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Immunology

Antibodies to RNA from autoimmune NZB/NZW mice recognize a similar antigenic determinant and show a large idiotypic diversity

(autoimmune disease/monoclonal antibodies/RNA sequence/idiotypes)

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ABSTRACT **We have identified two RNA-specific hybridoma autoantibodies in fusions of spleen cells from unimmunized NZB/NZW female mice with BALB/c myeloma cells. The two fusion experiments were carried out 2 years apart with different myeloma partners. Specificity analyses showed that the two monoclonal antibodies and the total RNA-binding IgG in NZB/NZW serum recognize a G,C-rich sequence of ribonucleotides. The isolated heavy and light chains of the two antibodies and their papain Fab fragments could be distinguished by $NaDodSO_4$/polyacrylamide gel electrophoresis. Rabbit anti-idiotypic antibodies prepared against the two monoclonal proteins showed unique specificities for the antigen-binding sites of their cognate autoantibodies. Moreover, the anti-idiotypic antisera had little effect on the RNA-binding capacity of the total IgG from NZB/NZW serum. These results suggest that a wide range of different idiotypes is involved in the autoimmune response to a similar antigenic determinant.**

Antibodies to native nucleic acids are found in the sera of patients with systemic lupus erythematosus (SLE), an autoimmune disease of unknown etiology (1). They also are found in several strains of mice (NZB/NZW F_1, MRL, BXSB), which serve as animal models for this disease (2).

The mechanisms leading to the production of nucleic acid-reactive antibodies remain obscure. Both antigenic stimulation (possibly by a virus) and polyclonal activation have been implicated. A particularly interesting question is concerned with the remarkable heterogeneity of SLE autoantibodies (1, 3). This heterogeneity could arise from multiple antigenic specificities of antibodies directed against a multideterminant macromolecule; alternatively, it could reflect the production of a large number of antibodies that bind to the same antigenic determinant with different affinities. In either case, the autoantibodies could be idiotypically related (i.e., sharing variable region structures) or unrelated. The answer to these questions would have a direct bearing on the development of certain therapeutic approaches to SLE. Shared idiotypic determinants would favor the use of suppressive anti-idiotypic antibodies (4, 5), whereas a limited number of antigenic specificities would promote the use of suitable tolerogens (6).

We have shown (3) that the frequency of autoantibodies reactive with native, single-stranded RNA in SLE sera is similar to that of anti-DNA antibodies, and their serum levels correlate well with disease activity. More recently, we have reported (7) the production of an RNA-binding monoclonal antibody (D4) from an unimmunized female NZB/NZW F_1 mouse by the cell-fusion technique (8). The antibody was shown to be directed against a G,C-rich sequence of ribonucleotides (9). We now describe a second monoclonal antibody (D44) that is derived from a different individual of the same mouse strain and possesses a very similar nucleotide specificity but different idiotypic structures.

MATERIALS AND METHODS

Production and Purification of Hybridoma Autoantibodies. Fusion of spleen cells from unimmunized 7-month-old NZB/NZW mice with the BALB/c drug-resistant myeloma cell lines 45.6TG1.7 (IgG2b, κ light chain producer) (7) and NSI/1-Ag4-1 (κ chain nonsecretor) (8) were carried out as described (7). Culture supernatants were screened by the nitrocellulose filter assay with *Escherichia coli* [^{3}H]rRNA (7). Positive cultures were cloned and injected intraperitoneally into NZB/NZW male mice, which were either untreated or subjected to total lymphoid irradiation and bone marrow transplantation with BALB/c cells. The latter procedure was performed by S. Slavin (Hadassah University Hospital) as described (10). Ascitic fluids were collected 10–20 days later and contained 5–10 mg of hybridoma antibody per ml. The monoclonal autoantibodies were purified by ammonium sulfate fractionation and affinity chromatography on protein A-Sepharose and RNA-Sepharose. Fab fragments were obtained by papain digestion and RNA-Sepharose affinity purification. The details of the purification steps are described elsewhere (11).

$NaDodSO_4$/Polyacrylamide Slab Gel Electrophoresis. The procedure has been described by Maizel (12). After reduction in 0.3 M 2-mercaptoethanol (90°C for 1 min), the samples were run at 35 V for 20 hr and stained with 0.25% Coomassie brilliant blue in aqueous solution containing 50% (vol/vol) methanol and 10% (vol/vol) acetic acid.

Preparation of Affinity-Purified Anti-Idiotype Antibodies. Pure Fab fragments (100 μg dissolved in 1 ml of phosphate-buffered saline) of D4 or D44 hybridoma antibodies were mixed with 1 ml of complete Freund's adjuvant (Difco) and injected subcutaneously into New Zealand White rabbits. Booster immunizations were given in complete and incomplete Freund's adjuvant 2 and 3 weeks, respectively, after the primary immunization, and blood was collected 1 week later. The antisera were absorbed first with Sepharose-bound FLOPC21, an IgG3 κ light chain myeloma protein of BALB/c origin (obtained from R. Asofsky, National Institutes of Health) and then with Sepharose-bound mouse IgG pooled from five different strains of mice and purified by conventional methods. Finally, each anti-idiotypic antiserum was bound to a Sepharose column to which

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Abbreviations: SLE, systemic lupus erythematosus; ELISA, enzyme-linked immunosorbent assay.

the corresponding antigen (D4 or D44) was covalently attached. The affinity-purified anti-idiotype antibodies were eluted with 3.5 M $MgCl_2$ and dialyzed against phosphate-buffered saline, hereafter referred to as saline. All affinity resins were prepared by coupling the suitable proteins to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

Solid-Phase Immunoassay for Idiotype–Anti-Idiotype Interaction. The enzyme-linked immunosorbent assay (ELISA) was carried out as follows. Purified antigen (0.3 μg/ml) in 0.1 M carbonate buffer (pH 9.6) was adsorbed to a U-shaped 96-well Flex vinyl plates (No. 22024, Cooke, Alexandria, VA) by incubating 0.3 ml of antigen solution in the wells for 30 min at 37°C and then overnight at 4°C. The protein was fixed to the plastic by treatment (1 hr at room temperature) with 0.25% glutaraldehyde (Sigma grade II) solution in water. The wells were washed three times for 3 min with saline containing 0.05% Tween 20. Sites not binding protein were blocked by treatment with 0.01% L-lysine in saline for 30 min at room temperature, followed by two washings with saline/Tween. Several dilutions of the tested rabbit antiserum in saline/Tween were allowed to react with the plastic-bound antigen for 1.5 hr at 37°C and then for 30 min at 4°C. The plates then were washed twice with saline/Tween and incubated overnight at room temperature with goat anti-rabbit IgG–alkaline phosphatase conjugate (Sigma; 0.3 ml per well, diluted 1:1,000 in saline/Tween). After two more washings in saline/Tween, the enzyme substrate [*p*-nitrophenyl phosphate (1 mg/ml) in 0.1 M glycine, pH 10.4/0.001 M $MgCl_2$; 0.3 ml per well] was added for 30 min at room temperature, and its conversion was measured spectrophotometrically at 405 nm.

Binding of RNA to Hybridoma Antibodies and Competition Experiments. Reaction mixtures contained 10 μl (0.3 μg; 4,000 cpm) of *E. coli* [^{3}H]rRNA (Miles) and 10 μl (3 μg) of D4 or D44 purified IgG in a final volume of 0.2 ml of saline containing 0.01 M $MgCl_2$. When indicated, various concentrations of nucleic acid inhibitors were included (9). In anti-idiotype assays, the hybridoma autoantibodies were incubated with different concentrations of purified anti-idiotypic antibodies for 30 min at room temperature before adding the radioactive RNA. The binding mixture was left for 1 hr at 4°C and then filtered through 0.22-μm nitrocellulose filters (Millipore). The filters were washed with saline containing 0.01 M $MgCl_2$, dried, and assayed in a toluene-based scintillation liquid (9).

Removal of IgG Heavy Chain Subclasses from Pooled NZB/NZW Serum. The IgG fraction of pooled sera from 15 female NZB/NZW mice was purified by ammonium sulfate fractionation and chromatography with DEAE-cellulose, and Sephadex G-200. Aliquots of the purified IgG were incubated with goat antisera to mouse IgG1, IgG2a, IgG2b, and IgG3. [The antisera were prepared as described (13) and were supplied by R. Asofsky, National Institutes of Health, Bethesda, MD.] After incubation for 4 days at 4°C, precipitates were removed by centrifugation, and each sample was tested for complete removal of the appropriate IgG subclass by Ouchterlony double diffusion in agar. The samples were then fractionated by ammonium sulfate at 40% saturation, and the immunoglobulin precipitates were dissolved in saline and tested for RNA-binding activity.

RESULTS

Isolation of Hybridoma Cell Lines. The RNA-binding monoclonal antibody D4 was produced in 1978 by fusion of spleen cells from an unimmunized autoimmune NZB/NZW female mouse to the IgG2b producer, MPC-11 drug-resistant myeloma cells. It was identified in screening of the hybridoma supernatants with [^{3}H]rRNA by the nitrocellulose filter assay. Two years later, a second RNA-reactive antibody clone was identified with the same screening technique. This time, cells from a female mouse with similar manifestations of disease were fused to a different myeloma partner, the κ light chain-nonsecretor NSI cells. After cloning, the cells were injected into male NZB/NZW mice and produced 3–10 mg of monoclonal antibodies per ml of ascitic fluid. The antibodies were purified by ammonium sulfate fractionation and affinity chromatography with protein A-Sepharose and RNA-Sepharose. They were shown by Ouchterlony analysis to be of the IgG3 subclass.

Fig. 1 shows a $NaDodSO_4$/polyacrylamide gradient gel electrophoretogram of the purified monoclonal autoantibodies. The protein A-Sepharose-purified D4 IgG (Fig. 1, lane 2) is a mixture of molecules containing two types of H chain (IgG2b and IgG3, unseparable in this gel system) and two types of L chain, of which the faster moving band belongs to the RNA-binding IgG (11). The purified D44 IgG (Fig. 1, lane 3) contains a single H chain with a slightly but reproducibly greater mobility than that of D4 and two closely spaced L chains; of these, the slower moving band is the product of the parent myeloma, as is evident from the electrophoretic pattern of IgG from a third (NZB/NZW–NSI) hybridoma, the anti-nuclear protein C12 antibody clone (Fig. 1, lane 4), and the faster moving band is the RNA-specific chain and is electrophoretically different from its D4 counterpart.

The dissimilarity between the two monoclonal RNA-specific autoantibodies is still better exemplified by the RNA-Sepharose-purified Fab fragments, which contain only RNA-binding immunoglobulin chains (11). Both the faster (L chains) and slower (Fd chains) moving bands of D4 Fab (Fig. 1, lane 7) are clearly different in electrophoretic mobility from the corresponding D44 bands (Fig. 1, lane 8).

Fine Antigenic Specificity of D4 and D44 Autoantibodies. We have presented evidence (9) for a sequence specificity of the D4 autoantibody—namely, a small (3–4 nucleotide) RNA sequence rich in G and C nucleotides. This finding was based on inhibition studies with synthetic polymers of different nucleotide compositions. It was later confirmed by direct binding of D4 to ribonuclease-derived fragments of native RNA and to isolated synthetic oligonucleotides (unpublished results). We subjected the second RNA-binding autoantibody (D44) to a similar competition analysis, using [^{3}H]RNA as the test antigen, and found that the two immunoglobulins had almost identical antigenic specificities. Similar to the D4 IgG (9), the D44 IgG

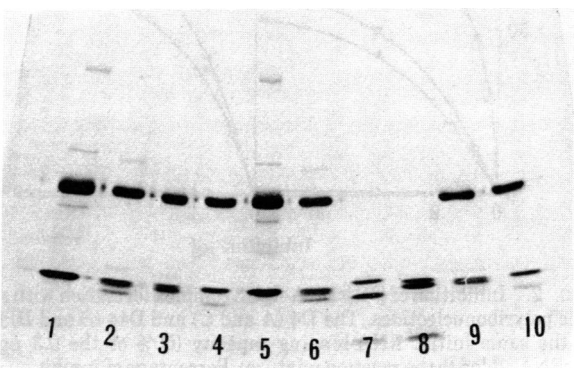

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of purified RNA-binding D4 and D44 proteins. The IgG immunoglobulins and their papain Fab fragments were reduced and run in a gradient gel containing 5–20% acrylamide. Lanes: 1, MPC-11 IgG; 2, D4 IgG; 3, D44 IgG; 4, C12 IgG; 5, MPC-11 IgG; 6, D4 IgG; 7, D4 Fab; 8, D44 Fab; 9, D44 IgG; 10, C12 IgG.

could not bind to a single- or double-stranded DNA. Likewise, no binding was observed to the synthetic homopolymers poly(A), poly(U), poly(C), poly(G), and poly(I) (not shown).

Among the six random copolymers containing two different nucleotides, poly(A,U), poly(A,C), poly(A,G), poly(G,U), poly(C,U), and poly(G,C), only the last polynucleotide had an inhibitory effect on the binding reaction between RNA and the two monoclonal autoantibodies (Fig. 2 *A* and *B*). An identical pattern of copolymer inhibition was observed with the total IgG fraction of pooled sera from 15 female NZB/NZW mice (not shown), where poly(G,C) was the only effective inhibitor.

When random heteropolymers containing three different ribonucleotides were tested in this competition assay, a similar pattern was obtained (Fig. 2 *C* and *D*) in which poly(G,C,U) bound most effectively to both monoclonal antibodies. Here, however, a subtle difference between the antigenic specificities of the two proteins was observed: whereas complex formation between D44 IgG and rRNA could only be inhibited by the G,C-containing heteropolymers poly(G,C,U) and poly(G,C,A), the rRNA complex with D4 IgG was also sensitive to inhibition to poly(G,A,U) (Fig. 2*C*). The polyclonal IgG fraction from pooled NZB/NZW sera showed a pattern of heteropolymer competition similar to that of the monoclonal antibody D4 (not shown).

Idiotypic Analysis of D4 and D44 Monoclonal Antibodies. Purified Fab fragments derived from D4 and D44 hybridoma antibodies (Fig. 1) were used as antigens in the production of anti-idiotypic antisera in rabbits. The rabbit antisera were made anti-idiotypic by solid-phase adsorption with purified mouse myelomas and total mouse IgG from several strains. Finally, the anti-iodiotypic antibodies were purified by absorption to and elution from Sepharose columns, to which the appropriate proteins (D4 or D44) were covalently bound.

Figs. 3 *A* and *B* show the binding properties of the two anti-idiotypic preparations to D4 and D44 proteins as measured by solid-phase ELISA. Each anti-idiotypic reagent showed a clear preference for its cognate antigen over the other monoclonal antibody. The two reagents bound only marginally to a third NZB/NZW hybridoma autoantibody specific for a nuclear protein (C12) and to the total IgG fraction from the pooled NZB/NZW sera.

Fig. 3 *C* and *D* show that the anti-idiotypic antibodies are directed at or close to the antigen binding sites of their target monoclonal antibodies. Incubation of the purified rabbit antibodies with D4 or D44 in roughly stoichiometric amounts abolished the capacity of the monoclonal antibodies to bind radioactive RNA. An average of one molecule of rabbit anti-idiotypic antibody per one molecule of hybridoma antibody was sufficient to cause 50% inhibition of RNA binding.

Rabbit anti-D4-idiotype was highly specific for the D4 active site and did not have any effect on the capacity of D44 IgG or pooled NZB/NZW IgG to bind RNA in the concentration range that was tested (Fig. 3*C*). The rabbit anti-D44 antibody showed a similar preference for the D44 binding site (Fig. 3*D*) but also had a measurable effect on the RNA-binding capacity of D4 and

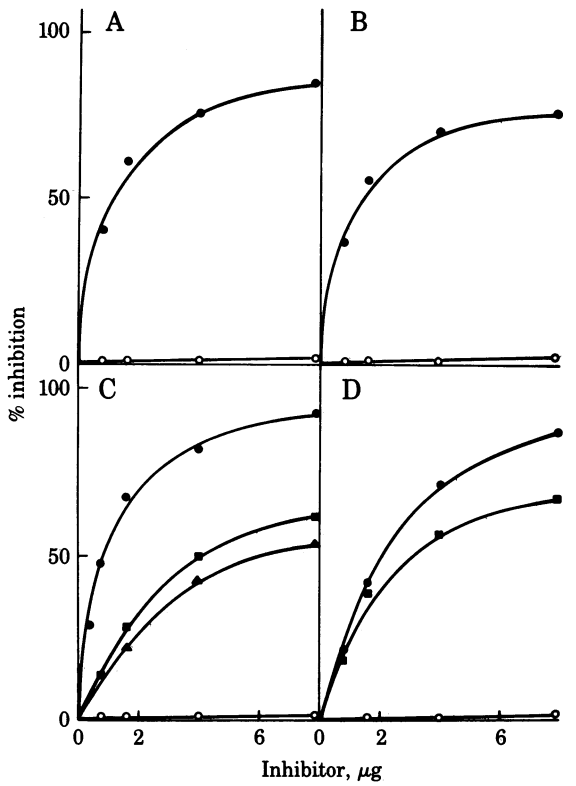

FIG. 2. Inhibition of [^{3}H]rRNA–IgG3 complex formation with synthetic polyribonucleotides. The D4 (*A* and *C*) and D44 (*B* and *D*) IgG had the same initial RNA-binding capacity (50% of the 0.3 μg of [^{3}H]rRNA added to the reaction mixture). Percentage of inhibition was calculated from the binding of the radiolabeled antigen in the presence and absence of inhibitor. (*A* and *B*) Competition experiments with copolymers containing two different nucleotides. ●, Poly(G,C); ○, poly(A,C), poly(G,U), poly(A,U), poly(C,U), and poly(A,G). (*C* and *D*) Competition experiments with heteropolymers containing three different nucleotides. ●, Poly(G,C,U); ■, poly(G,C,A); ▲, poly(G,A,U); ○, poly(A,C,U) in *C* or poly(A,C,U) and poly(G,A,U) in *D*.

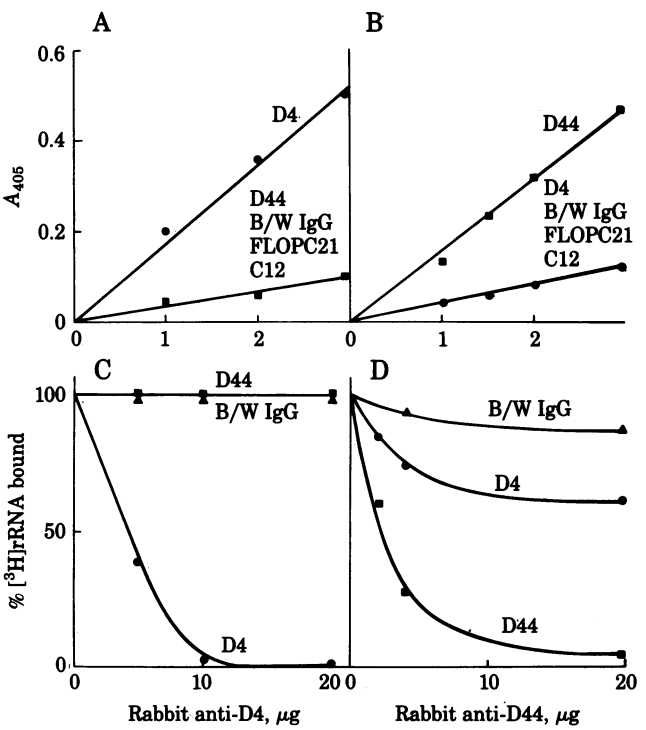

FIG. 3. Properties of rabbit anti-idiotypic antibodies produced against D4 and D44 hybridoma antibodies. (*A* and *B*) Direct reaction of rabbit anti-D4 and anti-D44 antibodies in solid-phase ELISA. The antigens absorbed to the solid-phase support were the two monoclonal autoantibodies D4 and D44, polyclonal IgG from pooled NZB/NZW sera (B/W IgG) IgG2a NZB/NZW hybridoma autoantibody specific for a nuclear protein (C12), and IgG3 myeloma of BALB/c origin (FLOPC21). (*C* and *D*) Binding of [^{3}H]rRNA to D4 (3 μg), D44 (3 μg), and polyclonal NZB/NZW IgG (70 μg) after their incubation with different amounts of rabbit anti-idiotypic antibodies. The D4, D44, and polyclonal IgG had the same initial RNA-binding capacity (50% of the 0.3 μg of [^{3}H]rRNA added to the reaction mixture).

to a lesser extent on NZB/NZW polyclonal IgG. An additional adsorption step of the rabbit anti-D44 antibodies with a RNA-nonbinding NZB/NZW hybridoma (C12-Sepharose) did not eliminate their crossreactivity with D4 and polyclonal IgG.

Subclass Distribution of RNA Binding IgG in NZB/NZW Serum. To test whether the IgG3 subclass has a particular role in RNA binding reactions, we used class-specific antisera to specifically remove each IgG heavy chain subclass from total IgG of pooled NZB/NZW sera. After removal of a particular subclass, the residual RNA-binding capacity of the polyclonal IgG was measured. Table 1 clearly shows that the IgG2a subclass and not IgG3 is mostly responsible for the RNA-binding phenomenon in female NZB/NZW serum. This result was confirmed qualitatively by Ouchterlony analysis of polyclonal NZB/NZW IgG, which was bound and eluted from an RNA-Sepharose column (not shown). A visible precipitin line was only formed between the RNA-specific IgG and anti-IgG2a antiserum.

DISCUSSION

In this report we describe some immunochemical properties of two RNA-binding hybridoma antibodies from different NZB/NZW mice and attempt to draw some general conclusions concerning the total RNA-reactive immunoglobulins in SLE. The finding that the two monoclonal antibodies belong to the relatively rare IgG3 heavy chain subclass of the mouse prompted us to test whether IgG3 autoantibodies are mostly responsible for RNA binding in NZB/NZW serum. This was found not to be the case (Table 1). Instead, most RNA-reactive antibodies were of the IgG2a subclass, which also has been found to be the major DNA-binding IgG subclass in NZB/NZW kidney eluates (14).

Analysis of the fine antigenic specificity of the two monoclonal antibodies and of polyclonal IgG from pooled sera has suggested that most RNA-reactive autoantibodies in NZB/NZW mice are directed against a very similar antigenic determinant composed of a short sequence of ribonucleotides. A similar specificity was shown by purified IgG fractions from three individual human SLE sera (15). Therefore, the large heterogeneity of autoantibodies to RNA observed in SLE sera (3) must be due mainly to the different affinities of the various antibodies rather than to different antigenic specificities.

The inhibition patterns of RNA complex formation with D4 and D44 by synthetic polynucleotides were strikingly similar. The only difference found in these competition experiments was the ability of the random polymer poly(G,A,U) to inhibit RNA binding to D4 and not to D44 IgG. We have suggested (9) that adenosine is capable of replacing cytidine to some extent due to the similarity in the structure of their pyrimidine rings. This type of degeneracy, which is shared by certain restriction enzymes, is not permitted by the D44 combining site, which strictly binds G,C-containing sequences. Subtle differences in antigenic specificity of antibodies elicited against small haptens have been well documented (e.g., heteroclitic antibodies) (16).

Polyacrylamide gel electrophoretic analysis of the two RNA-binding autoantibodies and their Fab fragments clearly demonstrated that their heavy and light chains are structurally different. Moreover, using active site-specific anti-idiotypic antibodies, we have shown that the two antibodies possess distinct protein structures at or near their antigen-binding sites. This was particularly evident in the case of anti-D4 antibodies, which were highly specific for the D4 private or individual idiotype. They did not have any inhibitory effect on the capacity of either D44 or polyclonal NZB/NZW IgG to bind RNA. The anti-D44 antiserum showed a clear preference for the D44 binding site but probably contained some antibodies directed against a public or crossreactive anti-RNA idiotype. This was demonstrated by the ability of these rabbit antibodies to partially influence the binding of RNA to either D4 or polyclonal NZB/NZW IgG. With anti-idiotypic antibodies produced against the dextran-binding MOPC104 and J558 proteins, it has been shown that both private and public idiotypes were active site-associated and were located in the hypervariable regions of the two proteins (17).

Table 1. Binding of [^{3}H]rRNA to polyclonal IgG from pooled NZB/NZW sera after removal of specific IgG heavy chain subclasses

NZB/NZW IgG	Residual [^{3}H]rRNA binding, %
Control	100
Minus IgG1	90 ± 10
Minus IgG2a	50 ± 10
Minus IgG2b	90 ± 10
Minus IgG3	90 ± 10

Our idiotypic analysis is in complete agreement with the results of Ju *et al.* (18), who studied the idiotypic specificities of 15 monoclonal anti-poly ($Glu^{60}Ala^{30}Tyr^{10}$) antibodies. This system is particularly relevant to the present study because the immunizing antigen is a linear synthetic polymer composed of three different L-amino acids. Complete idiotypic identity was not observed between any two hybridoma anti-poly-($Glu^{60}Ala^{30}Tyr^{10}$) antibodies. Thus, it was concluded that virtually every hybridoma cell line was derived from a unique B-cell clone.

Therefore, we conclude that the disease-associated RNA-specific autoantibodies in the sera of NZB/NZW F_1 mice are restricted in their antigenic specificities and not in their idiotypic repertoire. This conclusion supports the view that a well-defined antigen, rather than a nonspecific activation, is responsible for the formation of autoantibodies in these mice. Andrzejewski *et al.* (19) have reached somewhat different conclusions based on results with monoclonal anti-DNA autoantibodies derived from MRL/l mice. These antibodies showed great diversity in their preference for nucleic acid antigens. Some shared idiotypic structures were found between monoclonal antibodies with different antigenic specificities, but no private idiotypes could be demonstrated for antibodies with the same antigenic specificity. Whether this is a fundamental difference between anti-DNA and anti-RNA antibodies or between the autoimmune mechanisms in the two mouse models, remains to be further investigated. Recent studies on the pathogenesis of murine SLE in NZB/NZW and MRL mice (20, 21) have shown major differences in the cellular, serologic, and clinical manifestations of the disease in these two mouse strains. The MRL mouse exhibits a much wider spectrum of autoimmune specificities. Our initial experiments with polyclonal anti-RNA antibodies from MRL/l mice have, so far, failed to identify a common RNA antigenic determinant.

The results presented in this paper may be taken into account when two different approaches to treatment of autoimmune diseases are considered. The use of anti-idiotypic antibodies (4) or drug-conjugated anti-idiotypic reagents (5) to efficiently suppress autoantibody-producing cells will depend on the selection of suitable antisera or monoclonal antibodies specific for the crossreactive idiotypes. On the other hand, the construction of suitable tolerogens (6) may be facilitated by the restriction in antigen specificity.

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