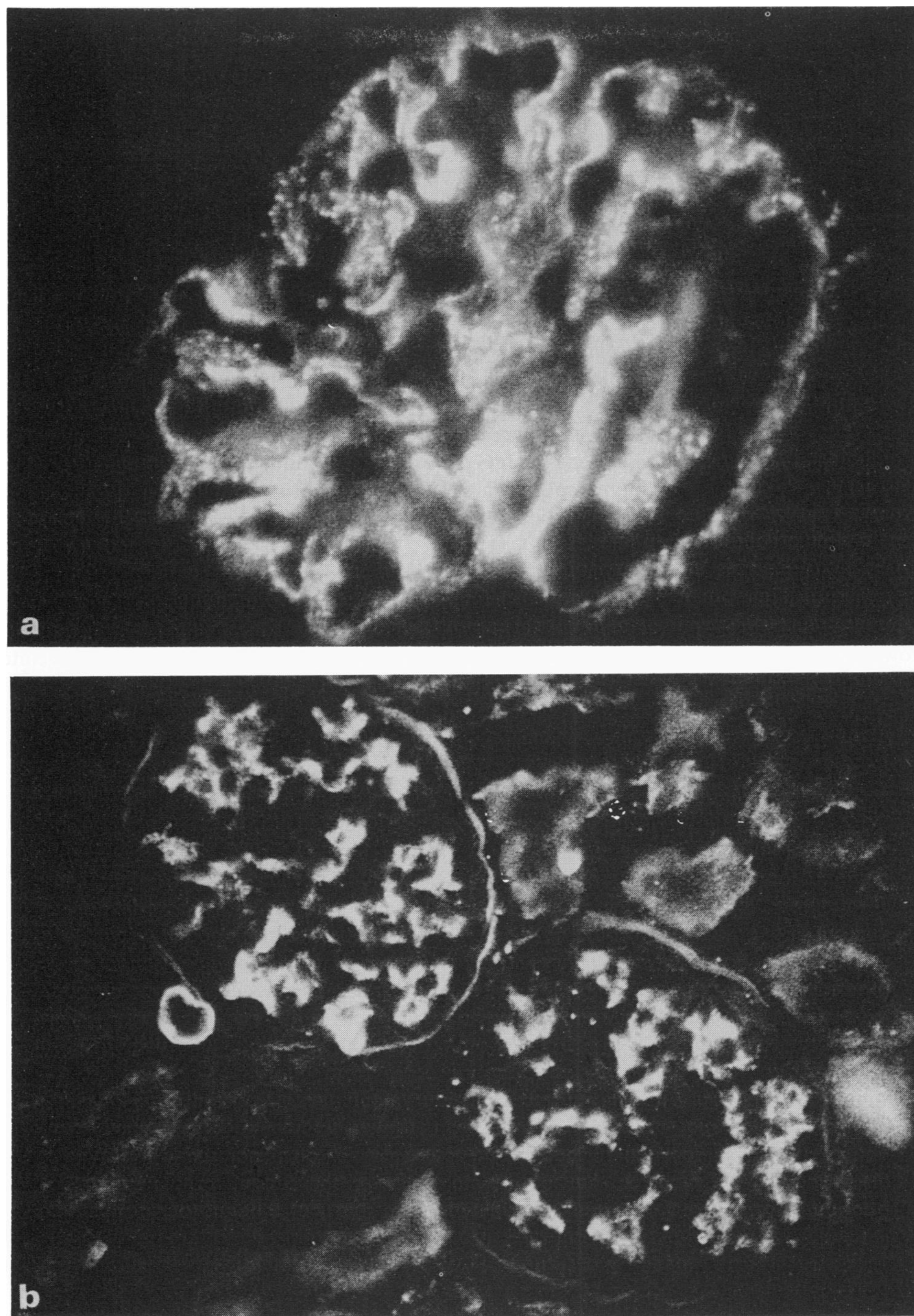

Fig. 2. Immunofluorescence characterization of the glomerular deposits of mice neonatally injected with F_1 hybrid spleen cells. (a) staining for IgG_1, showing deposits in mesangial area and along glomerular capillary walls with a granular pattern (× 1000). (b) staining for donor-type (A/J) immunoglobulins (× 600).

Table 3. Analysis of kidney eluate of chimeric mice

IgG_1	0·94*	Anti-SSDNA	1·46
IgG_{2b}	0·75	Anti-DSDNA	1·67
IgG_3	0·50	Rheumatoid factor	5·02

* Kidney/serum ratio (μl/g): volume of serum containing the same amount IgG_1, IgG_{2b}, IgG_3 or the same anti-SSDNA, anti-DSDNA and anti-mouse immunoglobulin activity as 1 g of kidney eluate (prepared from 17 chimeric mice).

membranous staining for IgG. Mesangial deposits of the four IgG subclasses were detected in all mice. While membranous deposits of IgG_1 were detected in those 11 mice, similar deposition of IgG_2a, IgG_2b and IgG_3 was detected respectively in 27%, 36% and 0% of them. Figure 2a shows an example of IgG_1 glomerular staining in chimeric mice. The analysis of the kidney eluate confirms the dominance of the IgG_1 subclass among the IgG deposited in the kidney (Table 3). Immunofluorescence study with anti-A/J allotype antiserum showed mesangial staining without membranous deposits in six out of 11 experimental mice. This mesangial staining was not observed in any of 10 controls animals.

Background staining of tubules and Bowman's capsule observed in both experimental and control mice was due to non-specific binding of fluorescent streptavidin. Anti-SSDNA and anti-DSDNA antibodies do not appear significantly concentrated in the eluate as shown in Table 3. However, rheumatoid factor was found to be concentrated 5-fold among immunoglobulins eluted from the kidney as compared with corresponding serum.

DISCUSSION

Our data first indicate that in mice injected at birth with semi-allogeneic cells, successful induction of B cell chimerism is necessary but not sufficient for autoimmunity to appear. Disappearance of chimerism was always followed by disappearance of serum anti-DNA activity, indicating a strict relationship between B cell chimerism and autoimmunity. On the other hand, the percentage of mice displaying autoimmune features increased in parallel with the number of cells injected at birth, although significant B cell chimerism was present in all these animals. It is thus possible that besides donor B cells, another cell type must be present within the neonatal inoculum for the occurrence of immunopathology. Indeed, our results show that donor T cells are required for the development of hypergammaglobulinemia, CIC and immune complex glomerulonephritis (ICGN) in chimeric mice. Thus, the number of T cells injected at birth could well be a limiting factor for the subsequent development of autoimmune disease. This could explain that semi-allogeneic T cell-enriched inocula containing only few B cells efficiently induced immunopathology when injected into newborn mice (Tateno *et al.*, 1985). The phenotype of the donor T cells and the lymphocytic interactions in which they participate are still unknown. It is possible that interactions between host and semi-allogeneic donor T cells are at play in chimeric mice. Along this line, interactions between unmodified and mercuric chloride-modified T cells appear critical in the pathogenesis of the autoimmune disease induced in rats by repeated injections of mercuric chloride (Pelletier *et al.*, 1986).

There is suggestive evidence that in tolerant mice activation of F_1 donor B cells leads to hypergammaglobulinaemia with a preferential production of anti-DNA antibodies (Luzuy *et al.*, 1986). We found that the presence of immune deposits in the glomeruli is always associated with B cell chimerism and autoantibodies. The dominance of the IgG_1 subclass among the immunoglobulins deposited in the kidneys suggests that the formation of nephritogenic antibodies is a T cell dependent phenomenon (Bankhurst, Lambert & Miescher, 1975). Thus the allo-help effect responsible for the production of autoantibodies could also be involved in the formation of

nephritogenic immune complexes. Indeed, the detection within the glomerular deposits of immunoglobulins bearing the A/J allotype indicates that F_1 hybrid donor B cells actively participate in the pathogenesis of the glomerular lesions. However, A/J immunoglobulins were detected only in mesangial areas and only in about half of the mice. This could be explained by the fact that IgG1, the isotype most represented in both granular and mesangial deposits displays no allotypic differences between A/J and Balb/c strains, and is thus not recognized by our anti-allotype antiserum. Of course, this allotypic study does not exclude an additional participation of host immunoglobulins in the deposits.

As both anti-DNA antibodies (Lambert & Dixon, 1968; Izui *et al.*, 1977; Rolink, Gleichmann & Gleichmann, 1983) and rheumatoid factor (Rossen *et al.*, 1975) have been implicated in the pathogenesis of ICGN, we measured these autoantibody activities in the immunoglobulins eluted from the kidneys. Anti-DNA antibodies do not seem to play a major role in the renal lesions whereas rheumatoid factor antibodies were found to be concentrated in the renal eluate. The specificity of the antibodies responsible for the formation of epimembranous deposits remains however to be determined.

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REFERENCES

Bankhurst, A.D., Lambert, P.H. & Miescher, P.A. (1975) Studies of the thymic dependence of the immunoglobulin classes of the mouse. *Proc. Soc. exp. Biol. Med.* **148,** 501.

Goldman, M., Feng, H.M., Engers, H., Hochman, A., Louis, J. & Lambert, P.H. (1983) Autoimmunity and immune complex disease after neonatal induction of transplantation tolerance in mice. *J. Immunol.* **131,** 251.

Hay, F.C., Nineham, L.J. & Roitt, I.M. (1976) Routine assay for the detection of immune complexes of known immunoglobulin class using solid phase C1q. *Clin. exp. Immunol.* **24,** 396.

Izui, S., Lambert, P.H., Fournie, G.J., Turler, H. & Miescher, P.A. (1977) Features of systemic lupus erythematosus in mice injected with bacterial lipopolysaccharides: identification of circulating DNA and renal localization of DNA-anti-DNA complexes. *J. exp. Med.* **145,** 1115.

Lambert, P.H. & Dixon, F.J. (1968) Pathogenesis of the glomerulonephritis in NZB/W mice. *J. exp. Med.* **127,** 507.

Lieberman, R. (1978) Genetics of IgCH (allotype) locus in the mouse. *Springer Semin. Immunopathol.* **1,** 7.

Luzuy, S., Merino, J., Engers, H., Izui, S. & Lambert, P.H. (1986) Autoimmunity after induction of neonatal tolerance to alloantigens: role of B cell chimerism and F_1 donor B cell activation. *J. Immunol.* **136,** 4420.

Pelletier, L., Pasquier, R., Hirsch, F., Sapin, C. & Druet, P. (1986) Autoreactive T cells in mercury-induced autoimmune disease: *in vitro* demonstration. *J. Immunol.* **137,** 2548.

Pisetsky, D.S. & Peters, D.V. (1981) A simple enzyme-linked immunosorbent assay for antibodies to native DNA. *J. Immunol. Meth.* **41,** 187.

Rolink, A.G., Gleichmann, H. & Gleichmann, E. (1983) Diseases caused by reactions of T lymphocytes to incompatible structures of the major histocompatibility complex. VII. Immune complex glomerulonephritis. *J. Immunol.* **130,** 209.

Rossen, R.D., Reisberg, M.A., Sharp, J.T., Sukiw, N., Schloeder, F.X., Hill, L.L. & Eknoyau, G. (1975) Antiglobulins and glomerulonephritis. *J. clin. Invest.* **56,** 427.

Tateno, M., Kondo, N., Itoh, T. & Yoshiki, T. (1985) Autoimmune disease and malignant lymphoma associated with host-versus-graft disease in mice. *Clin. exp. Immunol.* **62,** 535.