

ANTI-IDIOTYPIC ANTIBODIES TO THE COOMBS ANTIBODY IN NZB F₁ MICE*

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The network theory of the immune system proposes that idio-anti-idiotypic interactions provide important immunoregulatory controls (1). In many experimental systems, the passive administration of heterologous anti-idiotypic antibody has been shown to enhance (2) or suppress (3) immune responses. In addition, spontaneous auto-anti-idiotypic antibody has been detected after antigenic challenge (4), generally appearing coincident with a decline in antibody titer (5). These findings have suggested that network controls might similarly regulate the formation of pathological autoantibodies (6). Two recent studies support this hypothesis. In one, rabbit anti-(anti-thyroglobulin) idio-anti-idiotypic antibody prevented spontaneous autoimmune thyroid disease in Buffalo rats (7). In the other, anti-(anti-DNA)-idiotypic antibody was found in the serum of systemic lupus erythematosus (SLE)¹ patients when they were in remission (8).

In the current work, we have investigated the SLE mouse strain NZB for anti-(autoantibody)-idiotypic antibodies that might play an immunoregulatory role. With age, these mice spontaneously produce IgG anti-erythrocyte autoantibodies which mediate a severe Coombs-positive hemolytic anemia (9). The F₁ hybrids of NZB and several normal strains are known to develop a milder hemolytic disease with lower levels of Coombs antibodies (10). We have found that some of these hybrids spontaneously produce high titers of anti-(anti-erythrocyte)-idiotypic antibodies that specifically recognize the NZB Coombs idiotypes. This finding suggests that network idio-anti-idiotypic control might play a protective role in suppressing autoantibody formation in these animals.

Methods

Mice. NZB and CBA/J mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Some NZB mice used in early experiments came from Southwestern Medical School, Dallas, TX. (NZB × CBA)F₁ mice were bred in the animal facilities at the University of North Carolina.

Mice were bled from the retro-orbital plexus. To obtain erythrocyte (RBC) suspensions, blood was pipetted immediately into a large excess of Alsever's solution, centrifuged, and washed three times in saline before use. It was necessary to use fresh RBC for hemagglutination assays, as storage at 4°C overnight caused inconsistent results. Serum samples were obtained by

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¹ Abbreviations used in this paper: BSA, bovine serum albumin; MRBC, mouse RBC; RBC, erythrocytes; SLE, systemic lupus erythematosus; SRBC, sheep RBC.

allowing blood to clot at room temperature and separating by centrifugation. Serum was stored in aliquots at -20°C before use.

Hemagglutination Assays. For direct Coombs assays, washed RBC from 9–10-mo-old NZB mice were resuspended to 1% in normal saline containing 1% bovine serum albumin (BSA) (Sigmal Chemical Co., St. Louis, MO). For indirect hemagglutination assays, a 10% suspension of washed normal mouse RBC (MRBC) from CBA mice was incubated with an equal volume of a 1:10 dilution of NZB serum for 30 min at room temperature with rocking. In both assays, developing sera were diluted in BSA-normal saline in V-bottomed microtiter wells, and an equal volume of 1% RBC suspension was added. Hemagglutination was read after 1 h at room temperature. Titers represent the highest dilution giving $\geq 2+$ agglutination on a 0–4+ scale.

The developing antiserum used for screening for Coombs positivity was rabbit anti-mouse IgG, which was raised by immunization with an IgG myeloma and rendered gamma-specific by absorption with insolubilized IgM and lambda and kappa light chains. Detection of anti-RBC autoantibodies of differing subclasses was performed by using serial dilutions of rabbit antisera against mouse IgG1, IgG2a, IgG2b, and IgG3, all obtained from Litton Bionetics (Kensington, MD) and found to be monospecific by gel diffusion. (NZB \times CBA)F₁ hybrid sera were used as developing reagents in anti-idiotypic assays.

Hemagglutination Inhibition Assays. For confirmation of specificity, NZB sera were serially diluted and incubated for 30 min at room temperature with selected (CBA \times NZB)F₁ sera at a concentration fourfold greater than the agglutination endpoint. Appropriate Coombs-positive RBC were then added. After 60 min at room temperature, the hemagglutination inhibition titer was read as the highest NZB serum dilution to completely inhibit agglutination.

Absorptions. Sepharose conjugates of monospecific goat anti-mouse IgG and of anti-trinitrophenylated ovalbumin were kind gifts of Dr. Ellen Vitetta, University of Texas Health Science Center, Dallas, TX. Mouse IgG conjugated to Sepharose was provided by Dr. Stella Robinson (University of Texas Health Science Center). MRBC were coated with mouse IgG (Miles Laboratories, Elkhart, IN), by the chromium chloride technique (11). IgG-coated sheep RBC (SRBC) were prepared by immunizing CBA mice three times intraperitoneally with 0.1 ml of 10% SRBC at 10-d intervals, and incubating the resulting immune serum (which contained high titer IgG anti-SRBC antibody) with SRBC for 30 min at room temperature, followed by extensive washing.

Preparation of Pepsin Fragments of Coombs and Anti-Idiotypic Antibodies. 200 μl of each of four NZB indirect-Coombs-positive sera and four corresponding hybrid anti-idiotypic sera were precipitated with 45% saturated ammonium sulfate. These partially-purified IgG fractions were digested with 2% pepsin at 37°C for 18 h in 0.5M acetate, pH 4.5. The resultant F(ab')₂ fragments were absorbed with goat anti-mouse IgG (pFc'-specific)-Sepharose 4B. Absence of intact IgG and pFc' fragments was demonstrated in each case by double immunodiffusion analysis. Fab' fragments were prepared from these F(ab')₂ preparations by reduction with 0.01 M dithiothreitol and alkylation with a 10% M excess of iodoacetamide.

CBA RBC were coated with Fab and F(ab')₂ fragments of NZB IgG by incubating 10% washed CBA RBC with equal volume of a 1:20 dilution of these preparations (equivalent to a 1:40 dilution of whole serum). Indirect hemagglutination assays were done as described above.

Results

F₁ Sera Agglutinate NZB Erythrocytes. We initially observed that sera from certain (NZB \times CBA)F₁ mice agglutinated the RBC from some Coombs-positive NZB mice. RBC from Coombs-negative NZB mice were never agglutinated by any F₁ sera. Sera from NZB mice that had free-circulating anti-RBC antibodies (indirect Coombs positive) could be used to coat normal MRBC so that they would be agglutinated by the same F₁ sera that agglutinated the donor NZB RBC. The activity of the F₁ sera was age related, as 0 of 47 6–8-wk-old (CBA \times NZB)F₁ sera were able to agglutinate, compared with 15 of 84 4–6-mo-old sera. No difference was seen between F₁ offspring of either CBA or NZB mothers. Agglutinating activity was not seen in the NZB or CBA parents, nor was it found in the unrelated mouse strains BALB/c and C57BL.

TABLE I
Absorption of (CBA × NZB)*F*₁ Serum 57 Agglutinating Activity

Absorbant	Titer ⁻¹ vs. NZB E1 RBC	Titer ⁻¹ vs. NZB C2 RBC
None	12,800	6,400
Sepharose-goat anti-mouse IgG	<10	<10
Sepharose-goat anti-TNP-OVA*	6,400	6,400
Sepharose-goat anti-KLH‡	12,800	3,200
Mouse IgG-coated SRBC§	12,800	1,600
Mouse IgG-coated SRBC	25,600	6,400

* Trinitrophenylated ovalbumin.
‡ Keyhole limpet hemocyanin.
§ Coated with mouse anti-SRBC.
|| Coated using CrCl₃.

TABLE II
Reactivity of (NZB × CBA)*F*₁ Sera with NZB RBC (Reciprocal Titers)

<i>F</i> ₁ serum	NZB RBC											
	C1	C2	D2	E1	F1	F2	H1	H2	J1	J2	K1	K2
42	51,200	3,200	—*	—	—	—	—	—	—	—	—	—
45	1,280	—	1,280	—	—	—	—	—	—	—	—	—
47	12,800	—	51,200	—	—	NT‡	NT	NT	NT	NT	NT	NT
54	102,400	2,560	—	51,200	2,560	1,280	—	—	NT	NT	NT	NT
56	51,200	12,800	—	—	10,280	2,560	3,200	—	3,200	3,200	—	—
57	12,800	51,200	—	12,800	200	—	—	NT	NT	NT	NT	NT
63	640	—	320	—	—	—	NT	NT	NT	NT	NT	NT
66	102,400	51,200	2,560	51,200	—	—	NT	NT	NT	NT	NT	NT
96	—	—	—	12,800	—	—	—	—	—	—	—	—
143	—	—	1,600	—	—	—	—	—	12,800	—	—	—

* <50
‡ Not tested.

Nature of the F₁ Agglutinating Principle. The finding that *F*₁ sera would agglutinate only Coombs-positive MRBC suggested that an anti-antibody might be involved. To test this hypothesis, one positive *F*₁ serum (serum 57) was absorbed in several ways and tested against RBC from two Coombs-positive NZB mice (E1 and C2). As indicated in Table I, this particular combination of sera and cells gave high titers of agglutination. Absorption of the *F*₁ serum with a column of anti-mouse IgG completely removed the agglutinating activity, whereas control columns had no significant effect. This suggested that an antibody was responsible for the activity of the *F*₁ serum. One possibility was rheumatoid factor; however, serum 57 failed to agglutinate SRBC coated either with mouse anti-SRBC antibody or coated nonspecifically with normal mouse IgG (Table I). Mouse IgG bound to Sepharose also failed to remove the agglutinating activity from another *F*₁ serum (not shown). Similar absorption experiments with four other *F*₁ sera gave comparable results.

Specificity of Agglutinating Principle. One of the most striking features of the agglutination phenomenon was the specificity that individual *F*₁ sera showed for individual NZB RBC. A summary of titrations of 10 reacting *F*₁ sera with RBC from each of 12 Coombs-positive NZB mice is shown in Table II. Although certain sera and certain

TABLE III
Lack of Correlation Between Coombs Antibody Subclass and Agglutination by F₁ Serum

Serum	NZB RBC										
	A1	B1	B4	C1	C2	C4	D2	D3	F3	G1	G4
Rabbit anti-IgG	+	+	+	+	+	+	+	+	+	+	+
Rabbit anti-IgG1	+	-	-	+	+	+	+	+	-	-	+
Rabbit anti-IgG2a	+	+	-	+	+	+	+	+	+	-	-
Rabbit anti-IgG2b	-	+	+	+	+	+	+	+	-	+	-
Rabbit anti-IgG3	-	-	-	+	+	+	+	+	+	-	-
F ₁ serum 56	-	-	-	+	+	-	-	+	-	-	+
F ₁ serum 143	-	-	-	-	-	-	+	-	+	-	-

TABLE IV
Agglutination Inhibition by NZB Sera

Inhibiting serum	Reciprocal hemagglutination-inhibition titer
Serum 96 vs. E1 RBC	
C1	—*
C2	—
D2	—
E1	80
F1	—
H1	—
J1	—
Mouse IgG (1 mg/ml)	—
Serum 143 vs. J1 RBC	
C1	—
C2	—
D2	80
E1	—
F1	—
H1	—
J1	320
Mouse IgG (1 mg/ml)	—

* <10.

cells were more reactive than others, the overall pattern suggested individual specificities of a qualitative nature. For example, F₁ serum 42 agglutinated RBC C1 with a titer of 51,200, but failed to agglutinate erythrocytes J1. Conversely, serum 143 failed to agglutinate NZB RBC C1 but agglutinated RBC J1 with a titer of 12,800. It is unlikely that this individual specificity represented the reactivity of F₁ rheumatoid factors with selected subclasses of mouse IgG, as we found no obvious correlation of the pattern of reactivity of NZB erythrocytes with heterologous anti-subclass antisera compared with the pattern of reactivity with the (CBA × NZB)F₁ sera (Table III).

The individual specificity of the reactivities was confirmed by blocking experiments, as shown in Table IV. The reactivity of F₁ serum 96 vs. NZB E1 RBC (see Table II) could be inhibited by serum from mouse E1 but not by serum from six other Coombs-positive NZB mice, the RBC of which did not react with F₁ serum 96. Similarly,

TABLE V
Agglutination of Autoantibody-coated RBC by Whole or Pepsin-digested (NZB × CBA)F₁ Sera

F ₁ Reagent	Uncoated MRBC	Normal MRBC coated with NZB whole sera				Normal MRBC coated with NZB F(ab') ₂			
		NZB-5	NZB-12	NZB-22	NZB-25	NZB-5	NZB-12	NZB-22	NZB-25
		Mouse 2-5 whole serum*	-	-	+	-	+	-	+
Mouse 2-5 F(ab') ₂	-	-	+	-	+	-	+	-	+
Mouse 3-1 whole serum	-	-	+	-	-	-	+	-	-
Mouse 3-1 F(ab') ₂	-	-	-	-	-	-	+	-	-
Mouse 4-3 whole serum	-	+	-	+	-	+	+	+	-
Mouse 4-3 F(ab') ₂	-	+	+	+	-	+	+	+	-
Mouse 4-4 whole serum	-	+	+	+	-	+	+	-	-
Mouse 4-4 F(ab') ₂	-	-	+	-	-	+	+	-	-
Anti κ + anti λ‡	-	+	+	+	+	+	+	-	+
Buffer	-	-	-	-	-	-	-	-	-

* 1:20 dilution.

‡ Mixture of 1:50 dilution of monospecific goat anti-mouse κ and goat anti-mouse λ sera.

TABLE VI
Comparison of F(ab')₂ and Fab' Agglutination of F(ab')₂ and Fab'-coated Mouse RBC

Agglutinating fragment	F ₁ mouse	F(ab') ₂ coating reagent				Fab' coating reagent				
		Saline	NZB-5	NZB-12	NZB-22	NZB-25	NZB-5	NZB-12	NZB-22	NZB-25
F(ab') ₂ *	2-5	-	-	+	-	+	-	-	-	-
	3-1	-	-	+	-	-	-	-	-	-
	4-3	-	+	+	+	-	-	-	-	-
	4-4	-	+	+	+	-	-	-	-	-
Fab'‡	2-5	-	-	-	-	-	-	-	-	-
	3-1	-	-	-	-	-	-	-	-	-
	4-3	-	-	-	-	-	-	-	-	-
Anti κ + anti λ§	4-4	-	+/-	-	-	-	-	-	-	-
		-	+	+	+	+	+	+	+	+

* 1:20 dilution.

‡ 1:10 dilution.

§ 1:50 dilution.

serum 143 could be inhibited in its reactions with NZB J1 RBC by J1 serum, and also by serum from another NZB with which 143 reacted, D2. Thus, the individual NZB specificities seen by the F₁ sera were represented consistently both on the NZB RBC and in their serum.

Agglutination Required Fab'-Anti-Fab' Binding. If the agglutinating phenomenon we observed was due to idiotype-anti-idiotype interactions, the binding function of both the F₁ serum and the NZB Coombs antibody should be contained in the Fab portion of IgG. To demonstrate this, 45% ammonium sulphate fractions of four individual F₁ sera and four NZB sera containing antibodies that reacted with at least some of the chosen F₁ sera were all digested with pepsin and carefully purified free of intact IgG (see Methods). Due to the small volume involved, the resultant reagents could only be tested at a single dilution. The patterns of reactivity of the F₁ F(ab')₂ fragments were in general no different than those of the initial F₁ sera (Table V). In addition, the F₁ serum [or F(ab')₂] equally agglutinated normal MRBC coated with either NZB sera or with the F(ab')₂ fragments purified from these sera (Table V). Furthermore, as shown in Table VI, reduction and alkylation of the F₁ F(ab')₂ completely removed their agglutinating activities. Finally, normal MRBC coated with reduced and alkylated NZB F(ab')₂ (i.e., Fab') were not agglutinated with F₁ F(ab')₂ fragments,

but still could be agglutinated with heterologous antibodies to mouse light chains (Table VI).

Discussion

The present data indicate that certain NZB F₁ hybrids spontaneously produce antibodies directed against idiotypes of NZB Coombs anti-RBC antibodies. Evidence for the idiootype specificity of these antibodies derives from: (a) the selective reactivity of certain F₁ sera with only certain NZB RBC (Table II); (b) the inhibition of F₁ sera-NZB RBC agglutination by only those NZB sera from mice whose RBC were recognized by the F₁ sera (Table IV); (c) the fact that F₁ (F(ab')₂) fragments agglutinated RBC coated with F(ab')₂ fragments of the Coombs antibody, which indicated that Fab-Fab interactions were sufficient (Table V); and (d) the failure of F₁ sera to agglutinate MRBC coated with NZB Fab', which indicated that the F₁ sera recognized determinants near the antigen-binding site of the Coombs antibody. That the observed agglutination might be due to rheumatoid factor was ruled out by the patterns of serum reactivity, the failure to absorb out activity with mouse IgG in a variety of forms, and the specificity for the F(ab')₂ portion of the Coombs antibody molecule. In sum, then, all our data are consistent with the interpretation that the F₁-agglutinating activity is an anti-(anti-RBC)-idiotypic antibody. An unlikely alternative explanation would be that the F₁ sera recognize V-region subgroups (12). This possibility cannot be formally excluded, because it has been shown that such markers on anti-Rh antibodies can be blocked by antigen binding (13, 14). However, we believe it improbable that such anti-V-region subgroup antibodies would spontaneously arise in the F₁, and that they would show the high degree of specificity for individual NZB Coombs antibodies (Tables II and IV).

The observation of anti-idiotypic antibody to an autoantibody is in keeping with several experimental reports (15-17) of the formation of spontaneous anti-idiotype antibody against previously synthesized antibody directed against exogenous antigens. (NZB × CBA)F₁ mice and other NZB hybrids develop hemolytic disease less severe than that of their NZB parent (10). Furthermore, it has been reported that the Coombs antibody found in certain NZB hybrid mice is allotype restricted and therefore probably oligoclonal (18). The small amount and clonal restriction of NZB hybrid anti-RBC autoantibody may be the result of anti-idiotypic regulation (albeit imperfect) of these self-reactive specificities. It is of interest that we have not detected anti-idiotypic antibody against Coombs antibody either in NZB mice or in normal strains.

That a hemagglutination assay is capable of detecting anti-(anti-Coombs) idiotypic antibody may reveal something of the nature of the Coombs antibody. It is likely that one of the two Fab regions of the Coombs antibody must be relatively free for the anti-idiotypic antibody to recognize it. The fact that (anti-RBC)Fab'-coated MRBC can be agglutinated by anti-light chain antisera, but not by F₁ anti-idiotype sera is in accord with this interpretation. Such a mechanism argues for substantial univalent attachment of the Coombs antibody to the RBC surface, a hypothesis consistent with low avidity and the presence of free antibody in the serum. It also implies that the epitopes recognized by the Coombs antibody are widely spaced on RBC. Similar conclusions were reached in studies of the reactivity of idiotypic, V-region framework,

and constant-region antisera with RBC coated with $F(ab')_2$ or Fab' fragments of anti-Rh antibodies (13, 14).

The data thus far collected allow only a rough estimation of the size of the repertoire of the NZB Coombs antibody. Cross-reactivity using anti-idiotypic containing F_1 sera suggests several shared idiotypes; however, the complex pattern of reactivity (Table II) implies more than one anti-idiotypic specificity in some F_1 sera and more than one Coombs idiotypic on some NZB RBC. It will be important to quantitate anti-idiotypic antibody and Coombs antibody levels over time in individual mice to define the relationship between RBC autoantibody levels and the appearance of anti-idiotypic. With the availability of anti-idiotypic reagents to the NZB Coombs antibody, direct functional studies will also be possible to elucidate the role of such antibodies in regulation of autoantibody levels and autoimmune disease.

Summary

The F_1 hybrids of NZB and several normal mouse strains are known to produce less anti-erythrocyte (Coombs) autoantibody and develop a milder hemolytic anemia than their NZB parents. We have found that serum from some $(NZB \times CBA)F_1$ mice agglutinated erythrocytes from certain Coombs-positive NZB mice, often in extremely high titer, whereas other $(CBA \times NZB)F_1$ sera agglutinated erythrocytes from different individual NZB mice. The agglutination was due to antibody, but was not due to rheumatoid factor activity. Because $F(ab')_2$ fragments of the F_1 sera agglutinated erythrocytes coated with $F(ab')_2$ fragments of the appropriate NZB sera, the observed reactivity was probably caused by idiotypic-anti-idiotypic interactions. In addition, because F_1 sera could not agglutinate mouse erythrocytes coated with monovalent NZB Fab' fragments, the recognized idiotypic probably involved the antigen-binding site. Anti-idiotypic antibodies against anti-erythrocyte autoantibodies may play an important role in the regulation of autoantibody formation.

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