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Immunology

Immunoglobulin heavy chain constant region determines the pathogenicity and the antigen-binding activity of rheumatoid factor

(autoantibody/immunoglobulin switch variant/cryoglobulin/glomerulonephritis/vasculitis)

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ABSTRACT **An IgG3 monoclonal antibody, 6-19, derived from unmanipulated MRL/MpJ-*lpr/lpr* mice, exhibiting cryoglobulin and anti-IgG2a rheumatoid factor activities, induces skin leukocytoclastic vasculitis and glomerulonephritis when injected into normal mice. To determine the role of the γ3 heavy chain constant region in the generation of cryoglobulins and associated tissue lesions, we have established an IgG1 class switch variant, clone SS2F8, from the 6-19 hybridoma by sequential sublining. Here we report that the SS2F8 monoclonal antibody, which loses the cryoglobulin activity but retains the rheumatoid factor activity, fails to generate skin and glomerular lesions. The lack of pathogenicity of the IgG1 SS2F8 switch variant is not due to mutations in variable regions, since nucleotide sequence analysis shows no differences between both clones. In addition, we have observed that the IgG1 SS2F8 switch variant exhibits <10% of the rheumatoid factor activity, as compared with the IgG3 6-19 monoclonal antibody, suggesting that the self-associating property of the γ3 isotype promotes antibody-binding activity. The present study indicates that the cryoglobulin activity associated with the γ3 isotype is critically involved in the pathogenicity of 6-19 anti-IgG2a rheumatoid factor monoclonal antibody and highlights the pathogenic relevance of autoantibodies of the IgG3 subclass in murine systemic lupus erythematosus.**

It has long been suggested that the qualitative aspects of autoantibodies may be important for the pathogenesis of autoantibody-mediated cellular and tissue injuries. These include autoantibody's fine specificity, affinity, electrostatic charge, capacity to activate complement, and capacity to fix Fc or complement receptors. If so, the immunoglobulin class switching may be extremely important for the expression of pathogenicity of autoantibodies, since this process may be accompanied with changes in antibody affinity, in electrostatic charges, and in effector functions of antibodies. Consequently, the class switch of autoantibodies may result in a remarkable change of their pathogenic potential. However, until recently, this question could not be directly studied, because of the lack of suitable experimental models that easily reproduce some of the main pathological features usually observed in autoimmune diseases.

Recently we have shown, by using a panel of anti-IgG2a rheumatoid factor (RF) monoclonal antibodies (mAb) of various immunoglobulin isotypes, that only RF of the IgG3 subclass was able to induce, in normal mice, severe glomerulonephritis and skin leukocytoclastic vasculitis in association with cryoglobulinemia (1). However, because of the differences in the variable regions' amino acid sequences among these RF mAb, the differences in their pathogenic activities could be partly related to changes in affinity or electrostatic charge of autoantibodies, as a result of somatic mutations occurring concomitantly with the IgG class switching. Therefore, to better define the role of the γ3 isotype in the generation of RF-induced tissue lesions, in the present study we have developed an IgG1 class switch variant from one of the most pathogenic IgG3 anti-IgG2a RF mAb, 6-19 (1, 2), and assessed its pathogenic activity, in relation to the cryoglobulin activity. Results presented here indicate that the cryoglobulin activity associated with the γ3 isotype is critically involved in the pathogenicity of the 6-19 anti-IgG2a RF mAb.

MATERIALS AND METHODS

Mice and Sera. BALB/c mice were purchased from Bomholtgard (Ry, Denmark) and MRL/MpJ-+/+ mice were from The Jackson Laboratory. (MRL/MpJ-+/+ × BALB/c)F_1 (MRL×BALB) mice were bred in our own facilities. Blood samples collected from the retroorbital plexus were allowed to clot at 37°C for 2 hr. Cryoglobulins were separated from serum as described (1).

mAb. The IgG3 6-19 and 5-7B anti-IgG2a RF mAb with cryoglobulin activity were derived from unmanipulated MRL/MpJ-*lpr/lpr* mice (1, 2). The IgG1 6-19 class switch variant, clone SS2F8, was established from IgG3 6-19 hybridoma cells by the sequential sublining method (3). IgG2b anti-6-19 anti-idiotypic mAb and IgG3 anti-dinitrophenyl (DNP) mAb (E11M, 6H10, CB1, and CB6) were obtained as described (4, 5). Rat anti-mouse κ-chain mAb (H139.52.1) (6), rat anti-mouse γ3-chain mAb (H139.61.1) (6), mouse anti-rat κ-chain mAb (MARK-I) (7), and mouse IgG2a,λ1 anti-(4-hydroxy-3-iodo-5-nitrophenyl)acetyl (NIP) mAb (NIP-23) (8) were kindly provided by M. Pierres (Marseille, France), H. Bazin (Brussels), and A. Marshak-Rothstein (Boston). BALB/c J606 IgG3 myeloma protein was purchased from Litton Bionetics. Murine and rat mAb were purified from culture supernatants by protein A or MARK-I affinity column chromatography (7).

Preparation of F(ab')$_2$ Fragments. F(ab')$_2$ fragments of IgG3 and IgG1 mAb were obtained by digestion of purified mAb at 37°C for 15 min and 2 hr, respectively, with pepsin (Sigma) at a ratio of 33:1 in 0.1 M acetate buffer (pH 3.5) and subsequently neutralized with 0.5 M Tris, as described (4). Integrity of F(ab')$_2$ was assessed by SDS/polyacrylamide gel electrophoresis.

ELISA. Anti-IgG2a RF activities were determined by ELISA as described (1). Briefly, microtiter plates were initially coated with NIP-conjugated bovine serum albumin (5 μg/ml) and then with IgG2a,λ1 anti-NIP mAb (NIP-23; 1

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Abbreviations: RF, rheumatoid factor; mAb, monoclonal antibody-(ies); MRL×BALB, (MRL/MpJ-+/+ × BALB/c)F_1; DNP, 2,4-dinitrophenyl; NIP, (4-hydroxy-3-iodo-5-nitrophenyl)acetyl; Id, idiotype; NMS, normal mouse serum; V_H, variable region of the heavy chain; V_κ, variable region of the light chain; PAS, periodic acid/Schiff reagent; H&E, hematoxylin/eosin.

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μg/ml). Then, plates were incubated with appropriately diluted culture supernatants or sera, and the assay was developed with alkaline phosphatase-labeled rat anti-mouse κ-chain mAb (H139.52.1). Results are expressed as optical density at a 405-nm wavelength. The expression of 6-19 idiotype (Id) in serum was quantified by ELISA. Microtiter plates were coated with anti-6-19 anti-Id mAb (10 μg/ml). The assay was developed with alkaline phosphatase-labeled rat anti-γ3-chain mAb (H139.61.1) or rabbit anti-mouse IgG1. The specificity of this assay has been described (4). Anti-DNP activities were determined by ELISA using DNP-conjugated bovine serum albumin as antigen and alkaline phosphatase-labeled goat anti-mouse $F(ab')_2$ as second antibody. Concentrations of IgG3 and IgG1 in culture supernatants, sera, and cryoglobulins were determined by IgG subclass-specific ELISA as described (9).

IgG Fc–Fc Binding Test. Fifty microliters of various concentrations of 6-19 or SS2F8 mAb was incubated with 40 μl of ^{125}I-labeled 6-19, SS2F8, or J606 (20 ng) antibody at 4°C overnight in the presence of 10 μl of normal mouse serum (NMS). ^{125}I-labeled 6-19, SS2F8, or J606 bound to mAb was precipitated after 1 hr of incubation at 4°C with 1 ml of polyethylene glycol (M_r 6000: Merck) at a final concentration of 7.5% (10). The precipitates were washed once with 7.5% polyethylene glycol. Results are expressed as a percentage of ^{125}I-labeled 6-19, SS2F8, or J606 precipitated specifically after correction of the nonspecific precipitation (<10%) obtained in the presence of NMS.

cDNA Cloning and Sequencing. RNA was prepared from SS2F8 hybridoma by the LiCl method (11). The first strand of cDNA was synthesized with an oligo(dT) primer and 10 μg of total RNA (12). For amplification with *Taq* DNA polymerase (Perkin–Elmer/Cetus) (13), the following primers (5′ → 3′) were used: V_HFOR$_{6\text{-}19}$ (CACTGACTTTCACCATG) and C_γ (GGCCAGTGGATAGAC) for the heavy chain, and V_κFOR$_{6\text{-}19}$ (GTTGCCTCCTCAAAATG) and C_κ (TGGATGGTGGGAAGATG) for the light chain. Polymerase chain reaction (PCR) was performed following recommendations of the manufacturer (Perkin–Elmer/Cetus). The amplified fragments were purified on 2% agarose gel and inserted into pUC18-SmaI vector. The nucleotide sequences corresponding to the variable region of the heavy (V_H) and the light chain (V_κ) were determined by the dideoxynucleotide chain-termination method using M13 primers (14). Consensus sequences of independent clones derived from two different preparations of cDNA were compared with 6-19 V_H and V_κ sequences (15).

Histopathology. Kidney and skin samples were obtained at autopsy, and sections were stained with periodic acid/Schiff reagent (PAS) or hematoxylin/eosin (H&E).

RESULTS

Generation of IgG1 Class Switch Variant of IgG3 6-19 RF mAb. From the IgG3 6-19 hybridoma cell culture, we have isolated by sequential sublining a subclone, SS2F8, secreting IgG1, but not IgG3. This IgG1 SS2F8 antibody as well as the IgG3 6-19 mAb was specifically recognized by anti-6-19 anti-Id mAb (Fig. 1*A*). Quantitative analysis, as determined by the ability of purified 6-19 and SS2F8 mAb adsorbed on nitrocellulose membrane to bind alkaline phosphatase-labeled anti-Id mAb, revealed that both antibodies expressed comparable amounts of 6-19 Id (data not shown). To be certain that V_H and V_κ regions of the IgG1 SS2F8 switch variant were identical to those of 6-19 mAb, we performed cDNA cloning by PCR and DNA sequencing of SS2F8 V_H and V_κ regions. The consensus nucleotide sequence (three clones for V_H and two clones for V_κ from cDNA 1, and three clones for V_H and two clones for V_κ from cDNA 2) showed a complete identity to V_H and V_κ regions of 6-19 mAb (15) (data not shown). The absence of mutations in the variable regions in SS2F8 mAb enabled us to investigate the role of the γ3 isotype in the properties and pathogenicity of 6-19 mAb.

IgG1 SS2F8 RF Switch Variant Fails to Self-Associate and Cryoprecipitate. We have previously shown that murine IgG3 has the unique physicochemical property to self-associate through nonimmunological Fc–Fc interactions (2, 5, 10). When various amounts of 6-19 or SS2F8 mAb were incubated with radiolabeled 6-19, SS2F8, or J606 protein, 6-19 mAb exhibited a concentration-dependent increased binding to ^{125}I-labeled 6-19 or J606 IgG3 but not at all to labeled SS2F8 mAb (Fig. 1 *B* and *C*). In contrast, none of the radiolabeled antibodies was significantly precipitated in the presence of SS2F8 mAb. In addition, at the concentration of 1 mg/ml, ≈20% of 6-19 mAb was cryoprecipitated following 2 days of incubation at 4°C. However, SS2F8 mAb completely failed to exhibit any cryoglobulin activity even at a concentration of 10 mg/ml.

Anti-IgG2a RF Activity of IgG3 6-19 mAb Is Partially Fc Dependent. Although IgG1 SS2F8 mAb exhibited a significant anti-IgG2a RF activity, its specific activity was <0.1 of IgG3 6-19 mAb (Fig. 2*A*). Since similar results were obtained with freshly prepared culture supernatants, this striking decrease in anti-IgG2a activity of the IgG1 variant was not due to partial degradation or denaturation of SS2F8 mAb during the purification procedure. Moreover, anti-IgG2a RF activity of IgG3 6-19 mAb was similarly diminished following pepsin digestion (Fig. 2*B*). It should be mentioned that IgG3 6-19 and IgG1 SS2F8 mAb after pepsin treatment exhibited comparable RF activities (data not shown). As the V_H and V_κ regions of IgG3 6-19 and IgG1 SS2F8 mAb are identical, the observed difference of anti-IgG2a RF activity could be related to the unique self-associating property of the γ3 isotype, which may enhance the RF binding activity.

To further define the role of the IgG3 Fc region for antibody activity, DNP-binding activities of four IgG3 anti-DNP mAb, derived from mice immunized with either DNP-lipopolysaccharides (E11M and 6H10) or trinitrophenyl-keyhole limpet

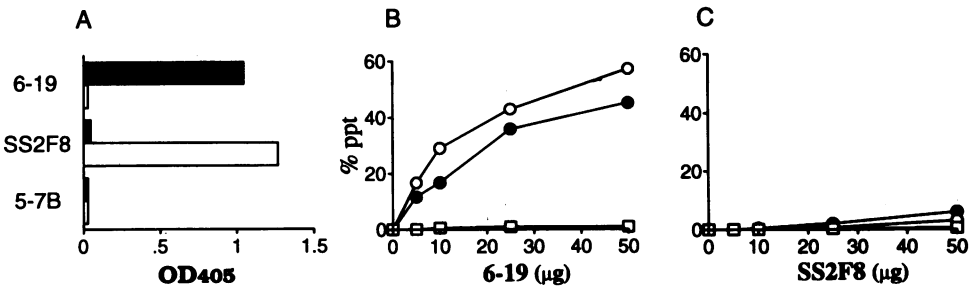

FIG. 1. (*A*) Expression of 6-19 Id in culture supernatants of 6-19, SS2F8, and 5-7B hybridomas. Plates were coated with anti-6-19 anti-Id mAb and developed with alkaline phosphatase-labeled rat anti-γ3 chain mAb (solid bars) or with rabbit anti-mouse IgG1 (open bars). Results are expressed as OD at 405 nm. (*B* and *C*) IgG Fc–Fc binding test. Various amounts (1–50 μg) of 6-19 mAb (*B*) or SS2F8 mAb (*C*) were incubated with 20 ng of ^{125}I-6-19 (○), ^{125}I-SS2F8 (□), or ^{125}I-J606 (●) in presence of 10 μl of NMS. Results are expressed as a percentage of ^{125}I-labeled 6-19, SS2F8, or J606 precipitated (% ppt) specifically.

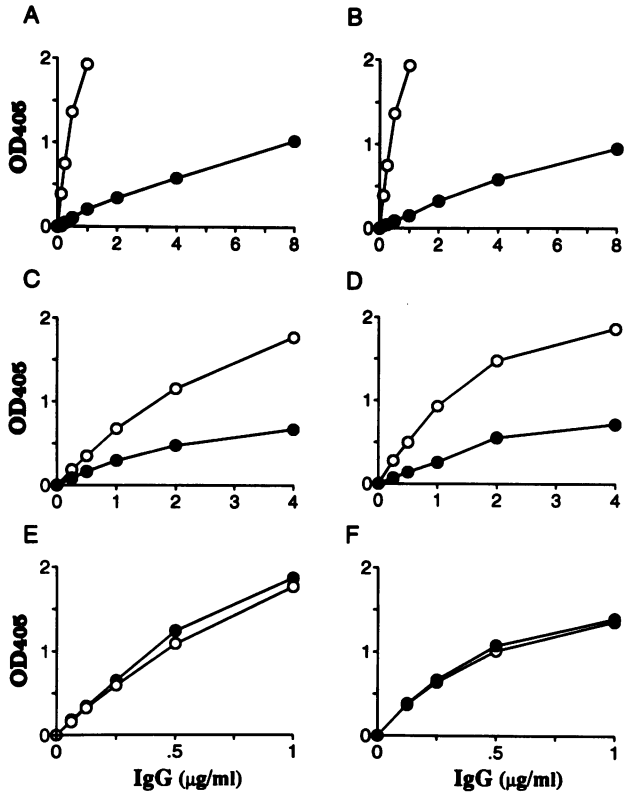

FIG. 2. (*A*) Anti-IgG2a RF activity of 6-19 (○) and SS2F8 (●) mAb. (*B*) RF activities of 6-19 mAb before (○) or after (●) pepsin digestion. (*C–F*) Anti-DNP activities of E11M (*C*), 6H10 (*D*), CB1 (*E*), and CB6 (*F*) mAb before (○) or after (●) pepsin digestion. Thirty-three micrograms of IgG3 mAb was incubated with 1 μg of pepsin in acetate buffer for 15 min at 37°C or with acetate buffer alone. After neutralizing with Tris, their RF or anti-DNP activities were determined by ELISA, using alkaline phosphatase-labeled rat anti-mouse κ-chain mAb or goat anti-mouse $F(ab')_2$ antibodies. Results are expressed as OD at 405 nm.

hemocyanin (CB1 and CB6) (5), were assessed before and after pepsin digestion. Although two mAb, E11M and 6H10, again exhibited a marked diminution (four to eight times) of anti-DNP activity after pepsin digestion, essentially identical DNP-binding activities were observed with CB1 and CB6 mAb (Fig. 2 *C–F*). It should be mentioned that the Fc dependency of IgG3 anti-DNP activity inversely correlated with anti-DNP affinities: DNP-binding affinities of CB1 (K_0 = 5.5×10^6 M^{-1}) and CB6 (2.4×10^6 M^{-1}) were higher than those of E11M (0.8×10^6 M^{-1}) and 6H10 (0.3×10^6 M^{-1}) (5).

IgG1 SS2F8 RF Switch Variant Fails to Induce Skin and Glomerular Lesions. To study *in vivo* the role of the γ3 isotype in the generation of tissue lesions (skin vasculitis and glomerulonephritis) induced by 6-19 RF mAb, MRL×BALB mice were injected with IgG3 6-19 or IgG1 SS2F8 hybridoma cells. None of the MRL×BALB mice injected with SS2F8 hybridoma cells developed the characteristic vascular purpura on the skin of their ears, tails, and foot pads or acute glomerulonephritis, as observed in mice receiving 6-19 hybridoma (Table 1; Fig. 3). Whereas substantial amounts of cryoglobulins were recoverable from sera of all 6-19 hybridoma-injected animals, no cryoglobulin activity was detectable at any moment in sera of SS2F8 hybridoma-injected mice (Table 1). Although sera from mice receiving SS2F8 hybridoma exhibited lower anti-IgG2a RF activities than those from 6-19 hybridoma-injected mice, serum levels of the 6-19 Id in SS2F8 hybridoma-injected mice were consistently higher than those of 6-19 hybridoma-injected mice. Relatively lower RF activities in mice injected with SS2F8 hybridoma were in keeping with the diminished RF activity of IgG1 SS2F8 mAb, as compared with IgG3 6-19 mAb.

DISCUSSION

The present study has demonstrated that, whereas 6-19 RF mAb of the IgG3 isotype induced severe cryoglobulin-associated tissue lesions (skin vasculitis and glomerulonephritis), its IgG1 class switch variant, bearing the identical V_H and V_κ regions, completely lost its pathogenic activity. This provides direct evidence that the pathogenicity of autoantibodies is dramatically influenced by the immunoglobulin constant region, supporting the importance of subpopulation(s) of autoantibodies in the pathogenesis of autoantibody-mediated tissue lesions.

We have recently demonstrated, using a panel of anti-IgG2a RF mAb of different immunoglobulin isotypes that only the RF mAb able to cryoprecipitate—all from the IgG3 subclass—can induce skin vasculitis and glomerulonephritis (1). However, it was difficult to exclude the possibility that differences in fine specificities, affinities, or sequences of the variable regions might account for the absence of pathogenicity observed with non-IgG3 RF mAb. In particular, the acquisition or loss of charged amino acid residues as a result of somatic mutations in autoantibodies following the immunoglobulin class switching (16) might influence the pathogenic potential of autoantibodies. In fact, it has been well demonstrated that the electrostatic charge of antibodies can be one of the factors that determine their pathogenicity, particularly nephritogenic activity (17). However, the present finding that the IgG1 switch variant of IgG3 6-19 RF mAb, which is the most pathogenic RF mAb we have examined so far (1), is not able to generate cryoglobulins and cause any tissue lesions, formally proves that the γ3 heavy chain constant region is critically involved in the development of

Table 1. Serum levels of 6-19 Id, cryoglobulins, and anti-IgG2a RF and skin and glomerular lesions in mice injected with 6-19 or SS2F8 hybridoma cells

mAb	*n*	Day of sacrifice	6-19 Id*	Cryoglobulins*	RF†	Skin vasculitis	Glomerulonephritis
6-19	10	8	3.3 ± 0.9	57.8 ± 19.7	1.38 ± 0.49	10/10	10/10
SS2F8	8	10	5.1 ± 2.8	<0.1	0.49 ± 0.13	0/8	0/8
Control‡	5		<0.1	<0.1	<0.1		

Hybridoma cells (10^7) were injected intraperitoneally into 3-month-old MRL×BALB female mice on day 0. *n*, Number of mice studied.

*Serum levels of 6-19 Id (mg/ml) and cryoglobulins (μg/ml) at sacrifice were determined by ELISA (mean ± 1 SD).

†Anti-IgG2a RF activities at sacrifice were determined by incubating 1:10,000 diluted serum on plates coated with NIP and then with IgG2a,λ1 anti-NIP mAb. The assay was developed with alkaline phosphatase-labeled rat anti-mouse κ-chain mAb. Results are expressed as OD at 405 nm (mean ± 1 SD).

‡Pristane-treated MRL×BALB mice of the same age and sex.

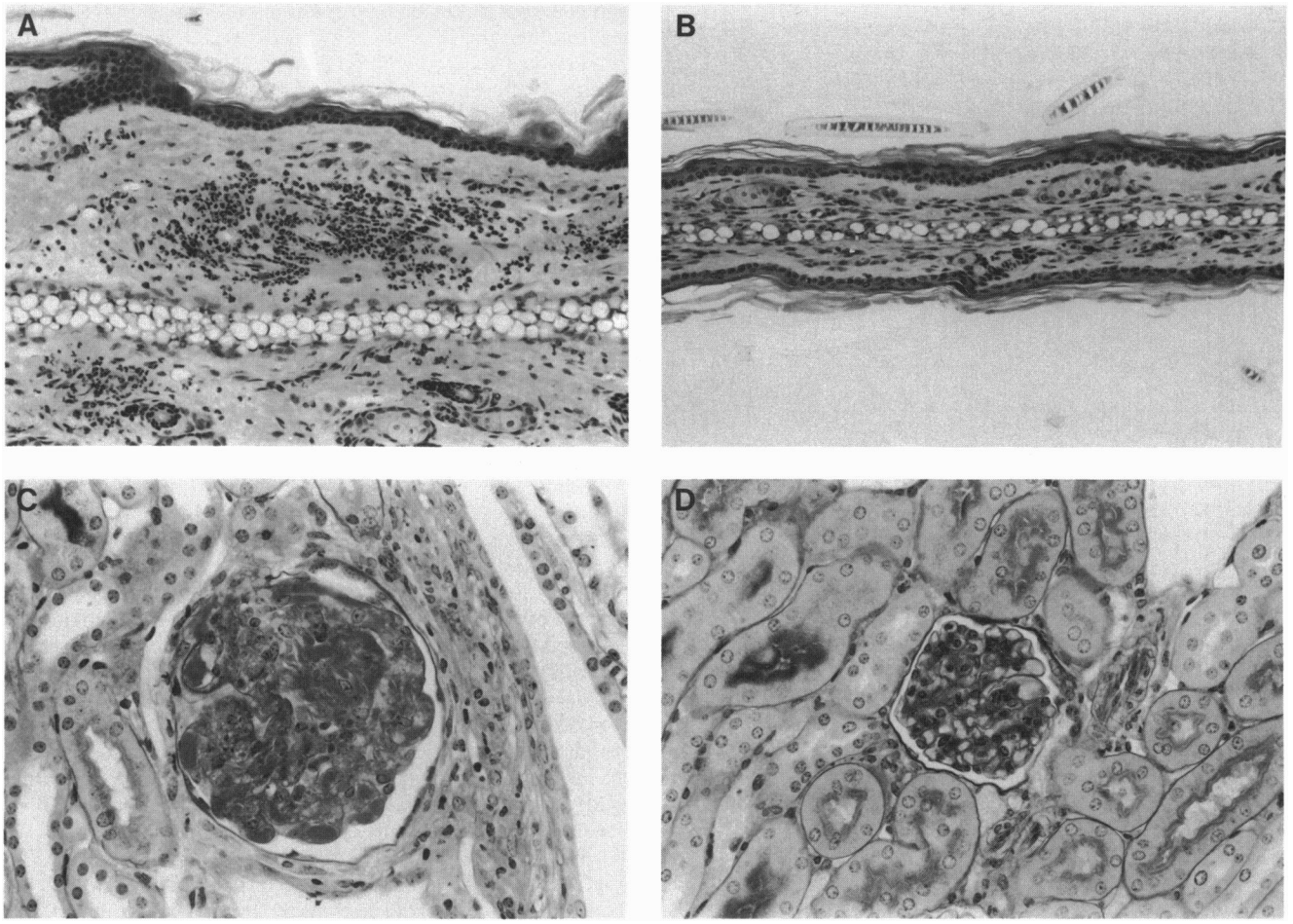

FIG. 3. (*A*) Representative histological appearance of skin lesions of ears 8 days after intraperitoneal injection of IgG3 6-19 RF hybridoma cells into MRL×BALB mice. Leukocytoclastic vasculitis was characterized by the infiltration of polymorphonuclear leukocytes and the extravasation of erythrocytes. (H&E, ×60.) (*B*) Absence of vascular lesions in skin of ears from mice injected with IgG1 SS2F8 hybridoma cells. (H&E, ×60.) (*C*) Representative histological appearance of glomerular lesions induced by IgG3 6-19 hybridoma cells, showing the voluminous deposition of PAS-positive materials that nearly occluded the capillary lumen. Note a marked enlargement of glomeruli as compared with those (*D*) of mice injected with IgG1 SS2F8 hybridoma cells. (PAS, ×120.) (*D*) Representative histological appearance of kidneys from mice injected with IgG1 SS2F8 hybridoma cells. Note the absence of significant glomerular changes. (PAS, ×120.)

skin vasculitis and glomerulonephritis induced by IgG3 RF mAb. However, it should be emphasized that the RF activity of 6-19 mAb plays an additional role in the cryoglobulin-associated cutaneous vascular lesions (15).

Particular properties of the IgG3 isotype are not only involved in cryoprecipitation but also play a significant role by increasing binding activity of antibodies. We have observed that the IgG1 switch variant, SS2F8, had <0.1 of the RF activity as compared with IgG3 6-19 mAb and that a similarly decreased binding activity was observed with the $F(ab')_2$ fragment of 6-19 mAb. In fact, recent studies have shown that an IgG3 anti-streptococcal group A carbohydrate mAb exhibited a higher binding activity than its IgG1 and IgG2b switch variants (18). This could well be explained by the unique self-associating property of IgG3 heavy chain constant region. An increased binding activity of self-aggregating IgG1 mAb caused by mutation in its heavy chain constant regions (19) further supports this idea. However, it should be mentioned that this Fc dependency of IgG3 antibody activity is not always apparent. Our studies on anti-DNP mAb have shown that IgG3 antibodies with lower affinities, derived from mice immunized with a T-cell-independent antigen, are more Fc dependent for antigen binding than those with higher affinities, obtained following immunization with a T-cell-dependent antigen. This is consistent with the Fc dependency of IgG3 anti-bacterial polysaccharide antibodies, which are likely to have a low affinity for multivalent polysaccharide antigens due to a T-cell-independent immune response (20). Thus, it is conceivable that the remarkable Fc dependency of the RF activity of 6-19 mAb can be the reflection of its low affinity; this is supported by the germ-line configuration of the variable region of its light chain, which is crucially involved in its RF activity (15).

The present study further documents the pathogenic importance of autoantibodies of the IgG3 subclass in murine systemic lupus erythematosus. It has been shown that (*i*) IgG3 monoclonal cryoglobulins of various specificities (RF, anti-DNA, etc.) derived from MRL/MpJ-*lpr*/*lpr* and (NZB × NZW)F_1 lupus-prone mice are able to generate "wire-loop" lesions, characteristically described for lupus nephritis (21, 22); (*ii*) IgG3 production was well correlated with the development of lupus nephritis in mice bearing the *lpr* mutation (23); and (*iii*) the *xid* mutation causing a defect of IgG3 production markedly reduced autoimmune pathology in three different lupus-prone mice (24–26). Finally, the complete loss of pathogenicity of autoantibodies by the IgG class switching is relevant to the understanding, monitoring, and treatment of patients with autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and related rheumatic diseases.

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