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T Cells and Macrophages in *Trypanosoma brucei*-Related Glomerulopathy

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In a previous study, susceptibility for *Trypanosoma brucei*-related glomerulopathy in mice was shown to be dependent on non-major histocompatibility complex genes. Glomerular disease in this model could not be explained by the production of autoantibodies alone. In order to analyze which part of the defense system, in addition to the B-cell compartment, is involved in the development of this infection-related glomerular disease, groups of athymic (BALB/c *rnu/rnu*), splenectomized, or macrophage-depleted BALB/c mice were inoculated with *T. brucei* parasites. Polyclonal B-cell activation, invariably observed in infected BALB/c mice, was absent in BALB/c *rnu/rnu* mice. Glomerular disease in athymic mice, however, as defined by albuminuria and deposition of immune complexes, was not different from that seen in euthymic infected BALB/c mice. Splenectomy prior to inoculation of parasites led to a decreased incidence of albuminuria in 40% of the animals, whereas splenectomy 21 days after inoculation reduced albuminuria significantly, suggesting a role for spleen cells in the induction of glomerular disease. After macrophage depletion with liposome-encapsulated dichlorodimethylene-diphosphonate, infected BALB/c mice developed significantly higher albuminuria levels for a period up to 2 weeks after depletion. Therefore, it was concluded that the development of *T. brucei*-related glomerular disease is independent of thymus-matured T cells, while the involvement of macrophages in the development of proteinuria is inhibitory rather than disease inducing. Spleen cells other than thymus-dependent T cells, B cells, and macrophages should be investigated for their role in the pathogenesis of this glomerulopathy.

Infection can be associated with the development of glomerular disease. Besides the well-known postinfectious glomerulonephritis associated with bacterial infections, a number of viral and parasitic infections are known to be related to the development of glomerular disease (2, 3, 7, 20, 23). In a previous study, we described an infection-related glomerular disease in BALB/c mice, induced by *Trypanosoma brucei brucei* (28). As this is an easily inducible, reproducible disease, with overt albuminuria, it is a suitable model to study the pathogenetic mechanisms of glomerular damage. In this model, polyclonal B-cell stimulation and glomerular deposition of immunoglobulins (Igs), in part directed against extracellular-matrix components, are observed (28). The susceptibility to developing glomerular disease in this model was shown to be dependent upon non-major histocompatibility complex genes (30). A relationship between the occurrence of the glomerulopathy, the susceptibility for infection, and the occurrence of polyclonal B-cell activation over time in the different mouse strains was found. The development of proteinuria, however, was not correlated with the production and glomerular deposition of known nephritogenic autoantibodies. An additional pathogenetic role for non-Ig humoral factors was considered (30). In order to establish which cells from the defense system, in addition to the B-cell compartment, determined the type of response and were needed for the development of proteinuria, disease was induced in groups of athymic (BALB/c *rnu/rnu*), splenectomized, or macrophage-depleted BALB/c mice. The development of this *T. brucei*-related glomerular disease is shown to be independent of thymus-matured T cells, while the role of macrophages in the development of proteinuria is inhibitory rather than disease inducing.

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MATERIALS AND METHODS

Animals. Female BALB/c mice (10 to 12 weeks old) were bred in our own facilities, while female BALB/c *rnu/rnu* mice were obtained from the TNO Institute for Vascular Research and Ageing in Leiden, The Netherlands.

Parasites. A stabilate of *T. brucei* (STIB 348c variant A) was obtained from the Department of Pathology of the Bernard-Nocht-Institut für Schiffs- und Tropenkrankheiten (Hamburg, Germany). This stabilate was expanded after a passage in an irradiated (5 Gy) BALB/c mouse. Blood from this animal containing 10^8 parasites per ml was diluted in glycerol and saline and cryopreserved at −70°C in batches of 40-μl samples.

Macrophage depletion and splenectomy. Macrophage depletion was produced as described elsewhere (26). In short, BALB/c mice were injected intravenously with 100 μl of a suspension of liposome-encapsulated dichloromethylene-diphosphonate (Cl_2MDP). The Cl_2MDP was a gift of Boehringer Mannheim GmbH, Mannheim, Germany. As a control, liposomes with encapsulated phosphate-buffered saline (PBS) were administered. Macrophage depletion was checked by immunohistological staining of splenic macrophages.

For splenectomy, mice were anesthetized with a mixture of 15% Aescoket (Aesculaap, Boxtel, The Netherlands), 9% Thalamonal (Janssen Pharmaceutica, Tilburg, The Netherlands), and 1.5% Hypnorm (Duphar, Amsterdam, The Netherlands). After an incision was made in the left flank, the splenic vasculature was ligated and the spleen was taken out. The abdominal wall was closed separately from the skin.

Histological methods. All mice were perfused via aortic puncture, in vivo with PBS for 2 min, prior to sacrifice. For immunofluorescence studies, specimens were snap frozen and stained as described elsewhere (28). Marginal metallophilic macrophages were detected with monoclonal antibody MOMA-1 (11), while marginal-zone macrophages were detected with monoclonal antibody ERTR9 (6). Both were kindly donated by G. Kraal (Department of Cell Biology, Free University, Amsterdam, The Netherlands). As a second step, fluorescein isothiocyanate-labeled rabbit anti-rat antibodies were used (DAKO, Glostrup, Denmark). For detection of glomerular Igs, fluorescein isothiocyanate-labeled goat anti-mouse IgG and goat anti-mouse IgM (DAKO) were used. Tissue specimens of kidney and spleen were fixed in 10% paraformaldehyde, embedded in paraffin, processed in the usual way, and stained with hematoxylin and eosin stain and periodic acid-Schiff reagent, according to standard methods.

Parasitemia. Parasites were counted in a smear of tail blood stained with Leishman's stain. The results were expressed as number of parasites per 100 erythrocytes. Hematocrit values were determined as a control for anemia.

Albuminuria. Mice were kept in metabolic cages for 18 h with free access to water and food. The albumin concentration in urine was measured by rocket electrophoresis, with anti-mouse albumin antibodies.

Preparation of antigens and antisera. Renal tubular epithelium antigens, glomerular basement membrane antigens, dipeptidyl-peptidase IV (CD26), and gp330 were prepared and characterized as described elsewhere (1, 8, 31). The type IV collagen, the antibodies directed against type IV collagen, and the antibodies against laminin were a generous gift of J. M. Foidart (Department of Biology, University of Liège, Belgium). Preparation and characterization of these antigens and antibodies have been described elsewhere (16, 24). The Engelberth Holm Swarm (EHS) mouse laminin was obtained from Sigma Chemical Co. Ltd., Dorset, England. The dinitrophenol coupled to rat serum albumin (DNP-RSA) was kindly provided by M. R. Daha (Department of Nephrology, University Hospital of Leiden, The Netherlands).

ELISA studies. Antibodies directed against EHS laminin, gp330, and DNP-RSA were measured to evaluate polyclonal B-cell activation. Sera of BALB/c *rnu/rnu* mice were tested additionally for the presence of antibodies directed against collagen type IV, glomerular basement membrane antigens, dipeptidyl-peptidase IV, and renal tubular epithelial antigens. Enzyme-linked immunosorbent assay (ELISA) studies were performed as described elsewhere (8, 17, 25). Antibodies were measured in four experimental and four control serum samples for each time point and for each group. All sera were tested in two dilutions (both in duplo). The results of the sera diluted 1/40 were used. Peroxidase-conjugated goat anti-mouse Ig (DAKOPATTS, Glostrup, Denmark) was applied as a second step. As a positive control for the coating procedure of the antigens, specific rabbit and goat antisera were used. As a negative control, each serum was tested in the same dilutions on bovine serum albumin-coated wells. In all studies, a standard dilution curve from a reference serum pool of BALB/c mice 42 days after inoculation with parasites was included. The reaction products were quantified after addition of tetramethylbenzidine (Sigma) by measuring extinction at 450 nm with a Titertek Multiscan ELISA reader (Amstelstad BV, Zwanenburg, The Netherlands).

Statistical analysis. To analyze the significance of the differences obtained in the various experiments, an unpaired Student's t test was used. Differences with a P value of <0.05 were considered to be significant. Results from group 7 (see Table 1), which were comparable to those of previous experiments with infected BALB/c mice, were used to test the significance of differences with the other groups.

TABLE 1. Animals used and treatments given

Group	Infection	n	Feature or treatment
1a	+	10	BALB/c *rnu/rnu*
1b	−	5	BALB/c *rnu/rnu*
2a	+	7	Splenectomy, day −14
2b	−	5	Splenectomy, day −14
3a	+	7	Splenectomy, day 21
3b	−	5	Splenectomy, day 21
4	+	9	Cl_2MDP liposomes, day 21
5	+	9	Cl_2MDP liposomes, days 21 and 28
6	+	9	Cl_2MDP liposomes, day 28
7	+	9	PBS liposomes, days 21 and 28
8	+	5	Cl_2MDP liposomes, day −1
9	+	5	PBS liposomes, day −1
10	−	6	Cl_2MDP liposomes, day −1

Individual mice were considered to be albuminuric when their albuminuria in 18 h exceeded the mean plus twice the standard deviation of control (noninfected) mice.

Experimental design. Disease was induced as described previously (28). To induce infection, mice were inoculated intraperitoneally with *T. brucei* parasites, approximately 100 parasites per mouse. They were treated 7 days after infection with 40 mg of diminazene aceturate (Berenil; Hoechst AG, Frankfurt am Main, Germany) per kg of body weight intraperitoneally, to obtain chronic infection as described previously (28). All noninfected mice were treated with diminazene aceturate only and served as controls. Albuminuria and parasitemia were measured before inoculation and at weekly intervals. Serum was obtained on day 0, day 28, and day 42. Day 0, the day of infection, was the start of the experiments, which were terminated on day 42.

Ten groups of mice were examined. These are shown in Table 1 with the number of animals used and the treatment given. For infected nude mice (BALB/c *rnu/rnu*; group 1a) and infected splenectomized mice (groups 2a and 3a), noninfected nude and noninfected splenectomized mice (groups 1b, 2b, and 3b) served as a control for the effect of the changed status. For experiments with mice infected and treated with Cl_2MDP liposomes to obtain macrophage depletion (groups 4, 5, and 6), control experiments were performed with infected mice treated with PBS liposomes (group 7) and noninfected mice treated with Cl_2MDP liposomes (group 10).

RESULTS

Parasitemia and survival. All groups of infected mice showed similar parasitemia curves with a few exceptions. Parasitemia levels on day 7 in nude mice tended to be higher, although not significantly ($P > 0.05$), than in the other groups (Fig. 1). On day 35, all macrophage-depleted groups and the group of mice splenectomized on day 21 showed higher parasitemia levels than nonsplenectomized or non-macrophage-depleted mice, i.e., group 7 ($P < 0.05$) (Fig. 1 and 2). In each of groups 5 and 7, one mouse died because of administration of liposomes. In all three macrophage-depleted groups (groups 4, 5, and 6), three mice died between day 28 and day 42. Of the BALB/c *rnu/rnu* mice, one died before the end of the experiment.

The role of thymus-matured T cells. Albuminuria in nude mice (group 1a) was detected initially on day 28. All infected

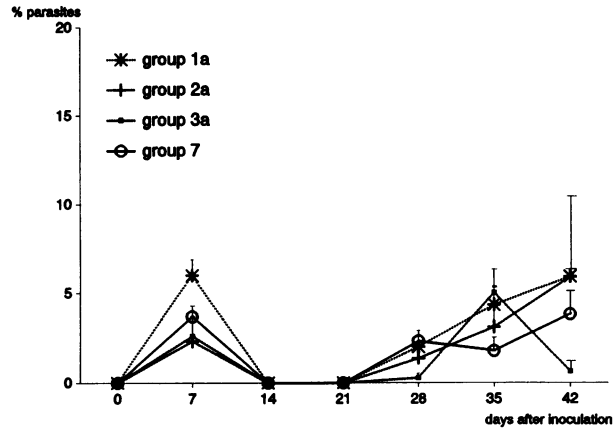

FIG. 1. Parasitemia in nude and splenectomized mice. Each point represents the mean of parasitemia values for at least seven mice. The standard error of the mean for each point is shown (bars). Group 1a, infected *rnu/rnu* mice; group 2a, infected mice splenectomized before infection; group 3a, infected mice splenectomized 21 days after infection; group 7, infected mice treated on days 21 and 28 with liposome-encapsulated PBS.

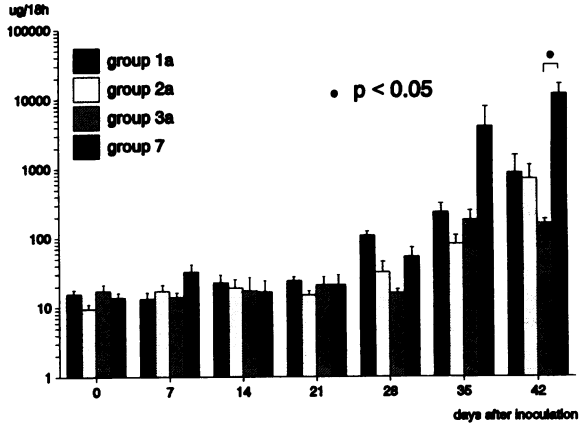

FIG. 3. Albuminuria in nude and splenectomized mice. Each bar represents the mean of albuminuria values for at least seven mice. The standard error of the mean for each point is shown (bars). Group 1a, infected *rnu/rnu* mice; group 2a, infected mice splenectomized before infection; group 3a, infected mice splenectomized 21 days after infection; group 7, infected mice treated on days 21 and 28 with liposome-encapsulated PBS.

nude mice were albuminuric on day 42. The level of the albuminuria on this day was not statistically different from that of nonsplenectomized, non-macrophage-depleted infected BALB/c mice (group 7; $P > 0.05$) (Fig. 3). Noninfected nude mice (group 1b) showed albuminuria levels within the normal range (20 to 80 μg/18 h). Immunofluorescence of renal tissue showed strong mesangial staining for IgG and IgM and mixed linear-granular staining for IgM alone along the glomerular capillary loop (Table 2). No staining for C3 was seen. In serum, no antibody production could be detected against laminin, collagen type IV, glomerular basement membrane antigens, renal tubular epithelial antigens, gp330, dipeptidyl-peptidase IV, or DNP-RSA, by comparison with noninfected intact BALB/c mice and noninfected BALB/c *rnu/rnu* mice.

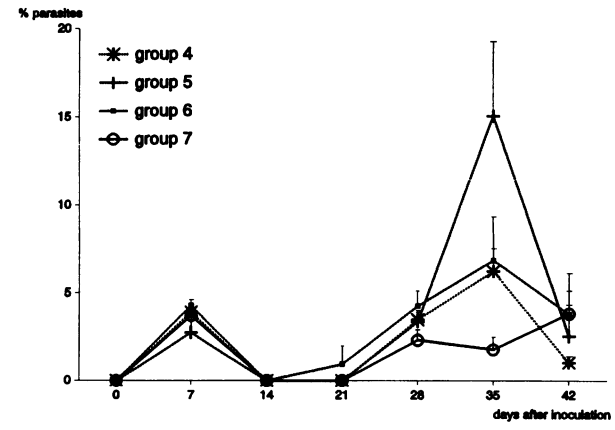

FIG. 2. Parasitemia in macrophage-depleted mice. Each point represents the mean of parasitemia values for at least seven mice. The standard error of the mean for each point is shown (bars). Group 4, infected mice treated on day 21 with liposome-encapsulated Cl_2MDP; group 5, infected mice treated on days 21 and 28 with liposome-encapsulated Cl_2MDP; group 6, infected mice treated on day 28 with liposome-encapsulated Cl_2MDP; group 7, infected mice treated on days 21 and 28 with liposome-encapsulated PBS.

The role of the spleen. The group of mice splenectomized before inoculation with parasites (group 2a) was devided into an albuminuric group (four mice) and a nonalbuminuric group (three mice). The difference of mean levels of albuminuria between those two groups on day 42 was significant ($P = 0.01$). The first albuminuria was detected on day 35. Immunofluorescence of renal tissue showed mesangial staining for IgM, IgG, and C3 and linear staining along the glomerular capillary loops for IgM in all infected mice (Table 2). Only three mice showed staining along the glomerular capillary loops for IgG. Two of these mice were albuminuric, and one was not. In all four albuminuric mice, however, glomerular, intracapillary, granular staining for IgG was seen. In all infected animals, antibodies to laminin, gp330, and DNP-RSA were detected in serum.

Mice splenectomized on day 21 after parasite inoculation (group 3a) all developed albuminuria. The level of the albuminuria in this group of mice, however, was significantly lower than that in infected BALB/c mice of group 7 ($P < 0.05$) (Fig. 3). In sera of mice of this group, antibodies directed against laminin, gp330, and DNP-RSA were detected as well.

Noninfected splenectomized mice (groups 2b and 3b) showed albuminuria within the normal range (20 to 80 μg/18

TABLE 2. Immunofluorescence and antibodies in serum[a]

Group(s)	Location(s) and strength(s) of immunofluorescence for:		Presence in serum of antibodies against:		
	IgM	IgG	Lam	gp330	DNP
1a	MS++ CL+	MS++	−	−	−
2a	MS++ CL+	MS+++ CL+	+	+	+
3a	MS++ CL+	MS++ CL+	+	+	+
4	MS++ CL+	MS+++ CL+	+	+	+
5	MS++ CL+	MS+++ CL+	+	+	+
6	MS++ CL+	MS+++ CL+	+	+	+
7	MS++ CL+	MS++ CL+	+	+	+
1b, 2b, 3b, and 10	MS+	MS+	−	−	−

[a] Abbreviations: lam, laminin; MS, mesangium; CL, glomerular capillary wall. Increasing numbers of plus signs indicate increasing strengths of fluorescence.

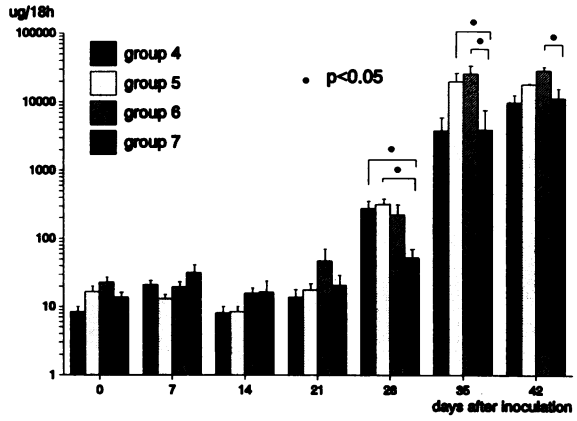

FIG. 4. Albuminuria in macrophage-depleted mice. Each bar represents the mean of albuminuria values for at least seven mice. The standard error of the mean for each point is shown (bars). Group 4, infected mice treated on day 21 with liposome-encapsulated Cl_2MDP; group 5, infected mice treated on days 21 and 28 with liposome-encapsulated Cl_2MDP; group 6, infected mice treated on day 28 with liposome-encapsulated Cl_2MDP; group 7, infected mice treated on days 21 and 28 with liposome-encapsulated PBS.

h). Slight mesangial staining for IgM was seen by immunofluorescence (Table 2). In sera of these mice, antibodies against laminin, gp330, or DNP-RSA were not detectable.

The role of macrophages. One and two weeks after macrophage depletion, infected mice (groups 4, 5, and 6) showed significantly higher albuminuria levels than infected BALB/c mice of group 7 (Fig. 4). Administration of liposome-encapsulated Cl_2MDP before inoculation with parasites (group 8) did not alter the course of the albuminuria from that of normal infected BALB/c mice (group 7). Neither did administration of liposome-encapsulated Cl_2MDP alone (group 10) induce albuminuria. Immunofluorescence of renal tissue from mice of all infected groups showed glomerular mesangial staining for IgM, IgG, and C3 and a mixed linear-granular fluorescence pattern for IgM and IgG along the glomerular capillary loop, as well as intracapillary granular staining for IgG (Table 2). In sera of mice of all infected groups, antibodies directed against laminin, gp330, and DNP-RSA were found. The antibody titers of different groups were compared, but no significant differences were observed.

Alterations in spleen morphology and architecture during infection. During infection with *T. brucei* in normal BALB/c mice, striking enlargement of the spleen was observed (Fig. 5A and D). On histologic examination, this enlargement was seen to be accompanied by deterioration of the normal splenic architecture with enlargement of both white and red pulp, massive increase of extramedullary hematopoiesis, and an increase in the number of blast-like cells (Fig. 5B and E). Immunofluorescence showed almost complete disappearance, close to vanishing, of the marginal zone (Fig. 5C and F) in spleens of infected mice, irrespective of macrophage depletion (groups 4 to 7). After macrophage depletion alone (group 10), a similar disappearance of this marginal zone was observed. Spleen weight in macrophage-depleted mice was not significantly different from spleen weight in control infected BALB/c mice (group 7).

DISCUSSION

This study was performed in order to analyze which part of the defense system, in addition to the B-cell compartment, is required for the development of *T. brucei*-related glomerular disease. A previously described experimental model in mice was employed, induced by *T. brucei* infection (28). Infected BALB/c mice, splenectomized or macrophage-depleted in various stages of disease, were studied, as well as infected athymic (BALB/c *nu/nu*) mice.

The glomerular disease observed in athymic mice was not different from the disease seen in euthymic infected BALB/c mice, with respect to the albuminuria and the glomerular deposition of immunoglobulins. Polyclonal B-cell activation, however, as measured by the presence of serum antibodies to self and non-self antigens, was not observed. This experiment showed that thymus-dependent T cells could not account for the development of proteinuria. Furthermore, these results support previously obtained results indicating that polyclonal B-cell activation was not needed for the development of glomerular disease (30).

The spleen is considered to be the main lymphoid organ involved in the response to blood-borne antigens (10). Moreover, the spleen is massively enlarged in mice with this *T. brucei* infection. In order to investigate the role of the spleen in the development of proteinuria, the effect of splenectomy was studied in this model. Splenectomy before inoculation with parasites led to a decreased incidence of albuminuria in 40% of the animals, whereas nonsplenectomized infected BALB/c mice invariably developed albuminuria (28, 30). Splenectomy performed 21 days after parasite inoculation resulted in significantly lower albuminuria. These results suggest a role for spleen cells.

The spleen enlargement during *T. brucei* infection is not fully understood. It is in part due to an increase in extramedullary hematopoiesis and in part due to a proportional increase in $CD4^+$ T cells, $CD8^+$ T cells, and B cells. Moreover, a relative increase in null cells is observed (12). These might be, for instance, macrophages, γδ T cells, or natural killer (NK) cells. Part of the splenic enlargement, however, can not be explained by an increase of nucleated cells and must be due to sequestration of erythrocytes or edema. The microscopic architecture of the spleen is severely disrupted, while the splenic marginal zone seems to be enlarged (Fig. 5B and E). The macrophages of this zone are involved in presentation of particulate blood-borne antigens and thus in stimulation of the immune system (10, 27). The number of macrophages was shown to be increased in spleens of infected mice (14). Consequently, because macrophages have been shown to play a crucial role in this infection (21, 22), experiments in macrophage-depleted mice were performed. Macrophage depletion, however, resulted in significantly higher albuminuria levels for a period up to 2 weeks after depletion. Moreover, as in noninfected mice treated with Cl_2MDP liposomes, marginal-zone macrophages had almost vanished in the course of the disease of PBS-liposome-treated infected mice. The difference in albuminuria levels described might be a reflection of loss of phagocytic capacity due to depletion of hepatic macrophages. These macrophages might be involved in phagocytosis of nephrotoxic products, such as transforming growth factor β, platelet-activating factors, or prostaglandins (18), secreted by cellular subsets of the spleen or by the trypanosome itself. These macrophages were shown to be only marginally involved in the clearance of the parasites (9). The increase in albuminuria, however, might also be a reflection of the loss of suppressive effect of macrophages, as splenic macrophages can mediate suppression by decreasing interleukin 2 receptor expression (21) or, especially if soluble blood-borne antigens are involved, by reducing antigen presentation (27).

We conclude that (i) the development of *Trypanosoma*

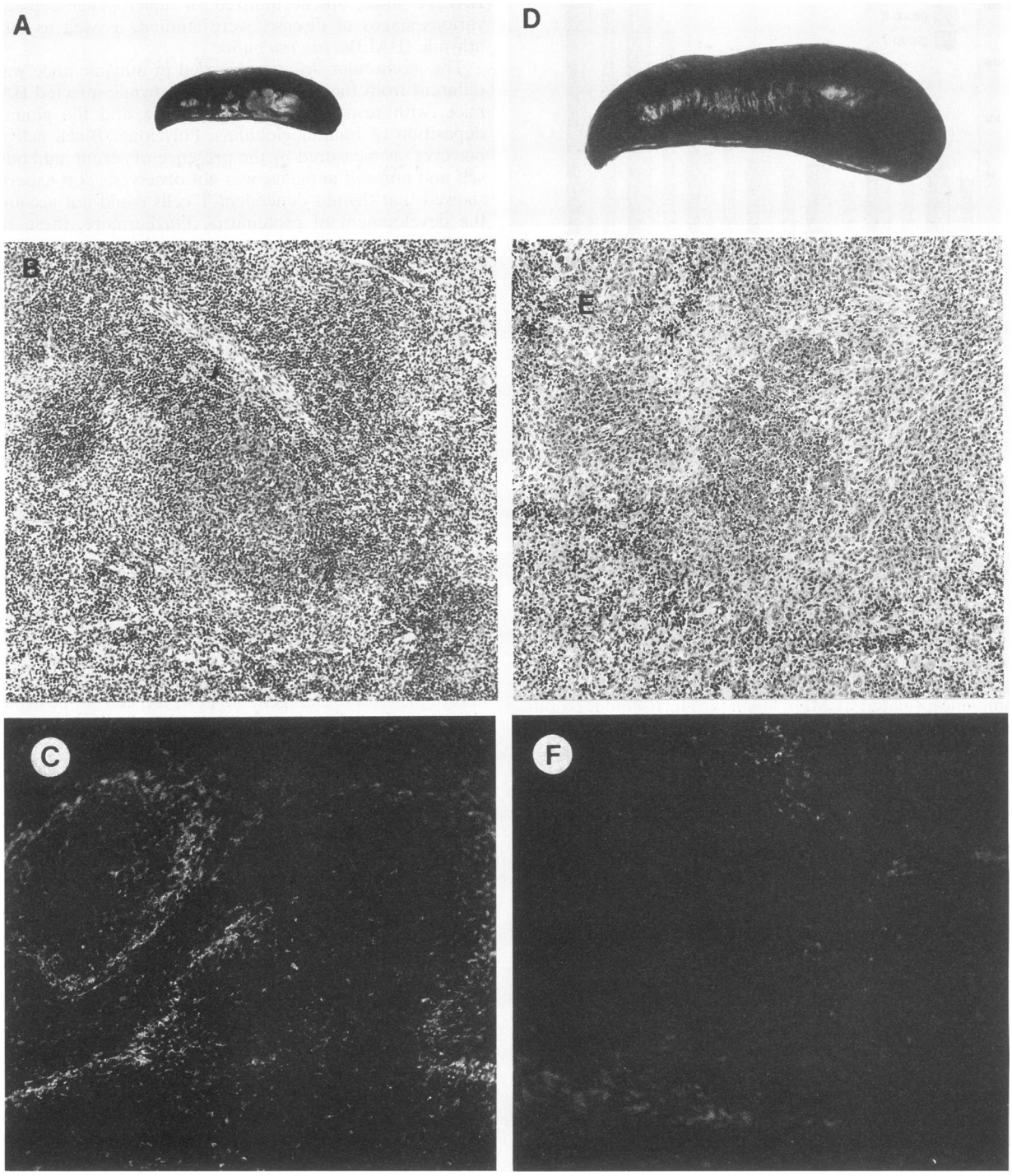

FIG. 5. Representative example of the macroscopy (A and D; magnification, ×2), microscopy (B and E; hematoxylin and eosin stain; magnification, ×100), and immunohistology (C and F; fluorescein isothiocyanate staining for marginal-zone macrophages with MOMA-1; magnification, ×100) of the spleen of a noninfected mouse (A, B, and C) and an infected BALB/c mouse (D, E, and F). The mean spleen weight (± standard deviation) of noninfected BALB/c mice was 140 ± 10 mg; the mean spleen weight of infected BALB/c mice was 1,220 ± 110 mg. Microscopic examination shows a disruption of the normal splenic architecture with seeming enlargement of the marginal zone, while immunofluorescence shows almost complete disappearance of marginal-zone macrophages.

brucei-related glomerular disease in the model used is independent of thymus-matured T cells; (ii) the function of macrophages in this model is shown to be inhibitory rather than disease inducing; and (iii) splenic cells other than thymus-dependent T cells, B cells, or macrophages might play a crucial role in the pathogenesis of this infection-related glomerulopathy.

Analogous to susceptibility to the development of glomerular disease, resistance to infection did not seem to be mediated by the humoral, antibody-related response (4, 13, 19). Susceptibility to infection was reported to be mediated by macrophages and $CD8^+$ cells, influenced by factors secreted by the trypanosome itself (15, 21, 22). It is a matter of speculation which cellular subset, present in the spleen, is responsible for the development of glomerular disease in this model. While macrophages have been excluded; natural killer cells and γδ T cells are candidates, especially since the latter were shown to be significantly increased in spleens of infected mice (5). Therefore, further experiments will be performed to reveal the role of γδ T cells in the development of this *T. brucei*-related glomerular disease. The mechanism by which such a cellular subset might be responsible for the induction of proteinuria is a matter of speculation. Many humoral factors other than Igs, such as cytokines or reactive oxygen species, might be secreted. These factors might induce proteinuria by directly changing the ultrastructure of the glomerular basement membrane or by causing glomerular cell dysfunction, as was described for glomerular endothelial cells in this model (29).

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