IDIOTYPE-ANTI-IDIOTYPE REGULATION

I. Immunization with a Levan-binding Myeloma Protein Leads to the Appearance of Auto-Anti-(Anti-Idiotype) Antibodies and to the Activation of Silent Clones*

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It is now clear that immune responses are regulated by cells and antibodies that are specific for idiotypic determinants as well as by cells and antibodies specific for conventional antigenic determinants (1–4). Indeed, idiotype (Id)[1]-based regulatory systems have the capacity to favor the use of certain clones of lymphocytes, quite independently of the capacity of those clones to bind antigen (5, 6). Id-based regulatory systems also offer the possibility of extended chains of complementary members (i.e., Id, anti-Id, anti-[anti-Id], etc.) and might serve to interlink clones that express similar Id even if they share essentially no antigen-binding activity. The implications of such regulatory networks have been pointed out by Jerne and others (7–9).

Efforts to explore the regulatory consequences of immunity to a given Id, anti-Id, or anti-(anti-Id) have recently been initiated. These studies have demonstrated that such immunity can markedly influence which members of the repertoire of antibodies specific for a given antigen are actually used (10–12).

We and our colleagues have studied an immune system particularly well suited to the examination of Id-based regulation. The system involves the response of BALB/c mice to bacterial levan (BL), a $\beta(2\rightarrow6)$ fructosan with $\beta(2\rightarrow1)$ branch points. The antibodies produced in response to BL consist of two broad families of molecules (13). One group of antibodies is specific for $\beta(2\rightarrow1)$ linkages and reacts with inulin (In), a $\beta(2\rightarrow1)$ fructosan, as well as with BL. Most of these anti-$\beta(2\rightarrow1)$ antibodies express cross-reactive idiotypes (the In-IdX) found on a series of In-binding myeloma proteins (14). The IgG anti-$\beta(2\rightarrow1)$ antibodies made in BALB/c mice express great homogeneity (15, 16). They are essentially all IgG_3 molecules and their isoelectric focusing (IEF) patterns are identical to that of J606, a BALB/c IgG_3 In-binding myeloma protein.[2] The expressed anti-In repertoire is controlled by *Sr-1*, an autosomal gene not linked to the *Igh-C* gene complex (16), and also by *Igh* genes (14, 16).

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[1] *Abbreviations used in this paper*: Ab_1 Id, idiotype of Ab_1; Ab_1, A48 myeloma protein; Ab_2, anti-A48 antibody; Ab_3, anti-(anti-A48) antibody; Ab_4, anti-(anti-[anti-A48]) antibody; BL, bacterial levan; CFA, complete Freund's adjuvant; FCS, fetal calf serum; HA, hemagglutinin; HI, HA inhibition; Id, idiotype; IdX, cross-reactive idiotype; IEF, isoelectric focusing; IFA, incomplete Freund's adjuvant; In, inulin; KLH, keyhole limpet hemocyanin; PFC, plaque-forming cell; RIA, radioimmunoassay.

[2] Stein, K. E., C. Bona, R. Lieberman, and W. E. Paul. Manuscript in preparation.

The other major family of anti-BL antibodies bind $\beta(2\rightarrow6)$ fructosan linkages. These antibodies are more heterogeneous by IEF analysis, although still comparatively simple (16). The Id identified on the $\beta(2\rightarrow6)$ fructosan-binding myeloma proteins ABPC48 (A48 or Ab_1) and UPC10 are not generally detected in the serum of mice immunized with BL (14). However, anti-BL molecules expressing the idiotypic determinant(s) of Ab_1 (Ab_1 Id) are part of the repertoire. They are found in the anti-BL antibody response of congenitally athymic (*nu/nu*) BALB/c mice pretreated with anti-In-IdX antibody, although not in the response of "normal" *nu/nu* mice (17).

In the present experiments, we have immunized BALB/c mice with Ab_1, BALB/c anti-A48 (Ab_2), and BALB/c anti-(anti-A48) (Ab_3), and have examined the effect of such immunity on the total anti-BL response and on the expression of Ab_1 in this response. Interestingly, mice immunized with Ab_2 that have produced Ab_3 express a substantial amount of Ab_1 in their anti-BL response. In contrast, mice producing Ab_2 and anti-(anti-[anti-A48]) (Ab_4) show a generalized inhibition of the total anti-BL response.

Strikingly, Ab_3 expresses idiotypic cross-reactivity with Ab_1 in that Ab_4 binds to Ab_1, and Ab_1 inhibits the binding of radiolabeled Ab_3 to Ab_4. Although this cross-reaction is of relatively low affinity, it is a feature of 60–70% of the Ab_3 molecules detected in this assay. This implies that the Id-anti-Id chain is not unidirectional and strongly suggests that immunization with Ab_2 causes the production of "Ab_1-like" molecules (Ab_3) because it binds to Ab_1-like idiotypic determinants on B lymphocytes.

Finally, we have encountered an immunization procedure in which administration of Ab_1 to BALB/c mice leads to the production of Ab_3. These Ab_3-producing mice resemble Ab_2-immunized mice in that, upon immunization with BL, they produce antibodies expressing the Ab_1 Id. This suggests that functionally important Id-anti-Id chains may be initiated in the course of "normal" immune responses.

Materials and Methods

Mice. BALB/c AnN mice, 8–12 wk old, were used in this study.

Antigens. BL from *Aerobacter levanicum* (ATCC 1552) was obtained as previously described (14).

Myeloma Proteins. Ab_1 and UPC10, $\beta(2\rightarrow6)$ fructosan-binding BALB/c myeloma proteins, and MOPC-384, a *Salmonella tranaroa* lipopolysaccharide-binding BALB/c myeloma protein, were the gifts of Dr. Michael Potter, National Cancer Institute, National Institutes of Health, Bethesda, Md. Ab_1 and MOPC-384 are IgA, κ-proteins; UPC10 is an IgG_{2a}, κ-protein.

Preparation of Anti-Id Antisera. Ab_2 antisera were prepared in A/He mice by immunization with Ab_1 myeloma protein and in BALB/c mice by immunization with an Ab_1-keyhole limpet hemocyanin (KLH) conjugate. Ab_3 antiserum was prepared in BALB/c mice by immunization with a KLH conjugate of affinity chromatography-purified BALB/c Ab_2 Id antibody. Similarly, Ab_4 antiserum was prepared by immunization of BALB/c mice with a KLH conjugate of purified BALB/c Ab_3 antibody. Purification of antibodies on Sepharose 4B-anti-Id columns, coupling of affinity chromatography-purified antibodies to KLH, and immunization schedules are as previously described (18).

Preparation of Coupled Sheep Erythrocytes (SRBC). An *O*-steroyl derivative of BL was prepared according to the technique of Hämmerling and Westphal (19) and then coated to SRBC as previously described (14). SRBC were coated with myeloma proteins and various affinity chromatography-purified antibodies by the chromic chloride method, using a concentration of 0.6–1.0 mg/ml of appropriate protein.

Determination of Hemagglutinin (HA) Titers. HA titers of antibodies specific for BL and for Id were determined in microplates by using SRBC coated with *O*-steroyl BL, myeloma proteins,

or affinity chromatography-purified antibodies. The titer recorded is $1/\log_2$ of the highest dilution of antisera giving agglutination.

Determination of Serum Id. An HA-inhibition (HI) method described previously (13, 14) was used to test sera for the presence of Id. The Ab_1 Id was studied by using both A/He and BALB/c Ab_2 antisera and Ab_1-coated SRBC. BALB/c Ab_3 antisera and affinity chromatography-purified Ab_2 antibody-coated SRBC were used to detect Id of Ab_2 antibodies. BALB/c Ab_4 antisera and affinity chromatography-purified Ab_3 antibody-coated SRBC were used to detect Id of Ab_3 antibodies.

Radioimmunoassay (RIA). Purified Ab_3 antibodies and Ab_1 myeloma protein were tritiated according to the method of Wilder et al. (20). Ab_3 antibodies were labeled with ^{125}I by the chloramine T method (21). The ability of BL to bind to ^3H-Ab_1 myeloma protein was determined in microtiter plates that had been incubated for 18 h with 50 μg BL, followed by three washings with saline. The plates were incubated for 1 h with 50% fetal calf serum (FCS) and, after three washings, incubated for 3 h with ^3H-Ab_1 (10,000 cpm/50 μl). The ability of ^3H-Ab_1, ^3H-Ab_3, and ^{125}I-Ab_3 to bind Ab_2 antibodies or Ab_4 immunoglobulin (Ig) was determined with the use of microplates that had been incubated with various amounts of purified Ab_2 antibodies or with the Ig fraction of Ab_4 antiserum for 18 h at 4°C. After washing and incubation with 50% FCS as described above, the plates were incubated for 3 h with ^3H-Ab_1, ^3H-Ab_3 (~5,000 cpm/50 μl), or ^{125}I-Ab_3 (39,000 cpm/50 μl). To determine the capacity of various proteins or sera to inhibit the binding of radiolabeled Ab_3 antibodies or of ^3H-Ab_1 to Ab_2 or Ab_4, plates coated with Ab_2 antibodies or with Ab_4 Ig were incubated for at least 3 h with various dilutions of inhibitor and washed before the addition of radiolabeled antibodies (22). Radioactivity on plates was measured in a liquid-scintillation spectrometer.

Plaque-forming Cell (PFC) Assay. The number of PFC-secreting antibodies specific for BL was determined according to a previously described technique (13). PFC secreting anti-BL antibodies carrying the Ab_1 Id were enumerated by the addition of BALB/c (1:300) or A/He (1:150) anti-A48 Id antisera to agarose. The number of PFC obtained in the presence of these sera was subtracted from that obtained if no inhibitory serum was present to give the number of Ab_1 Id^+ anti-BL PFC.

Results

Characterization of Id. A series of anti-Id antibodies mimicking an Id-anti-Id chain, or pathway, comprising Ab_2, Ab_3, and Ab_4 antibodies was induced by immunization of syngeneic mice with KLH conjugates of Ab_1, Ab_2, and Ab_3 antibodies, respectively. The presence of these anti-Id antibodies was determined by HA and RIA assays. Thus, Ab_2 antiserum agglutinated both Ab_1-SRBC and Ab_3-SRBC (Table I). The Ab_1-SRBC agglutinating activity of Ab_2 antiserum was completely removed by

TABLE I

HA Titers of Ab_2, Ab_3, and Ab_4 Antisera

Sera	HA titers* on SRBC coated with						
	Ab_1			Ab_2		Ab_3	
	Unadsorbed	Ab_1‡	Ab_3	Unadsorbed	Ab_2	Unadsorbed	Ab_3
Nonimmune	0	ND§	ND	0	ND	0	ND
Anti-A48-KLH (Ab_2)	10	0	2	0	0	9	2
Anti-(anti-A48)-KLH (Ab_3)	0	0	ND	10	0	0	0
Anti-(anti-[anti-A48])-KLH (Ab_4)	3	2	0	0	0	4	0
Rabbit anti-mouse κ	>12	ND	ND	>12	ND	>12	ND

* HA titers in $\log_2$ units.

‡ Sera adsorbed with Sepharose 4B conjugated with Ab_1, purified Ab_2, or purified Ab_3.

§ Not done.

absorption of the antiserum with Ab_1-Sepharose beads and largely, although not completely, with Ab_3-Sepharose beads. Ab_3 antiserum agglutinated Ab_2-SRBC.

The antiserum containing Ab_4 antibodies agglutinated Ab_3-SRBC, as expected. However, it also agglutinated Ab_1-SRBC. Interestingly, the capacity of Ab_4 serum to agglutinate Ab_1-SRBC was completely removed by absorption with Ab_3-Sepharose beads but only partially removed by Ab_1-Sepharose beads (Table I). These results indicate that Ab_4 can bind to Ab_1 but suggest that this binding is of relatively low affinity. To study this in greater detail, we examined the binding of ^{125}I-Ab_3 by both Ab_2- and Ab_4-coated microplates and compared this with the binding of ^{3}H-Ab_1 by similar microplates (Table II). We observed that ^{125}I-Ab_3 and ^{3}H-Ab_1 bound to both Ab_2 and Ab_4 plates. Controls using BALB/c Ig and Ab_3 plates indicated the specificity of this binding. The ratio of amount of ^{3}H-Ab_1 bound by the Ab_4 and Ab_2 plates and the ratio of ^{125}I-Ab_3 bound by these plates were similar. Because all Ab_1 and Ab_3 molecules should be specific for Ab_2, the similarity of this ratio suggests that the fraction of the Ab_1 molecules specific for Ab_4 is similar to the fraction of Ab_3 molecules specific for Ab_4. Furthermore, Ab_1 can inhibit the binding of 60–70% of radiolabeled Ab_3 to Ab_4 plates. Control proteins such as MOPC-384 and UPC10 cause little or no inhibition. One such inhibition experiment is illustrated in Fig. 1. Comparable results have been obtained in three additional experiments, using ^{3}H-Ab_3 as well as ^{125}I-Ab_3.

The relative affinity of Ab_4 for Ab_1 can be estimated from the concentration of Ab_1 required to inhibit binding of ^{125}I-Ab_3 or of ^{3}H-Ab_1 to Ab_4 microplates (Table III). We found that 2.2 μg/ml of Ab_1 was needed to inhibit 50% of the binding of ^{125}I-Ab_3 to Ab_4 plates, whereas only 0.006 μg/ml of Ab_3 was needed for comparable inhibition. This suggests that Ab_4 binds to Ab_3 with substantially greater affinity than it binds to Ab_1. On the other hand, a concentration of 0.03 μg/ml of Ab_1 was sufficient to inhibit 50% of the binding of ^{3}H-Ab_1 to Ab_4 microplates. This is consistent with a relative affinity of the binding of Ab_4 to Ab_1, which is greater than that indicated by Ab_1 inhibition of binding of Ab_4 to Ab_3 plates, although still lower than the relative affinity of binding of Ab_4 to Ab_3. At this time, we cannot fully reconcile these differing estimates of the relative affinity of Ab_4 for Ab_1.

The interactions of these anti-Id antibodies were also studied by an HI assay. The agglutination of Ab_1 SRBC by A/He and BALB/c Ab_2 was inhibited by Ab_1 myeloma protein (Table IV). The agglutination of Ab_1-coated SRBC by BALB/c Ab_2 antibod-

TABLE II

Binding of Radioactive Ab_1 and Ab_3 to Plates Coated with Ab_2 and Ab_4

Microplates coated with	Bound ligand	
	^{3}H-Ab_1	^{125}I-Ab_3
	cpm	
BALB/c Ig (10 μg/ml)	105	1,130 ± 111
Ab_3 (10 μg/ml)	110 ± 8	ND*
Ab_2 (10 μg/ml)	1,386 ± 142	22,944 ± 1,374
Ab_4 (150 μg Ig/ml)	524 ± 19	8,173 ± 271
Specific binding to Ab_4/specific to Ab_2‡	0.33	0.32

* Not done.

‡ Binding to Ab_4 plate − binding to control plates/binding to Ab_2 plate − binding to control plates.

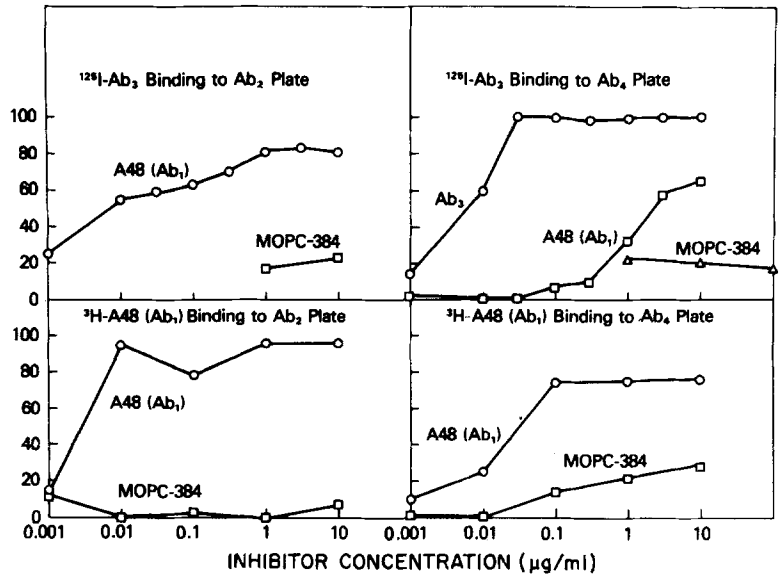

FIG. 1. Inhibition of binding of ^{3}H-Ab$_1$ and ^{125}I-Ab$_3$ to Ab$_2$ and Ab$_4$ microplates. Microplates were coated with Ab$_2$ (10 μg/ml purified antibody) and Ab$_4$ (150 μg/ml Ig) as described in Materials and Methods. Plates were preincubated with various concentrations of Ab$_1$, Ab$_3$, or MOPC-384, and washed. ^{125}I-Ab$_3$ or ^{3}H-Ab$_1$ were then added and binding measured. Percent inhibition was calculated as follows:

$$\% \text{ inhibition} = 100\left(1 - \frac{\text{cpm bound after addition of inhibitor} - \text{cpm bound to control plate}}{\text{cpm bound with no inhibitor} - \text{cpm bound to control plate}}\right).$$

Control plates for ^{3}H-Ab$_1$ were coated with BALB/c Ig and with Ab$_2$ Ig; control plates for ^{125}I-Ab$_3$ were coated with BALB/c Ig. The cpm bound to control plates and to Ab$_2$ and Ab$_4$ plates are presented in Table II.

TABLE III

Inhibition of Binding of Radioactive Ab$_1$ and Ab$_3$ to Plates Coated with Ab$_2$ and Ab$_4$

Microplate coated with	Ligand	[Inhibitor] for 50% inhibition		
		Ab$_1$	Ab$_3$	MOPC-384
			μg/ml	
Ab$_2$ (10 μg/ml)	^{3}H-Ab$_1$	0.003	ND*	>100
Ab$_2$	^{125}I-Ab$_3$	0.007	ND	>100
Ab$_4$ (150 μg Ig/ml)	^{3}H-Ab$_1$	0.03	ND	>100
Ab$_4$	^{125}I-Ab$_3$	2.2	0.006	>100

* Not done.

ies was also inhibited by Ab$_3$. On the other hand, Ab$_3$ did not inhibit the ability of A/He anti-Ab$_1$ to agglutinate Ab$_1$-SRBC. These results indicate that A/He Ab$_2$ lacks idiotypic determinant(s) borne by BALB/c Ab$_2$ antibodies or, as discussed later, that A/He Ab$_2$ is directed to different idiotypic determinants on Ab$_1$ than are BALB/c Ab$_2$ antibodies.

The agglutination of BALB/c Ab$_2$-SRBC by Ab$_3$ was inhibited by BALB/c Ab$_2$ and by Ab$_4$ (Table IV). The inhibitory ability of BALB/c Ab$_2$ was shared by BAB.14 and C.B20 Ab$_2$ antibodies but not by A/He and AL/N antibodies (data not shown).

TABLE IV
HI Titers of Ab_2 Id, Ab_3 Id, and Ab_4 Id Antisera

Inhibitory serum	A/He Ab_2 + Ab_1-SRBC	BALB/c		
		Ab_2 + Ab_1-SRBC	Ab_3 + Ab_2-SRBC	Ab_4 + Ab_3-SRBC
	log_2 U			
Nonimmune serum	0	0	0	0
Ab_1 (3 mg/ml)	8	8	0	1
Anti-A48-KLH (Ab_2)	0	0	8	0
Anti-(anti-A48)-KLH (Ab_3)	0	8	0	7
Anti-(anti-[anti-A48]-KLH) (Ab_4)	0	0	6	0

TABLE V
Anti-BL HA Titer

Sera	Number of mice	HA titer (BL-coated SRBC)
		log_2 U
Normal	2	2.5
Ab_1 (3 mg/ml)	—	11.0
Anti-A48-KLH (Ab_2)	5	1.8
Anti-(anti-A48)-KLH (Ab_3)	9	2.1
Anti-(anti-[anti-A48Id])-KLH (Ab_4)	3	2.3

The agglutination of Ab_3-SRBC by Ab_4 was inhibited by Ab_3, as anticipated. In addition, it was inhibited by high concentrations of Ab_1. This is similar to the finding that relatively high concentrations of Ab_1 are required to inhibit the binding of radiolabeled Ab_3 to Ab_4-coated plates. Both results indicate that Ab_4 recognizes Ab_3 and Ab_1, although its affinity for Ab_1 is lower than that for Ab_3.

The Ab_2, Ab_3, and Ab_4 antisera did not react with BL to any greater extent than did normal serum (Table V). The low anti-BL HA titer of these antisera and of the sera of normal nonimmunized BALB/c mice presumably represents the presence of "natural" anti-BL antibodies due to immunization with environmental antigens (22, 23). This phenomenon is common for several polysaccharide antigens borne by saprophytic intestinal flora.

These results indicate that through intentional immunization a set of complementary anti-Id antibodies can be produced and that each differs from the others. In particular, Ab_3 differs from Ab_1 in that it lacks the capacity to agglutinate BL-SRBC (Table V) and to bind to BL-coated plates (data not shown). Ab_4 differs from Ab_2 in that its ability to agglutinate Ab_1-SRBC is ineffectively removed by Ab_1-Sepharose and its ability to bind both radiolabeled Ab_3 and Ab_3-SRBC requires high concentrations of Ab_1 for inhibition. Nonetheless, this analysis indicates that the majority of Ab_3 molecules express an IdX which cross-reacts with an Id found on Ab_1. This result is quite unexpected in a "linear" Ab_1-Ab.i2-Ab_3-Ab_4 system, but is comparable to a previous result of Wikler et al. (24) in a similar rabbit system.

Immunization with Ab_1 Causes the Production of Ab_3. The previous experiments demonstrate the characteristics of members of an Id-anti-Id chain induced by intentional immunization with a KLH conjugate of the immediately preceding member of that

chain. In order to determine whether the chain normally develops beyond the first step after immunization with native Ab_1, we immunized BALB/c mice with Ab_1, using two different protocols, and tested for the appearance of both Ab_2 and Ab_3.

One group of seven mice was immunized eight times with Ab_1 not conjugated to KLH. The immunization protocol consisted of an initial immunization in complete Freund's adjuvant (CFA), followed by one immunization in incomplete Freund's adjuvant (IFA), and then by six weekly immunizations in saline. These mice were bled weekly after the completion of the entire protocol. Each of the mice of this group made Ab_2, which was found over an 8-wk period after completion of immunization. Ab_3 was not detected in any of these mice during this period (Fig. 2).

In five of the mice of this group, the Ab_2 observed differed from the Ab_2 found in BALB/c mice after immunization with Ab_1 conjugated to KLH (see Table I). The Ab_2 of these five mice failed to agglutinate Ab_3-SRBC. In two of the seven mice, the sera could agglutinate both Ab_1-SRBC and Ab_3-SRBC, although the titers varied substantially during the 8-wk period. Furthermore, these two mice may also have produced Ab_4. We suggest this because adsorption of their sera with Ab_1-Sepharose only partially removed the ability to agglutinate Ab_3-SRBC, whereas absorption with Ab_3-Sepharose removed it completely (Table VI).

A second group consisting of four mice was immunized twice with Ab_1, once in CFA and once in IFA. These mice expressed a low serum titer of hemagglutinating Ab_2 antibodies 1 wk after the completion of immunization (Fig. 3). By 3 wk after completion of immunization, three of these mice had no detectable Ab_2 serum titer and one mouse had a very low titer in its serum. At this time, each of these mice displayed a significant Ab_3 titer. By 5 wk, the Ab_3 titer had fallen in all but one of the mice and was replaced by Ab_2, but by 8 wk, Ab_3 was again found in the absence of detectable Ab_2. Thus, mice immunized in this way with Ab_1 show a prompt appearance of Ab_2 and then show an inverse fluctuation in Ab_2 and Ab_3 levels. However, we have not examined the second peak (at 5 wk) of Ab_2 HA activity to determine whether it represents an Ab_2 or Ab_4, or a mixture of Ab_2 and Ab_4.

Our data indicate the appearance of auto-Ab_3 and possibly of auto-Ab_4 in mice immunized with Ab_1. This is consistent with the development of an extended chain of anti-Id reactions in individual animals.

Activation of Ab_1 Id^+ Anti-BL Precursors in Mice Immune to Ab_2. Normal BALB/c mice, as well as BALB/c mice that had been immunized with Ab_2 Id antibodies, have low serum anti-BL HA titers and a small number of anti-BL PFC (Table VII). No Ab_1

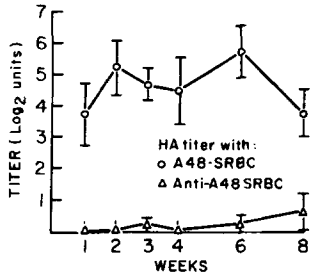

FIG. 2. HA titer of sera of BALB/c mice immunized with Ab_1 not conjugated to KLH, according to the eight-immunization protocol. HA titer with A48-SRBC (Ab_1) is interpreted to represent Ab_2 titer; HA titer with anti-A48-SRBC (Ab_2-SRBC) is interpreted to represent Ab_3 titer.

TABLE VI

*HA Titers of Serum from BALB/c Mice That Had Received Eight Immunizations with Ab_1**

Serum		HA titer	
Time after completion of immunization	Adsorption‡	Ab_1-SRBC	Ab_3-SRBC
wk		*log_2 U*	
1	—	4	6
	Ab_1	1	4
5	—	4	4
	Ab_1	1	3
	Ab_3	2	0
	MOPC-384	3	3

* Sera from two mice that produced Ab_2 antibody that agglutinated Ab_3-SRBC were tested.

‡ Serum (0.2 ml) was adsorbed with 0.1 ml of Sepharose 4B beads to which the indicated protein had been conjugated.

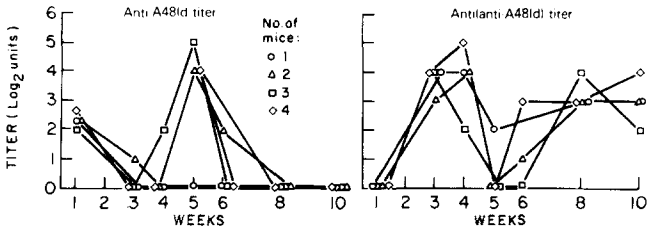

FIG. 3. HA titer of sera of four individual BALB/c mice immunized with Ab_1, not conjugated to KLH, according to the two-immunization protocol. Anti-A48Id (Ab_2 Id) titer represents HA titer with Ab_1-SRBC; anti-(anti-A48Id) (Ab_3 Id) HA titer represents HA titer with Ab_2-SRBC. Serum titers of individual mice are denoted as follows: mouse 1-○; mouse 2-△; mouse 3-□; mouse 4-◇.

TABLE VII

Anti-BL Response of Mice Immune to Ab_2

Pretreatment	Immunization with BL*	Number of mice	Response				
			HA (BL-SRBC)	HI Ab_1-SRBC + BALB/c Ab_2	RIA‡ Ab_1	BL PFC	
						Total PFC/spleen	Ab_1 Id
					μg/ml		%
None	−	2	2.5 ± 0.7	0	<0.1	24 ± 11	4 ± 5
Ab_2-KLH	−	4	3.8 ± 0.3	0	<0.1	44 ± 33	5 ± 3
None	+	5	6.0 ± 1.2	0	<0.1	330 ± 34	9 ± 6
Ab_2-KLH	+	9	7.8 ± 3.2	1.8 ± 0.7	4.8 ± 1.6	765 ± 433	32 ± 20
Ab_1 (8 immunizations)	+	6	3.3 ± 1.1	0.9 ± 1.2	<0.1	435 ± 102	18 ± 10
Ab_1 (2 immunizations)	+	4	10.8 ± 1.3	2.8 ± 0.5	10.3 ± 1.8	1,192 ± 180	47 ± 13

* Mice were immunized with 10 μg BL 10 wk after completion of pretreatment. Responses were measured 5 d after BL immunization.

‡ Results calculated based on the capacity of various concentrations of Ab_1 to inhibit binding of ^{3}H-Ab_1 to plates coated with purified Ab_2 (10 μg/ml).

Id^+ antibodies could be detected by either HI, RIA, or PFC assays in these mice. Immunization with BL leads to the development of a significant titer of anti-BL antibodies and anti-BL PFC. As we have previously shown, Ab_1 Id^+ anti-BL antibodies are not detected in this response (17). BALB/c mice that had been immunized with Ab_2-KLH developed a vigorous response upon immunization with BL, and a substantial fraction of their anti-BL antibodies expressed the Ab_1 Id. Immunized mice producing Ab_2 (and possibly Ab_4), as a result of immunization with Ab_1 using the eight-injection protocol, developed an anti-BL response of lower magnitude than did normal mice immunized with BL. They failed to express Ab_1 Id. In contrast, mice that had produced Ab_3 as a result of immunization with Ab_1 using the two-injection protocol expressed a marked increase in their total anti-BL response and developed a considerable amount of Ab_1 Id^+ anti-BL antibodies upon immunization with BL. Thus, in these animals, the presence of immunity to Ab_2 before immunization with BL was associated with the expression of the Ab_1 Id in the anti-BL response. In contrast, the presence of Ab_2 itself was associated with a failure of expression of Ab_1 Id and a decrease in the total anti-BL response.

Further, we examined the kinetics of the response to BL of mice producing Ab_2, Ab_3, and Ab_4 as a result of immunization with KLH conjugates of Ab_1, Ab_2, and Ab_3, respectively. As noted above, Ab_2 mice immunized with BL developed a lower anti-BL response compared to BL-immunized normal BALB/c mice. The titer in such Ab_2 mice did not approach normal levels until 20 d after immunization (Fig. 4). Ab_3 mice immunized with BL exhibited a total anti-BL response quite similar in amount to that of normal mice. Most interestingly, Ab_4 mice showed a degree of suppression of their total anti-BL response that was at least as profound as that of Ab_2 mice. These results suggest that Ab_2 and Ab_4 express a functional similarity and reinforce serologic evidence that indicates that Ab_4 resembles Ab_2 in that it binds Ab_1.

Discussion

Our results demonstrate that a chain of complementary anti-Id antibodies comprising four members (Ab_1, Ab_2, Ab_3, and Ab_4) can be generated by immunization of syngeneic mice with KLH conjugates of the immediately preceding member of the chain. Thus, these results formally demonstrate that in syngeneic animals an extended Id-anti-Id regulatory system is possible. A similar four-member chain (Ab_1–Ab_4) has

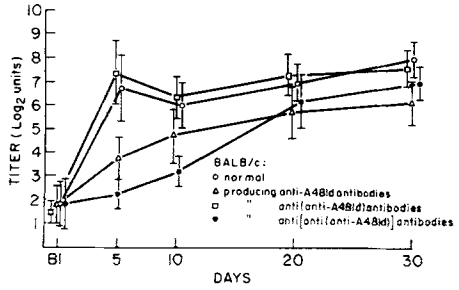

FIG. 4. Anti-BL HA titers of sera of BALB/c mice preimmunized with Ab_1 KLH (Δ-producing anti-A48Id [Ab_2 Id] antibodies); anti-A48-KLH (□, producing anti-[anti-A48Id] [Ab_3] antibodies); or anti-(anti-A48) [Ab_3]-KLH (●-producing anti-[anti-(anti-A48)] [Ab_4]) antibodies. Titers of nonpreimmunized mice designated with O. All mice were immunized with 10 μg BL 10 wk after completion of preimmunization. Each point represents average ± SD of three mice.

been described by Wikler et al. (24) in allotype-matched rabbits, and three-member chains (Ab_1–Ab_3) have been described previously in syngeneic mice (18) and allotype-matched rabbits (10).

We observed that immunization with unconjugated Ab_1, under certain circumstances, leads to the occurrence of auto-Ab_3, as well as auto-Ab_2. Furthermore, our results suggest that such immunization may occasionally lead to the production of auto-Ab_4. These observations indicate that the chain developed by stepwise immunization with KLH conjugates can be observed under normal conditions. Prior evidence that initial events in chain formation occur has been obtained. Thus, it has been reported that the immunization with conventional antigens leads to the occurrence of auto-anti-Id antibodies (25–27), auto-anti-Id PFC (13, 28), and auto-anti-Id-binding cells (29–31). Naturally occurring Id-specific T cells (2), as well as Id-specific T cells generated after immunization with Id-bearing antibodies (30, 32), with Id-bearing antibodies coupled to syngeneic cells (31, 33), or with anti-Id antibodies (30, 34), have also been described.

The presence of immunity to Ab_1, Ab_2, or Ab_3 in mice has a significant effect on the amount and nature of the antibody response to BL, which is the antigen that is the putative initiator of the Id-anti-Id chain. Thus, in mice that have produced Ab_3, either because of immunization with KLH-Ab_2 or after responses to Ab_1, we observed that the primary response to BL contained a major fraction of antibodies that express the Ab_1 Id. This Id is not detected in the response to BL of normal or *nu/nu* mice of the Igh^a type, nor is it found in the response of intact BALB/c mice that have been pretreated with antibody to the In-IdX (35). The only previous situation in which we observed the appearance of measurable amounts of the Ab_1 Id in anti-BL antibodies was in the response to BL of *nu/nu* BALB/c mice that had previously been treated with anti-In-IdX (17). If such mice are given T lymphocytes at the time of BL immunization, the Ab_1 Id is not observed in their antibody response. This suggests that the expression of Ab_1 Id is under some type of T lymphocyte-dependent control and that the Ab_1 Id is probably not a major member of the anti-BL repertoire, because suppression of the anti-$\beta(2\rightarrow1)$ component of the anti-BL response is required for its expression even in the absence of T cells.

In this context, it is interesting to consider why immunity to Ab_2 should lead to the development of an Ab_1 Id^+ anti-BL response. One obvious possibility is that Ab_3, which is anti-(anti-A48Id), could eliminate suppressor T lymphocytes that express Ab_2 (anti-A48Id) specificity. Indeed, we have shown in the MOPC-460-trinitrophenyl (TNP) system that mice immunized with Ab_2 (anti-MOPC-460) lack 460Id-specific suppressor T cells, which are found in normal mice and which regulate the activation of the precursors of $460Id^+$ anti-TNP antibody-secreting cells (12). However, if elimination of suppressor T cells specific for Ab_1 Id (i.e., Ab_2-bearing suppressors) is the only reason that immunity to Ab_2 leads to expression of Ab_1, one might anticipate that *nu/nu* mice immunized with BL would express substantial Ab_1 Id in their anti-BL response. As we noted above, this is not the case. A second possibility is that Ab_3 could be regarded as consisting of the entire set of antibodies that can react with Ab_2. Because Ab_2 is anti-A48Id, Ab_3 would be expected to include Ab_1. However, mice immunized with Ab_2-KLH, but not with BL, have no greater anti-BL serum antibody titer than do normal mice. Thus, Ab_1 cannot be a very important component of Ab_3. Nonetheless, precursors of Ab_1 (i.e., $A48Id^+$ anti-BL) antibody-secreting cells appear

to have been primed by immunization with Ab_2, because BL immunization leads to their activation and to the appearance of Ab_1 Id in the anti-BL response of Ab_2-KLH primed mice, and of those Ab_1-immunized mice that produced Ab_3. It seems likely to us that both of these mechanisms contribute to the regulation of the Ab_1 Id^+ anti-BL response in mice that have produced Ab_3.

One of the most interesting results to emerge from these studies and those of Wikler et al. (24) is the apparent asymmetry of the chain of anti-Id. Thus, we observed that Ab_4 resembles Ab_2 in that both bind to Ab_1 and to Ab_3, and that both lead to a suppression of anti-BL antibody response. Although Ab_4 and Ab_2 are not identical, because their affinities for Ab_1 are different, it is particularly important to note that the majority of the Ab_4 molecules tested in our assays bind Ab_1. This was shown by the fact the Ab_1 inhibited the binding of >60% of radioactive Ab_3 by an Ab_4 plate and by the capacity of high concentrations of Ab_1 to inhibit hemagglutination of Ab_3-SRBC by Ab_4. In contrast, Ab_3 and Ab_1 differ in that Ab_3 fails to recognize BL, whereas Ab_1 is a BL-binding myeloma protein. In the studies of Wikler et al. (24), a four-member chain was initiated with antibody to the polysaccharide from *Micrococcus lysodeikticus*. In these experiments, Ab_4 and Ab_2 both bound to Ab_1, but Ab_3 failed to bind to *M. lysodeikticus* polysaccharide antigen.

It is possible that the chain is only seemingly asymmetrical and the appearance of asymmetry is due to a relatively low affinity of Ab_1 for the polysaccharide antigens (BL and *M. lysodeikticus* polysaccharide) used in these systems. If Ab_3 were cross-reactive with Ab_1 but had an energy of binding which was 1 or 2 kcal lower than Ab_1, it might fail to bind antigen under the conditions used. In contrast, it is likely that the Ab_2-Ab_1 interactions, representing antibody-protein antigen interactions, are of relatively high affinity, and that the binding by cross-reactive Ab_4 would be detectable even if the energy of interaction of Ab_4 for Ab_1 was considerably less than that of Ab_2 for Ab_1. In fact, there are three reported instances of Ab_3 displaying some Ab_1-like antigen-binding activity. These are in systems in which the initiating antigens are insulin-binding receptor (36), retinol-binding receptor (36), and tobacco mosaic virus capsid protein (37). However, our observation that the majority of Ab_4 molecules bind Ab_1 suggests that this is an important feature of Id-anti-Id chains.

Consequently, we wish to suggest an explanation for the observed lack of symmetry in this chain of anti-Id which represents a distinctive view of the Id-anti-Id regulatory system. In considering this proposal, we first wish to outline what we believe to be a major difficulty in certain of the network concepts. In the most simplified and extreme case, one imagines a physiologic network to be initiated by the antibody produced in response to antigenic determinants. Such antigenic determinants, in accordance with the Jerne nomenclature, are termed epitopes (7). Anti-epitope antibodies (Ab_1) express Id that are currently recognized to actually consist of a series of distinctive determinants located on different portions of the variable region. These individual idiotypic determinants have been designated idiotopes. Because each Ab_1 may express at least two and probably several idiotopes (38), there should be at least two and probably several Ab_2 generated in response to each Ab_1 (39). Similarly, each Ab_2 should stimulate the appearance of several Ab_3 and each of these, in turn, should stimulate the appearance of several Ab_4. This would suggest that the number of distinct molecules in the set of Ab_2, Ab_3, . . . Ab_n antibodies must be very much larger than the set of distinct Ab_1 antibodies, and that the bulk of the antibody repertoire is

devoted to recognition of idiotopes. This concentration on idiotopes might be more illusory than real if most anti-idiotope antibodies also recognized some epitope, presumably because of a structural similarity between the particular idiotope and the particular epitope (40). Thus, the set of anti-idiotope antibodies and anti-epitope antibodies would be the same set. This concept, in its extreme form, is tenable only if the expressed idiotopes of Ig cross-react with virtually every possible antigenic determinant, including polysaccharides, lipids, and simple organic haptens. We regard this as quite unlikely in chemical terms. A second finding not well explained by linear or expanding idiotope-anti-idiotope chains is the capacity of Ab_4 to bind Ab_1.

We wish to propose a very different idea to explain idiotope-anti-idiotope interactions (Fig. 5). In our model, the set of anti-epitopes (Ab_1) and anti-idiotopes (Ab_2) are functionally distinct. Thus, we suggest that, although antibodies to conventional antigenic determinants bear many idiotopes, only a limited number of these idiotopes function in eliciting responses in autologous or syngeneic animals. We designate such determinants "regulatory idiotopes." Thus, the anti-idiotope antibodies that develop in autologous or syngeneic systems will be directed to a limited number of determinants. It should be obvious that Ab_2 idiotopes will be similar to each other in that each binds the Ab_1 Id. They may be sufficiently heterogeneous to display a large number of regulatory idiotopes, each expressed on only a small fraction of the Ab_2 molecules. Alternatively, the set of Ab_2 may differ from the set of Ab_1 in that it fails to express regulatory idiotopes. In either case, intentional or natural immunization with Ab_2 will be relatively efficient in activating those B cells that bear the regulatory

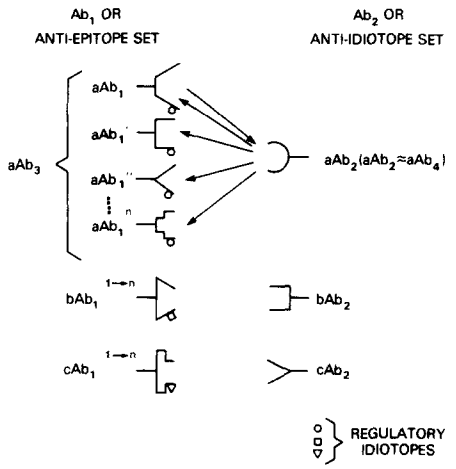

FIG. 5. The regulatory idiotope concept. Ab_1 of the a type (*a* Ab_1) represents an anti-epitope antibody which possesses a regulatory idiotope (the *a* Ab_1 idiotope). Immunization of syngeneic animals with *a* Ab_1 causes the production of the complementary anti-idiotope antibody *a* Ab_2, which displays any of its individual idiotopes at low concentration or because *a* Ab_2 lacks a regulatory idiotope. Immunization of syngeneic mice with *a* Ab_2 activates B lymphocytes that bear receptors that express the *a* Ab_1 idiotope or related idiotopes (i.e., *a* Ab_1', *a* Ab_1'' . . . *a* Ab_1^n). Because *a* Ab_2 lacks a regulatory idiotope, immunization with it fails to cause activation of anti-(anti-idiotope) antibodies. Thus, *a* Ab_3 is really the collection of molecules that bear the *a* Ab_1 and related idiotopes. Immunization with *a* Ab_3 thus elicits the production of antibodies specific for the *a* Ab_1 family of regulatory idiotopes. Thus, *a* Ab_2 and *a* Ab_4 will resemble each other. The *a* Ab_1-*a* Ab_2 system is one of a series of distinct complementary systems which include the *b* Ab_1-*b* Ab_2, *c* Ab_1-*c* Ab_2, . . . *z* Ab_1-*z* Ab_2 systems.

idiotopes for which Ab_2 is specific (the Ab_1 regulatory Id), but will cause limited induction of anti-Ab_2, either because no single Ab_2 Id is present at sufficiently high concentration to stimulate anti-Id antibodies, or because Ab_2 molecules do not display regulatory Id. Therefore, for purposes of immunization, Ab_2 functions mainly as an anti-Id antibody rather than as an antigen. The antibodies raised against Ab_2 should resemble each other in that each will express the Ab_1 regulatory idiotope or determinants that cross-react with this idiotope. We suggest that the number of such idiotopes is relatively small and that antibodies of very different specificities may share cross-reactive regulatory idiotopes. Thus, Ab_3 should more correctly be regarded as a collection of molecules possessing the Ab_1-type regulatory idiotope (i.e., Ab_1, Ab_1' . . . Ab_1^n), of which Ab_1 will be only one member of a relatively large family. Thus, the antigen-binding activity of Ab_3 might be difficult to detect. On the other hand, immunization with antigen (e.g., BL) should reveal that Ab_1-bearing epitope-specific precursors have been "primed" by Ab_2 immunization. That is, Ab_1 should be represented in the anti-epitope response to a larger extent than in animals not immunized with Ab_2. When Ab_3 antibodies are used in immunization, the resultant Ab_4 should bind Ab_1 because Ab_1 and Ab_3 express cross-reactive regulatory idiotopes. Thus, Ab_4 should be similar to Ab_2, because both are directed against the Ab_1 regulatory idiotope.

This model is consistent with several findings that have been reported previously. First, the concept that in syngeneic immunizations only the regulatory idiotopes are antigenic is consistent with the Lieberman-Potter rule (42). This states that it is much more difficult to produce anti-Id antibodies against myeloma proteins when the mouse immunized is of the same *Igh-C* type as the donor of the myeloma protein than when the immunized animal and the donor are of different *Igh-C* types. Second, the concept that Ab_3 consist of molecules bearing idiotopes cross-reactive with Ab_1 but that the serum of Ab_3 mice has little antigen (e.g., BL)-binding activity is consistent with reports that Id, such as the A5AId, may be found on molecules capable of binding a given antigen (e.g., Streptococcus A carbohydrate), as well as molecules that have no detectable capacity to bind that antigen (43).

Obviously, a model of this sort is best stated in extreme terms both for ease of description and precision of predictions. Nonetheless, one must consider certain problems. First, Ab_4 binds Ab_3 with much higher affinity than it binds Ab_1. Thus, the Ab_1 family (i.e., those molecules that bear regulatory idiotopes cross-reactive with that of Ab_1) must be moderately heterogeneous. For simplicity, one would prefer a very limited degree of heterogeneity in the Ab_1 idiotope. However, this heterogeneity might be explained by influences on the conformation of the regulatory idiotope by the structure of other portions of the hypervariable regions. Second, we demonstrated that Ab_1 could inhibit the binding of 60–70% of radioactive Ab_3 to Ab_4 plates. This suggests that a fraction of the Ab_3 molecules may lack the Ab_1 regulatory idiotopes, and that immunization with Ab_2 actually elicits some anti-Ab_2 antibodies. However, it should be pointed out that the immunization scheme, involving Ab_2-KLH conjugates, is not physiologic and may allow responses to idiotopes not normally immunogenic in spontaneous autoimmunization.

Finally, one might speculate about whether regulatory idiotopes are a feature of all anti-epitope antibodies (i.e., all Ab_1) or only of a subset of Ab_1. In particular, regulatory idiotopes might be a feature only of those Ab_1 idiotopes that are capable

of becoming dominant Id, possibly because it is these determinants that call forth Id-specific T cell regulatory responses, as well as anti-idiotope (Ab_2) antibodies. Indeed, Ab_1 bearing anti-BL antibodies appear to be under the regulatory control of T lymphocytes because their expression in the anti-BL response of *nu/nu* BALB/c mice, pretreated with antibody to the IdX of anti-In, is inhibited by T lymphocytes. Thus, if the number of distinct regulatory idiotopes is relatively small, the existence of relatively large clones of regulatory T lymphocytes specific for these idiotopes might explain the powerful effects of Id-determined T lymphocyte regulation which have been increasingly observed in recent years.

Finally, this anti-epitope-anti-idiotope concept or, more simply, this +,− concept of the Id-anti-Id regulatory system does have some network features in that many apparently unrelated Ab_1 idiotopes can be affected by the action of a single Ab_2. However, in the model we propose, the effect of a single Ab_1-Ab_2 system (i.e., the *a* Ab_1-*a* Ab_2 system) should be limited to the Ab_1 possessing a common regulatory idiotope (the *a* Ab_1 idiotope) and as such should be relatively isolated from other Ab_1-Ab_2 systems (e.g., the *b* Ab_1-*b* Ab_2, *c* Ab_1-*c* Ab_2, . . . *z* Ab_1-*z* Ab_2 systems) (Fig. 5). One might describe this system as a "ping-pong" effect, in which the Ab_1 side has many players and the Ab_2 side has few.

Summary

BALB/c mice immunized multiple times with ABPC48 (A48 or Ab_1), a BALB/c bacterial levan (BL)-binding myeloma protein, produce anti-Ab_1 antibodies (Ab_2). Immunization with only two doses of Ab_1 often leads to the production of anti-(antiA48) (Ab_3) as does immunization with hemocyanin conjugates of Ab_2. Finally, immunization with hemocyanin conjugates of Ab_3 leads to the production of anti-(anti-[anti-A48]) (Ab_4). Normal BALB/c mice immunized with BL produce an anti-BL antibody response containing no detectable Ab_1 idiotype (Id)-bearing molecules. Mice producing Ab_3 express substantial amounts of Ab_1 Id in their anti-BL response whereas mice producing Ab_2 and Ab_4 show a generalized inhibition in their anti-BL response. These results indicate that states of immunity within an idiotypic chain may have marked effects on antibody responses to the antigen (i.e., BL) which is the putative initiator of the chain. Strikingly, the chain itself has an interesting feature. That is, Ab_3 and Ab_1 share a cross-reactive Id in that both are bound by Ab_4 and Ab_2. We propose a model of Id-anti-Id systems to explain this unexpected result. This is based on the concept of regulatory idiotopes on Ab_1 molecules which initiate Ab_2 (anti-idiotope) responses. In contrast, Ab_2 molecules generally fail to initiate anti-Ab_2 Id responses either because any individual idiotope is present at very low concentration or because Ab_2 molecules tend to lack regulatory idiotopes. Thus, Ab_2 molecules immunize syngeneic animals because they interact with cells bearing Ab_1-like regulatory idiotopes. Thus, Ab_3 will share regulatory idiotopes with Ab_1. Ab_4 and Ab_2 will share the ability to bind the Ab_1-like regulatory idiotope.

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