

## IDIOTYPE-ANTIIDIOTYPE REGULATION

### IV. Expression of Common Regulatory Idiotopes on Fructosan-binding and Non-fructosan-binding Monoclonal Immunoglobulin\*

BY BURT GOLDBERG, WILLIAM E. PAUL, AND CONSTANTIN A. BONA<sup>‡</sup>

*Department of Microbiology, Mount Sinai School of Medicine, New York 10029; Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205*

We have recently proposed that the immune system's idiotypic repertoire includes a particular class of idiotopes that play an integral role in the regulation of immune responses (1, 2). Hence, these idiotopes are designated as the regulatory idiotopes and can be defined by the following criteria: (a) they function as autoimmunogens; (b) they are shared by various members of an idiotype (Id)<sup>1</sup> network pathway, which may consequently include antibodies with distinct antigenic specificities; (c) they are recognized by regulatory T cells that control the expression of clones expressing them. These clones possess the potential of becoming dominantly expressed in an immune response because of the possible mediation of these regulatory T cells that are either present before immunization or may be easily elicited during the course of an immune response (3).

The notion that this special class of idiotopes exists was based on our study of the immunochemical properties of several members of an idiotypic network pathway, which was initiated by the immunization of normal BALB/c mice with ABPC-48 (A48), a BALB/c levan-binding myeloma protein. Immunization with A48 induced the synthesis of anti-A48 Id antibodies (Ab<sub>2</sub>). In turn, immunization with Ab<sub>2</sub> induced the synthesis of anti-(anti-A48 Id) (Ab<sub>3</sub>), which expressed A48 idiotopes. When Ab<sub>3</sub> was used as an immunogen, it induced an antibody population termed anti-(anti[anti-A48 Id]) (Ab<sub>4</sub>), which bound not only to Ab<sub>3</sub> but A48 as well. To explain this result, we postulated that Ab<sub>1</sub> molecules must express an idiotope that is a member of a special set of regulatory idiotopes, the only strongly immunogenic idiotopes in a syngeneic or autologous system. The Ab<sub>2</sub> evoked by immunization with this Ab<sub>1</sub> does not bear any regulatory idiotopes. Therefore, any subsequent immunization with Ab<sub>2</sub> will activate only those cells that express the regulatory idiotopes of Ab<sub>1</sub>, so the resulting Ab<sub>3</sub>

\* Supported in part by grant IM-275 from the American Cancer Society and grant PCM 8110578 from the National Science Foundation.

<sup>‡</sup> To whom correspondence should be addressed at the Department of Microbiology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029.

<sup>1</sup> *Abbreviations used in this paper:* A48, ABPC-48; Ab<sub>2</sub>, anti-A48 Id antibody; Ab<sub>3</sub>, anti-(anti-A48 Id)antibody; Ab<sub>4</sub>, anti-(anti[anti-A48 Id]) antibody; BL, bacterial levan; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; Inu, inulin; KLH, keyhole limpet hemocyanin; MAbs, monoclonal antibody; PBS, phosphate-buffered saline; PC, phosphocholine; RIA, radioimmunoassay; SRBC, sheep erythrocytes.

response will express those Ab<sub>1</sub> idiotopes as well. Thus, the Ab<sub>4</sub> induced by immunization with Ab<sub>3</sub> is primarily made up of Ab<sub>2</sub>-like antiregulatory idiotope molecules. Indeed, both Ab<sub>2</sub> and Ab<sub>4</sub> in our experiments bind to Ab<sub>1</sub> as well as Ab<sub>3</sub> (1).

The A48 Id is normally not expressed in detectable quantities in the sera of BALB/c mice that have been immunized with bacterial levan (BL), a polyfructosan containing primarily  $\beta(2-6)$  linkages. Under some circumstances, *nu/nu* BALB/c mice will produce A48 Id-bearing anti-BL molecules as we have previously described (4). However, A48 Id-bearing anti-BL clones can be activated to expression in BL-immunized BALB/c mice, by any one of three distinct treatments administered before antigenic challenge. These include: (a) administration of A48 Id-bearing monoclonal proteins to neonatal mice (5); (b) administration of minute amounts of anti-A48 Id antibodies to neonatal mice (6); and (c) hyperimmunization of adult mice with anti-A48 Id antibodies conjugated to an immunogenic carrier such as keyhole limpet hemocyanin (KLH) (1).

To thoroughly examine the properties of the members of the A48 Id network pathway, we prepared a panel of A48 Id<sup>+</sup> monoclonal antibodies (MAb) from mice treated in each of the ways enumerated above. Furthermore, to test the prediction that regulatory idiotopes are shared by antibodies of many specificities, we screened a sample of 198 mouse and 80 human myeloma proteins for expression of the A48 Id. We found A48 Id expressed on one of these proteins, MOPC-167, a phosphocholine (PC)-binding mouse monoclonal protein.

### Materials and Methods

*Animals.* 1-d-old BALB/c mice were obtained from breeding our colony at the Mount Sinai School of Medicine, New York. 8–12-wk-old BALB/c, A/J, and SJL mice were obtained from The Jackson Laboratory, Bar Harbor, ME.

*Antigens.* BL from *Aerobacter laevenicum* was prepared according to a previously described technique (7). Inulin (Inu) (Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, CA), was coupled to bovine serum albumin (BSA) according to Chien et al. (8).

*Cell Fusion.* Hybridomas were produced as described by Nowinsky et al. (9) using the SP2/0 plasmacytoma cell line. Polyethylene glycol 1000 (Sigma Chemical Co., St. Louis, MO), was used as the fusing agent. Cloning was performed by the limiting dilution method (9) using 10<sup>5</sup> BALB/c thymocytes per well as the feeder layer.

*Myeloma Proteins and Monoclonal Antibodies.* Table I lists the isotype and origin of some of the myeloma proteins and monoclonal antibodies used in this paper. The W3082 myeloma protein was a gift of Mrs. R. Lieberman (National Institutes of Health). The panel of 198 murine myeloma proteins that were screened for the A48 Id was kindly donated by Dr. M. Potter (NIH). Likewise, the 80 human myeloma proteins screened were gathered from our own collection and those of Dr. H. Kunkel (The Rockefeller University, New York) and Dr. D. Capra (University of Texas, Dallas).

*Labelling of Antibodies.* MAb were labeled with <sup>3</sup>H amino acids in vitro according to the procedure of Kummer et al. (10) and modified by Inman (personal communication). <sup>125</sup>I-goat anti-mouse Ig was obtained from New England Nuclear, Boston, MA. Alkaline phosphatase labeling was carried out according to the methods previously described (5).

*Labeling of Sheep Erythrocytes (SRBC) with BL, Inu, and PC.* O-stearoyl derivatives of BL and Inu were prepared according to the method of Hammerling and Westphal (11), and were used to coat SRBC as previously described (7). PC-SRBC were prepared by the method of Cosenza (12).

*Preparation of Anti-Id Antibodies.* Anti-Id antibodies specific for A48, 1-5-1, 2-11-3,

and 3-76-42 were obtained by immunization of BALB/c mice with the Mab conjugated to KLH. The coupling of monoclonal proteins with KLH and the schedule of immunization have been described elsewhere (1). SJL anti-UPC-10 Id antibodies were prepared as described by Lieberman et al. (7). These anti-Id sera were depleted of anti-IgG<sub>2a</sub> allotype antibodies by adsorption on a column of Sepharose 4B conjugated with the BALB/c IgG<sub>2a</sub> myeloma protein RPC-5. Anti-Id antibodies were affinity purified by elution from Sepharose 4B columns conjugated with the monoclonal Ig used for immunization.

*Radioimmunoassay (RIA) Binding to Antigen.* The capacity of the monoclonal proteins to bind to various polysaccharides was studied as follows: microtiter plates were coated for 1 h at 37°C with BL (50 µg/ml) or Inu-bovine serum albumin (BSA) (5 µg/ml) in 0.02 M borate buffer, pH 8.2. Plates with <sup>125</sup>I-goat anti-mouse Ig antibodies (~50,000 cpm) (New England Nuclear). After extensive washing, the radioactivity bound to individual wells was measured in a gamma counter.

*Determination of Idiotypes.* Microtiter plates were coated for 1 h at 37°C with purified anti-Id antibodies (25 µg/ml) in 0.02 M borate buffer, pH 8.2, washed three times with saline, and then incubated for 1 h at 4°C with 3% BSA. After three more washings, the microplates were incubated for 18 h at 4°C with <sup>3</sup>H-labeled MAb (i.e., A48, UPC-10, 1-5-1, 2-11-3, and 3-76-42). After extensive washing, radioactivity bound to microtiter wells was measured in a liquid scintillation counter. In other experiments, microtiter plates coated with anti-A48 Id antibodies were incubated with A48, MOPC-167, MOPC-511, MOPC-460 as previously described and then incubated for 18 h at 4°C with <sup>3</sup>H-goat anti-mouse IgA antibodies. These latter four myeloma proteins are all IgA<sub>k</sub>.

*Enzyme-linked Immunosorbent Assay (ELISA).* The binding ability of MAb was also measured by ELISA. Microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were incubated for 18 h at 4°C with BL (100 µg/ml) or Inu-BSA (5 µg/ml). After three washings with PBS containing 0.5% Tween, the microtiter plates were incubated for 18 h at 4°C with the MAb (10 µg/ml). After three further washings, the plates were incubated with alkaline phosphatase-labeled goat anti-mouse Ig antibodies (1:200) (New England Nuclear). The capacity of A48 Id-bearing molecules to inhibit the binding of A48 by anti-A48 was also measured by ELISA. Microtiter plates were coated with purified anti-A48 Id antibodies (5 µg/ml) for 18 h at 4°C and washed as previously described; various dilutions of inhibitors were then incubated for 3 h at 4°C. After three washings, alkaline phosphatase-labeled A48(1:100) was added for 18 h at 4°C to the microtiter plates. Microplates to which alkaline phosphatase-labeled antibodies had been added were washed three times with PBS-Tween 0.5%, and then incubated for 1 h at 37°C with *p*-nitrophenyl-phosphate in ethanolamine buffer pH 9.2, as previously described (9). The reaction was stopped by addition of 25 µl of 3 N NaOH to each well. The extent of the reaction was determined by measuring the absorbance of each well at 405 nm in a micro ELISA spectrophotometer (Dynatech Laboratories, Inc.).

## Results

*Antigenic Specificity of Monoclonal Antibodies.* Hybridomas were prepared by the fusion of SP2/0 cells with spleen cells from three individual groups of mice that had been pretreated in the following ways: (a) injected with 10 µg A48 monoclonal protein at birth (series 1); (b) injected with 10 ng of anti-A48 Id antibodies at birth (series 2); or (c) immunized with a KLH-anti-A48 Id antibody conjugate, emulsified in complete Freund's adjuvant as an adult (series 3). All mice were immunized with 20 µg BL 1 mo after the completion of the pretreatment and their spleen cells were fused 3 d after antigenic challenge.

Hybridomas arising from each set of fusions were screened for the ability of their culture supernatants to bind to BL as well as to inhibit the binding of <sup>3</sup>H-A48 to anti-A48-coated plates in the radioimmunoassay (RIA). Among 49 MAb screened, 19 expressed the A48 Id as assessed by these criteria. These MAb

were of the  $\mu$  or  $\gamma 3$  isotypes (Table I). Among the 14 MAb obtained from mice pretreated at birth with A48 or anti-A48 Id, 8 bound to BL but not to Inu, whereas the remaining 6 bound both BL and Inu as assessed by hemagglutinin (HA) assay, RIA, and ELISA (Table II). Since Inu contains only  $\beta(2-1)$  linkages, those MAb that bind BL but not Inu should be considered specific for  $\beta(2-6)$  linkages. The MAb which bind to both BL and Inu might be specific for  $\beta(2-6)$  linkages only since both BL and Inu contain such linkages. However, it has been shown that Inu-binding myeloma proteins display  $\beta(2-1)$ ,  $\beta(2-6)$  cross-reactivity (4). Since the MAb in our series bound Inu quite weakly, we think it more likely that these antibodies are  $\beta 2-6$  specific and  $\beta 2-1$  cross-reactive.

Among the five A48 Id<sup>+</sup> MAb of series 3 (those prepared from adult mice hyperimmunized with an anti-A48-KLH conjugate), three lack binding activity for both BL and Inu, one displayed a low HA and ELISA titer to BL only, and one had a low binding titer for both BL and Inu. The BL-binding MAb (3-76-1 and 3-76-38) may be regarded as expressing A48-like idiotopes. Since they bind BL considerably less well than A48, they must be considered as different from Ab<sub>1</sub>, which initiated this pathway. The 3-76-12, 3-76-29, and 3-76-42 MAb may

TABLE I  
*Origin and Isotypes of Monoclonal Antibodies*

	Isotype	Origin of antibodies
ABPC-48	$\alpha$	BALB/c myeloma proteins
UPC-10	$\gamma 2\alpha$	
W3082	$\alpha$	
Series 1		
1-5-1	$\mu$	Obtained by fusion of SP2/0 cells with spleen cells from BALB/c mice treated at birth with 10 $\mu$ g A48 monoclonal protein and immunized with BL.
1-3-1	$\mu$	
Series 2		
2-1-3	$\gamma 3$	Obtained by fusion of SP2/0 cells with spleen cells from BL-immunized BALB/c mice treated at birth with 10 ng syngeneic anti-A48 Id antibodies.
2-18-4	$\mu$	
2-17-2	$\mu$	
2-11-3	$\mu$	
2-1-13	$\gamma 3$	
2-1-1	$\mu$	
1-9-1	$\mu$	
2-14-1	$\gamma 3$	
2-28-1	$\mu$	
2-12-3	$\mu$	
2-7-3	$\mu$	
Series 3		
3-76-1	$\gamma 3$	Obtained by fusion of SP2/0 cells with spleen cells from BL-immunized BALB/c mice producing anti-(anti-A48 Id) antibodies subsequent to immunization with anti-A48 Id-KLH conjugate in complete Freund's adjuvant.
3-76-12	$\mu$	
3-76-29	$\mu$	
3-76-38	$\mu$	
3-76-42	$\mu$	

TABLE II  
Antigenic Specificity of Monoclonal Antibodies

Monoclonal antibodies	BL			Inu		
	HA*	RIA <sup>‡</sup>	ELISA <sup>§</sup>	HA	RIA	ELISA
APBC-48	7	1,062 + 167	1.68	0	0	0.16
UPC-10	9	2,144 + 71	1.80	0	0	0.03
W3082	7	1,888 + 308	1.78	>12	2,895 + 99	1.76
MOPC-167	0	0	0.11	0	0	0.13
1-5-1	4	958 + 48	1.75	0	22 + 78	0.01
1-3-1	12	1,170 + 58	1.87	0	187 + 48	0.03
2-1-1	5	1,279 + 33	1.58	1	221 + 42	0.06
2-1-3	5	1,444 + 267	1.83	3	393 + 131	0.16
2-1-13	3	909 + 223	1.59	0	0	0.03
2-7-3	6	1,057 + 115	1.55	2	285 + 14	0.27
2-9-1	5	1,159 + 24	1.61	0	0	0.07
2-11-3	10	ND	ND	4	ND	ND
2-12-5	8	1,173 + 125	1.61	1	157 + 82	0.21
2-14-1	7	1,089 + 47	1.75	0	36 + 37	0.02
2-17-2	7	1,106 + 68	1.19	0	54 + 37	0.10
2-18-3	1	610 + 27	1.60	0	124 + 55	0
2-28-3	2	920 + 119	1.40	2	299 + 10	0.30
2-32-3	4	1,036 + 29	1.58	0	187 + 77	0.10
3-76-1	1	466 + 148	0.31	0	0	0
3-76-12	0	208 + 28	0.14	0	43 + 24	0
3-76-24	0	194 + 89	0.13	0	180 + 70	0
3-76-38	1	289 + 21	0.34	1	117 + 33	0.15
3-76-42	0	78 + 34	0.02	0	0	0

\* In log<sub>2</sub> units. ND, not done.

<sup>‡</sup> In cpm, mean + SD of triplicates.

<sup>§</sup> OD at 405 nm.

be either molecules bearing an A48-like Id which lack BL binding activity or antibodies specific for the idiotopes of anti-A48 Id antibodies (i.e., anti-anti-A48[Ab<sub>3</sub>] molecules).

*Expression of A48 Id on Monoclonal Antibodies.* The presence of A48 Id on MAb was further studied in a competitive inhibition RIA. The data depicted in Fig. 1 shows the degree of inhibition of the binding of radiolabeled A48 to syngeneic anti-A48 Id antibody-coated plates, caused by the various MAb: A48; W3082; an Inu-binding myeloma protein known to express the cross-reactive (IdX) B, A, and G; and MOPC-21. W3082 and MOPC-21 monoclonal proteins did not inhibit the binding of <sup>3</sup>H-A48 to anti-A48 Id. Two MAb from series 1, six MAb from series 2, and three MAb from series 3 caused inhibition at low concentrations (0.5–5 μg). Among the latter three, only one had BL binding activity. The capacity of the two non-BL-binding MAb to inhibit in this assay could be explained either by their true expression of A48 Id or by their being specific for idiotopes on anti-A48.

*A48 Id Represents a Family of Idiotopes.* Legrain et al. (13) has suggested that the A48 Id is composed of several idiotopes. We investigated this possibility using two approaches. In the first, we compared the binding of <sup>3</sup>H-A48, <sup>3</sup>H-

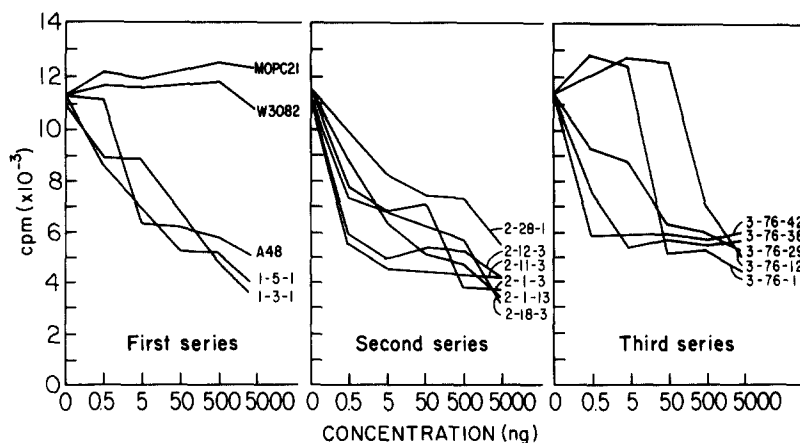


FIGURE 1. Inhibition of binding of  $^3\text{H}$ -A48 to anti-A48 Id antibodies by series 1, 2, and 3 MAb, and A48, MOPC-21, and W3082 monoclonal proteins.

TABLE III

Percentage of Binding of  $^3\text{H}$ -A48, UPC-10, 1-5-1, 2-11-3, and 3-76-42 Monoclonal Proteins to Anti-Id Antibodies

Anti-Id antibodies	Percent of binding				
	A48	UPC-10	1-5-1	2-11-3	3-76-42
BALB/c anti-A48	+100	+(61)	+(98)	+(161)	+(29)
SJL anti-UPC-10	+(64)	+(100)	+(98)	+(36)	+(43)
BALB/c anti-1-5-1	+(30)	+(62)	+(100)	+(74)	-(0)
BALB/c anti-2-11-3	+(42)	+(48)	+(64)	+(100)	-(0)
BALB/c anti-3-76-42	+(20)	+(63)	$\pm$ (5)	+(10)	+(100)

In this experiment microtiter plates were coated with anti-Id antibodies (25  $\mu\text{g}/\text{ml}$ ) and then with  $^3\text{H}$ -labeled monoclonal proteins. The amount of radioactivity bound to their corresponding anti-Id antibodies was considered 100% (see italics) and was compared with the binding to different anti-Id antibodies. The percentage was calculated as follows:  $[(\text{cpm binding to different anti-Id}) - (\text{cpm binding to BSA}) / (\text{cpm binding to corresponding anti-Id} - \text{cpm binding to BSA})] \times 100$ . Idiotopes were recognized by various anti-Id antibodies on monoclonal antibodies.

UPC-10, and a representative  $^3\text{H}$ -MAb for each series (i.e., 1-5-1, 2-11-3, and 3-76-42) to a series of plates coated with anti-Id antibodies generated to each of these monoclonal proteins. Since the radioactive ligand is a monoclonal protein, the critical parameter is whether it was bound to anti-Id-coated plates or not. However, we observed that different anti-Id bound different percentages of ligand under our test conditions. This could be explained either by differences in the affinity of the anti-Id for the MAb or to the polyclonal nature of the anti-Id, which contains a mixture of molecules only some of which are specific for the MAb being tested. We observed (Table III) that anti-A48 binds to A48, UPC-10, 1-5-1, 2-11-3, and 3-76-42, while anti-1-5-1 and -2-11-3 binds to A48, UPC-10, 1-5-1, and 2-11-3 but not to 3-76-42. This could indicate that A48 and UPC-10 bear at least two determinants (A48<sub>1</sub> and A48<sub>2</sub>), one of which (i.e., A48<sub>1</sub>) is not expressed on 3-76-42. Alternatively, 3-76-42 might be an anti-(anti-A48) that does not recognize the idiotopes of anti-1-5-1 or anti-2-11-13. The capacity of anti-3-76-42 to bind weakly to 1-5-1 and 2-11-3 is consistent with

both possibilities. Another possibility is that 1-5-1 and 2-11-3 could express A48<sub>1</sub> and A48<sub>2</sub> sites although anti-1-5-1 and anti-2-11-3 lack antibodies specific for the A48<sub>2</sub> site. Finally, anti-3-76-42 could be a mixture of anti-Ab<sub>3</sub> (i.e., Ab<sub>4</sub>) and Ab<sub>2</sub>-like (i.e., anti-A48-like) molecules. It would thus bind 3-76-42 well and A48, 1-5-1, and 2-11-3, poorly. An interesting result is that an SJL anti-UPC-10 antibody binds 3-76-42 well and conversely anti-3-76-42 binds UPC-10 well. Again, this is consistent with either 3-76-42 bearing an A48-Id or being an anti-(anti-A48 Id) antibody. However, for the latter to be correct, one must postulate that SJL anti-UPC-10 shares idiotypes with BALB/c anti-A48 since 3-76-42 binds both.

Further information on the heterogeneity of the idiotopes expressed on A48 and on the other MAb was obtained by studying the capacity of BL to inhibit the binding of radiolabeled MAb to anti-Id antibody-coated plates. The data in Fig. 2 show that the binding of A48 to anti-A48 Id antibodies was only partially inhibited by BL, indicating that these anti-Id antibodies recognized at least two families of idiotopes, one associated with the combining site (antigen-inhibitible) and the other located outside the combining site (noninhibited by antigen). Anti-1-5-1 Id antibodies also recognizes both antigen-inhibitible and nonantigen-inhibitible determinants on A48. An analysis of the binding of these anti-Id antibodies to UPC-10 indicated that anti-A48 recognized only the antigen-inhibitible sites of UPC-10, whereas anti-1-5-1 recognized A48 and UPC-10 antigen-inhibitible idiotypes.

So far, these results indicate that A48 must express at least one antigen-inhibitible site. However, further studies revealed that while anti-A48 recognizes an UPC-10 antigen-inhibitible site, anti-UPC-10, which also recognizes an UPC-10 antigen-inhibitible site, fails to recognize an A48 antigen-inhibitible site. These results are consistent with the presence of two antigen-inhibitible sites on A48, one shared with UPC-10 and the other not. However, these results can also be explained by the absence of reciprocally cross-reactive antibody in the anti-UPC-10 Id which implies that an antigen-inhibitible site exists that is shared by A48, UPC-10, and 1-5-1. By the same line of reasoning, we are led to the

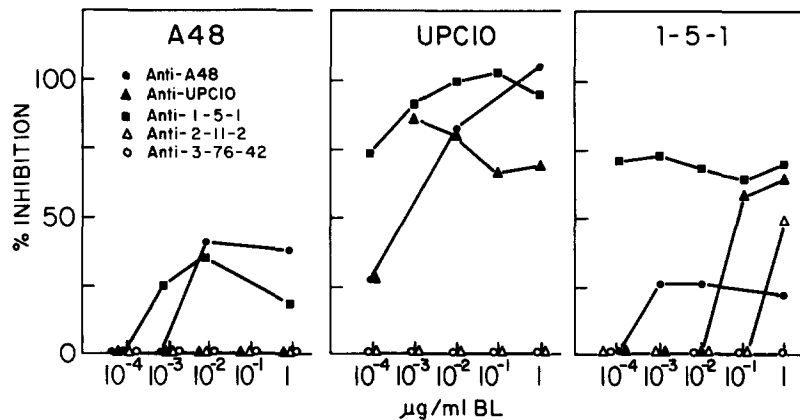


FIGURE 2. Inhibition of binding of <sup>3</sup>H-labeled A48, UPC-10, and 1-5-1 to various anti-Id antibodies by BL.

conclusion that UPC-10 may have two antigen-inhibitable idiotopes, only one of which is shared by A48 but both are expressed on 1-5-1. Neither anti-2-11-3 nor anti-3-76-42 identifies antigen-inhibitable sites on A48, UPC-10, or 1-5-1. Furthermore, only anti-2-11-3 Id antibodies detect an antigen-inhibitable site on 2-11-3. Since 3-76-42 does not bind BL, the ability of antigen to inhibit the binding of anti-Id antibodies to these molecules could not be tested. The conclusions of the analysis of the expression of various idiotopes on A48, UPC-10, and three MAb are illustrated in Table IV.

*Search for Expression of A48 Id on a Sample of 198 Mouse and 80 Human Myeloma Proteins.* Thus far, A48 Id have been shown to be associated with MAb that bind to BL and Inu as well as those which bind to BL only. Nonetheless, the series of MAb that bind BL do so relatively poorly. A more stringent test of the A48 Id as regulatory idiotopes would be their expression on MAb that lack BL- or Inu-binding activity, since it is possible that the three MAb of series 3 which lack antigen-binding activity are antibodies to idiotopes of anti-A48-Id antibodies (i.e., Ab<sub>3</sub>) rather than A48 Id-bearing molecules. Thus, we screened a sample of 198 mouse and 80 human myeloma proteins for their expression of the A48 Id, by their ability to inhibit the binding of A48 to anti-A48 Id antibody-coated plates by RIA and ELISA. Only MOPC-167, a mouse IgA<sub>k</sub> PC-binding myeloma protein gave a significant inhibitor (Table V). As shown in Table VI, MOPC-

TABLE IV  
Summary of Antigen Inhibition of Id-Anti-Id Interaction

	A48	UPC-10	1-5-1	2-11-3	3-76-42	Conclusion: idiotopes identified		
Anti-A48	P*	C	P	N	N	A48I <sup>‡</sup>	A48 NI	U10 I
Anti-UPC-10	N	P	P	N	N	A48I	U10 I	U10 NI
Anti-1-5-1	P	C	P	N	N	A48I	A48 NI	U10 I
Anti-2-11-3	N	N	N	N	N	A48 NI	U10 NI	
Anti-3-76-42	N	N	N	N	N	A48 NI	U10 NI	

\* C, complete; P, partial; N, noninhibition.

<sup>‡</sup> I, antigen inhibitable; NI, antigen-noninhibitable idiotope(s).

TABLE V  
Search for the Expression of A48 Id on Murine and Human Myeloma Proteins

Myeloma proteins	Antigen-binding activity						A48 Idiotype		ELISA
	BL		Inu		PC		HA inhibition	RIA inhibition	
	HA	RIA	HA	RIA	HA	RIA			
A48	11	4,824 + 694	0	171 + 296	0	0	8	5*	96 <sup>‡</sup>
W3082	9	2,529 + 102	23	7,016 + 806	0	0	0	>5,000	0
MOPC-167	0	682 + 389	0	0	7	2,538 + 136	0	50	52
Other 197 murine <sup>§</sup> and 80 human monoclonal proteins		ND		ND		ND	0	>5,000	>10

HA and HA inhibition titer are expressed as log<sub>2</sub> units. Each protein was used as 1 mg/ml. RIA: Microtiter plates were coated with BL 50 µg/ml, Inu-BSA 5 µg, PC-BSA 5 µg, respectively, and incubated with 10 µg/ml monoclonal proteins. The binding was measured with <sup>125</sup>I-goat anti-mouse Ig (10,000 cpm) and the nonspecific binding to BSA-coated plates was subtracted from each value.

\* ng of monoclonal protein required to give 50% inhibition of binding of radiolabeled A48 to anti-A48 Id-coated plates.

<sup>‡</sup> Percentage of inhibition of alkaline phosphatase-labeled A48 to anti-A48 Id antibodies obtained with 0.5 µg monoclonal protein.

<sup>§</sup> 197 murine monoclonal proteins sample includes EPC-109, UPC-61, W3082, AMPC1, J606, all β2-6 and β2-1 fructosan-binding proteins that express G, B, and A cross-reactive idiotypes (4).



TABLE VI  
*Direct Binding of A48 and MOPC-167 to Anti-A48 Id Antibodies*

Microtiter plates coated with:	Ligand	Binding of <sup>3</sup> H-anti-IgA
		<i>cpm</i>
Nil (BSA)	—	123 ± 50
Anti-A48	A48	2,856 ± 142
	MOPC-167	2,783 ± 47
	MOPC-511	273 ± 25
	MOPC-460	398 ± 118
Anti-A48 absorbed on A48-conjugated Sepharose beads	A48	91 ± 78
	MOPC-167	0
Anti-A48 absorbed on 167-conjugated Sepharose beads	A48	317 ± 80
	MOPC-167	0

Microtiter plates were coated with purified anti-A48 Id antibodies followed by three washings in saline. The plates were incubated for 1 h with 3% BSA and, after three washings, incubated for 18 h at 4°C with various myeloma proteins, followed by three washings in saline and incubation with <sup>3</sup>H-goat anti-IgA. The direct binding of <sup>3</sup>H-goat anti-IgA to anti-A48 Id antibodies was subtracted from each value.

167 bound to anti-A48 Id antibody-coated plates to the same extent as A48, as assessed by the subsequent binding of purified <sup>3</sup>H-goat anti-IgA antibody. By contrast, MOPC-511, another IgA<sub>k</sub> PC-binding myeloma protein failed to bind to anti-A48 Id antibodies. Furthermore, the binding activity of anti-A48 for A48 and for MOPC-167 was lost, if the anti-Id absorbed on either A48- or MOPC-167-conjugated Sepharose beads.

PC-chloride failed to inhibit the binding of alkaline phosphatase-labeled MOPC-167 to anti-A48 Id antibodies, indicating that the idiotope(s) of MOPC-167 recognized by anti-A48 Id is not associated with the combining site. On the other hand, strong inhibition was obtained with 0.1–10 μg A48 and 3-76-42 MAb in this ELISA, suggesting that the A48<sub>2</sub>, the nonantigen-inhibitable idiotope expressed on A48 and 3-76-42 is shared with MOPC-167 (Fig. 3). These results clearly indicate that A48 regulatory idiotopes can be expressed on antibodies with different antigenic specificities.

### Discussion

We have previously proposed that antibodies able to achieve clonal dominance generally express a special type of idiotope (a regulatory idiotope) for which specific regulatory T cells and/or antibodies either preexisted or could be rapidly produced. Such regulatory idiotopes, we proposed, were expressed on antibodies at a much higher frequency than conventional idiotopes and could be found on antibodies of distinct specificities (1, 2).

We were led to this proposal by observations in our study of a “network pathway” initiated by immunization with the fructosan-binding myeloma protein

## REGULATORY IDIOTOPES

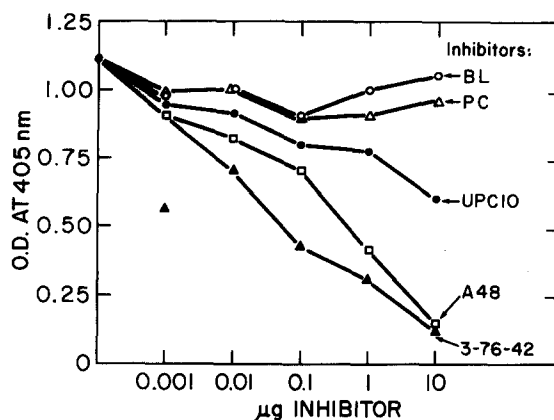


FIGURE 3. Inhibition of binding of alkaline phosphatase-labeled MOPC-167 to anti-A48 Id antibodies of PC-CL, BL, A48, UPC-10, and 3-76-42 monoclonal proteins.

A48 (1) and by similar results of Wikler et al. (14). In these two systems, both the anti-Id ( $Ab_2$ ) and anti-(anti-[anti-Id]) ( $Ab_4$ ) molecules bound to the Id-bearing molecule ( $Ab_1$ ) used to initiate the network pathway. We concluded that  $Ab_4$  should consist of a majority of  $Ab_1$ -binding molecules if the immunogen against which it was produced ( $Ab_3$ ) resembled  $Ab_1$  (1). In turn, this suggested that immunization with  $Ab_2$  preferentially activated molecules bearing  $Ab_1$ -like idiotopes rather than anti- $Ab_2$  paratopes. This, we reasoned, would indicate that B cells bearing  $Ab_1$ -like idiotopes were present at a higher frequency (or could more easily be activated) than anti- $Ab_2$ -bearing B cells.

The study of the A48 Id, both as expressed on  $Ab_1$  molecules (A48 itself) as well as on the other members of the A48 network pathway was therefore of considerable importance. The results presented in this communication demonstrate a sharing in expression of idiotopes among A48, UPC-10, and MOPC-167. The shared idiope of the fructosan-binding proteins A48 and UPC-10, although not previously appreciated (5) is not surprising. Both are members of the  $V_H$ III subgroup and, in addition, the nucleotide sequences of the expressed genes of these two proteins indicated that a strong homology exists among amino acids 32-97 of the  $V_H$  regions. The sequence of the first 31 amino acids of A48 is not available (15). The UPC-10  $V_H$  gene, which has been completely analyzed, appears to be derived from the  $V_H$ 441 germ line gene. The MOPC-173  $V_H$  gene also derives from this germline gene and its also expresses some of A48-U10 idiotopes (Table VII). The UPC-10 and MOPC-173  $V_H$  genes differ at only six positions. Finally, the D genes of A48, UPC-10, and MOPC-173 are quite different from one another, as are the J genes which encode the C terminal portion of each variable region (16). These results would suggest that the idiotypic sharing of A48-UPC-10 and MOPC-173 depends, at least in part, on  $V_H$  gene similarities, although the contribution of L chain remains to be evaluated.

MOPC-167, a PC-binding myeloma protein, also expresses A48 Id. The extent of its idiotypic sharing with A48 is illustrated by the finding that absorbing anti-A48 antibodies with Sepharose 4B beads to which MOPC-167 has been conjugated, removes ~90% of its A48-binding capacity. MOPC-167 also uses a  $V_H$ III

TABLE VII  
*Expression of A48 Id on MOPC-173 Monoclonal Proteins*

Microtiter plates coated with:	Alkaline phosphatase-labeled ligands (1:500)		
	A48	UPC-10	MOPC-173
BSA	0*	0	0.02
BL (10 µg/ml)	1.40	1.76	0
Trinitrophenyl/BSA (5 µg/ml)	0	0.03	0
Polyclonal anti-A48 (25 µg/ml)	1.31	0.13	0.08
Monoclonal anti-A48 (25 µg/ml) (IDA 10)	1.29	1.75	0.11
Polyclonal anti-UPC-10 (25 µg/ml)	0.79	1.75	1.95
Polyclonal anti-MOPC-173 (25 µg/ml)	0	0.07	1.97
Monoclonal anti-460 (25 µg/ml) (FD.5-1.3)	0	0	0.04

Polyclonal and monoclonal anti-A48 Id antibodies are syngeneic antibodies prepared in BALB/c mice; polyclonal anti-UPC-10 Id is an allogeneic antibody prepared in SJL mice; polyclonal anti-MOPC-173 and monoclonal anti-460 antibodies are allogeneic antibodies produced in A/J mice.

\* OD at 405 nm.

subgroup H chain. However, the third CDR, the D region and the J<sub>H</sub> region of A48 and MOPC-167, are very different from one another. Indeed, MOPC-167, and UPC-10 show substantial homology only in the FR1 segment of the V<sub>H</sub> gene (17) suggesting that similarities in this part of the V<sub>H</sub> gene and/or the light chain are critical to idiotypic sharing. MOPC-511, which lacks A48 Id, has strong homology to MOPC-167 in its V<sub>H</sub> gene but differs from MOPC-167 in only six positions in the amino acid sequence of its light chain (18). Although the structural analysis of these molecules (A48, UPC-10, MOPC-173, MOPC-167) is not sufficiently complete to allow the precise mapping of the A48-Id, rapid progress in this area should provide important information in the near future.

We attempted to obtain greater insight into the idiotopes of the A48-Id by the analysis of MAb generated from animals which had been pretreated with various components of the A48 network pathway and prior to their being immunized with BL. All MAb of the first two series (i.e., from donors pretreated as neonates with A48 or anti-A48) that expressed the capacity to bind to anti-A48 were also capable of binding BL. This result strongly indicates that these molecules express an A48 Id. The combined analysis of the binding specificities of anti-Id antibodies generated to each of these MAb, and the degree to which hapten inhibits the binding of these anti-Id to the MAb indicate that A48 expresses several idiotopes. A minimum estimate is that there is at least one, and probably two hapten-inhibitable idiotopes, and one noninhibitable idiotope.

The analysis of a non-BL-binding MAb from series 3 is potentially more important since it should allow a more rigorous demonstration of the regulatory idiotope concept. Our results cannot distinguish between the alternatives that 3-76-42, a non-antigen-binding MAb, bears an A48 Id or is specific for an idiotope of anti-A48. The regulatory idiotope concept would predict that MAb such as

3-76-42 should be mainly of the former type. In fact, Legrain and Buttin (19) have recently analyzed a series of seven hybridomas which produced non-antigen-binding MAb, produced from animals that had been immunized with monoclonal anti-A48 Id conjugated to KLH. They found that mRNA from five hybridomas hybridized with a V<sub>H</sub>-441 cloned germ line DNA, suggesting that they were A48-like rather than anti-anti-A48 Id molecules.

This interesting result strongly affirms the validity of the regulatory idiotope concept since the donor of the spleen cells used to form the hybridomas had not been boosted with BL. Thus, no antigenic stimulation favoring the expansion of A48 Id-bearing B cells over anti-A48 Id-specific B cells was provided.

Others (D. L. Sacks, G. H. Kelsoe, and D. H. Sachs, personal communication) have recently pointed out that immunization with anti-A48 Id antibodies would be more likely to lead to stimulation of A48 Id<sup>+</sup> B cells than of anti-(anti-A48 Id) B cells even if the A48 Id was a conventional idiotope. They reasoned that anti-A48 Id antibodies share specificity for A48 Id but display idiotypic heterogeneity. Thus, in immunization with anti-A48 Id, the concentration of Anti-A48 paratopes is high but the concentration of any individual anti-A48 Id is low. Thus, A48 Id-bearing B cells would be expected to be activated more efficiently than anti-A48 Id-specific B cells. The Legrain-Buttin experiment described above (18) constitutes an especially important test of the regulatory idiotope concept since they used MAb for immunization and, thus, paratope and idiotope concentration should be identical. The observation that the mRNA from five MAb expressing A48 Id hybridized with a V<sub>H</sub>441 germ line gene probe, and that two MAb specific for individual idiotopes of anti-A48 Id antibody did not hybridize, indicate that A48 Id<sup>+</sup> B cells are activated in preference to anti-A48 Id-specific B cells. Had the two types of B cell appeared in equal concentration, it would have suggested that unique regulatory idiotopes need not be postulated. We must note, however, that since only seven MAb from that immunization were studied, the result obtained can only be regarded as partial support for the regulatory idiotope concept.

Finally, a second important type of support for the regulatory idiotope concept is our detection of one myeloma protein expressing the A48 Id in a screen of 198 non-BL-binding myelomas. This suggests that the A48 Id may be expressed relatively frequently. Again, we must add that no firm conclusion may be reached from only a single positive monoclonal protein among a small sample (198) of monoclonal proteins.

The finding that the A48 Id<sup>+</sup> non-BL-binding protein is specific for PC suggests that a regulatory connection may exist between the antifructosan and anti-PC responses. This provides a valuable opportunity to examine consequences of immunization by one antigen on an idiotypically connected system. These studies are now in progress.

### Summary

The ABPC-48 Id (A48 Id) is normally not expressed in detectable amounts in the serum of BALB/c mice that have been immunized with bacterial levan (BL). However, A48 Id-bearing anti-BL clones can be activated in BL-immunized mice by three distinct prior treatments: (a) administration of A48 Id-bearing mono-

clonal proteins to newborn mice; (b) administration of minute amounts of anti-Id antibodies to newborn mice; and (c) production of anti-(anti-A48 Id) antibodies (Abs), in adult mice. From these three groups of mice, eighteen monoclonal antibodies (MAB) expressing A48 Id were obtained. Regarding the binding specificity, these MAB can be divided into three groups: one that binds only BL, the second that binds BL and displays low cross-reactivity for inulin, and the third that lacks BL- and inulin-binding activity. This latter group was obtained only from adult mice immunized with anti-A48 Id-KLH conjugate. Immunochemical analysis of these MAB has shown that the A48 Id is made up of several idiotopes, some of them associated with the combining site and others nonantigen inhibitable.

Comparisons of the amino acid sequence of the UPC-10 and A48 V<sub>H</sub> regions, and the distribution of the A48 Id family on A48, UPC-10, and three MAB, suggested that A48 regulatory idiotypes can be located on the framework segment of V<sub>H</sub> region. Furthermore, we screened 198 mouse and 80 human myeloma proteins for their ability to express A48 Id. Of these, only MOPC-167, an IgA<sub>k</sub> phosphocholine (PC)-binding myeloma protein, gave a significant inhibition of binding of labeled A48 to anti-A48 Id antibodies by radioimmunoassay and enzyme-linked immunosorbent assay. In addition, the binding of labeled MOPC-167 to anti-A48 Id antibodies was not inhibited by PC but was inhibited by A48 and 3-76-42 MAB bearing A48, UPC-10 non-antigen-inhibitable idiotopes. These results extend our prediction that the regulatory idiotopes can be expressed not only on antibodies specific for a family of antigens or members of the same network pathway, but also can be shared by antibodies with different antigenic specificity.

*Received for publication 14 March 1983 and in revised form 21 April 1983.*

### References

1. Bona, C. A., E. Heber-Katz, and W. E. Paul. 1981. Idiotypic-anti-idiotypic regulation. I. Immunization with a levan-binding myeloma protein leads to the appearance of auto-anti-(anti-idiotypic) antibodies and to the activation of silent clones. *J. Exp. Med.* 153:951.
2. Paul, W. E., and C. Bona. 1982. Regulatory idiotopes and immune networks: hypothesis. *Immunol. Today* 3:230.
3. Bona, C. A. Regulatory idiotopes. In *Idiotypic*. H. Kohler, J. Urbain, and P. A. Cazenave, editors. Academic Press, Inc., New York. In press.
4. Lieberman, R., C. A. Bona, C. C. Chien, K. E. Stein, and W. E. Paul. 1979. Genetic and Cellular regulation of the expression of specific antibody idiotypes in the anti-polyfructosan immune response. *Ann. Immunol. (Paris)* 130C:247.
5. Rubinstein, L. J., M. Yeh, and C. A. Bona. 1982. Idiotypic-anti-idiotypic network. II. Activation of silent clones by treatment at birth with idiotypes is associated with the expansion of idiotypic-specific helper T cells. *J. Exp. Med.* 156:506.
6. Hiernaux, J., C. A. Bona, and P. J. Baker. 1981. Neonatal treatment with low doses of anti-Id antibodies leads to the expression of a silent clone. *J. Exp. Med.* 153:1004.
7. Lieberman, R., M. Potter, H. Humphrey, and C. C. Chien. 1979. Idiotypic of inulin-binding myeloma proteins controlled by genes linked to the allotype locus of the mice. *J. Immunol.* 17:2111.

8. Chien, C. C., R. Lieberman and J. K. Inman. 1979. Preparation of functionalized derivation of inulin: conjugation of erythrocytes for hemagglutination and plaque-forming assays. *J. Immunol. Methods* 26:39.
9. Hiernaux, J., and C. A. Bona. 1982. Shared idiotypes among monoclonal antibodies specific for different immunodominant sugars of lipopolysaccharide of different gram-negative bacteria. *Proc. Natl. Acad. Sci. USA*. 79:1616.
10. Kummer, W., E. Thiel, I. Doxiadis, M. Eulitz, S. Sladoljev, and S. Thielfelder. 1981. Tritium radiolabeling of antibodies to high specific activity with *N*-succinimidyl(<sup>2</sup>-<sup>3</sup>H) propionate: use in detection of monoclonal antibodies. *J. Immunol. Methods* 42:367.
11. Hammerling, U., and O. Westphal. 1967. Synthesis and use of *O*-steroyl polysaccharides in passive hemagglutination and hemolysis. *Eur. J. Biochem.* 1:46.
12. Cosenza, H., and H. Kohler. 1972. Specific suppression of the antibody response by antibodies to receptor. *Proc. Natl. Acad. Sci. USA*. 69:2701.
13. Legrain, P., D. Voegtle, G. Buttin, and P. A. Cazenave. 1981. Idiotypic interactions and the control of the anti- $\beta(2\rightarrow6)$  poly-fructosan response in the mouse: specificity and idiotypic of anti-ABPC48 anti-idiotypic monoclonal antibodies. *Eur. J. Immunol.* 11:678.
14. Wikler, M., J. D. Frassen, C. Collignon, O. Leo, B. Mariame, P. van Walls, D. de Groote, and J. Urbain. 1979. Idiotypic regulation of the immune system. *J. Exp. Med.* 150:184.
15. Auffrey, Ch., J. L. Sikorav, R. Olo, and F. Rougeon. 1981. Correlation between D region structure and antigen-binding specificity: evidence from the comparison of closely related immunoglobulin V<sub>H</sub> sequence. *Ann. Immunol.* 132D:77.
16. Olo, R., C. Auffrey, J. L. Sikorav, and F. Rougeon. 1981. Mouse heavy chain variable region: nucleotide sequence of a germ line V<sub>H</sub> gene segment. *Nucleic Acid Res.* 9:4009.
17. Kabat, E. A., T. T. Wu, and H. Bilofsky. 1974. Sequence of Immunoglobulin Chains. U. S. Public Health Service, National Institutes of Health.
18. Selsing, E., and V. Storb. 1981. Somatic mutation of immunoglobulin light-chain variable-region genes. *Cell.* 25:47.
19. Legrain, P., and G. Buttin. 1983. The level of expression and molecular distribution of ABPC48 idiotypes in levan or anti-idiotypic primed BALB/c mice. *Ann. NY Acad. Sci.* In press.