IMMUNOCHEMICAL AND MOLECULAR CHARACTERIZATION OF REGULATORY IDIOTOPES EXPRESSED BY MONOCLONAL ANTIBODIES EXHIBITING OR LACKING β2-6 FRUCTOSAN BINDING ACTIVITY

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We recently proposed that a special category of idiotopes called regulatory idiotopes play an important role in idiotype-driven events in an autologous system (1, 2). Regulatory idiotopes are defined by three criteria: the ability to function as autoimmunogens, the potential to become dominantly expressed because they are recognized by regulatory T cells, and their shared expression by antibodies with different antigenic specificities (3).

The regulatory idiotope concept originated from an immunochemical analysis of four members of an idiotypic network pathway initiated by ABPC-48 (A48), a monoclonal protein with β2-6 fructosan binding activity. By studying polyclonal populations of syngeneic Ab_2 (anti-Id), Ab_3 [anti(anti-Id)], and Ab_4 (anti[anti(anti-Id)]) antibodies, we found that Ab_3 antibodies share A48 idiotopes because Ab_2 and Ab_4 bind to both A48 and Ab_3 antibodies (1). To explain these results, we proposed that Ab_1 antibodies bear a special set of idiotopes which are immunogenic in an autologous system and which elicit the production of Ab_2. Parenteral administration of these Ab_2 antibodies would lead to the activation of Ab_3 clones that express mainly these regulatory idiotopes. Hence, the immunization with Ab_3 antibodies would lead primarily to the production of anti–regulatory idiotope antibodies.

This concept suggests that, through such an "idiotype cascade immunization," clones with the predicted idiotopes are activated. A corollary is that those clones activated by anti-Id antibodies will display the same antigen specificity as the Ab_1 which initiated the cascade. Furthermore, the idiotypes borne by such clones may be phenotypic markers of the same germline heavy chain variable region (V_H)[1] gene from which is derived the V_H genes encoding the specificity of these antibodies. This last characteristic represents an important trait that differen-

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[1] *Abbreviations used in this paper:* A48, ABPC-48; BL, bacterial levan; BSA, bovine serum albumin; C_κ, constant region of the kappa light chain; ELISA, enzyme-linked immunosorbent assay; GL, grass (rye) levan; Id, idiotype; KLH, keyhole limpet hemocyanin; μ, IgM; PBS, phosphate-buffered saline; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; V, variable region; V_H, variable region of the immunoglobulin heavy chain; V_κ, variable region of the κ light chain.

tiates the regulatory idiotopes from "conventional" idiotopes (as described by Oudin) that result from noninheritable somatic recombination or mutational events.

We have recently shown (4) that the A48-Id is shared by UPC-10 and other monoclonal antibodies, some of which exhibit β2-6 fructosan–binding activity while others, such as the MOPC-173 myeloma protein, do not. The V_H region expressed by A48 and UPC-10 derives from the $V_H^{441\text{-}4}$ germline gene (5), which belongs to the small X-24 V gene family ($V_H^{441\text{-}4}$ and V_H^{X24}) located at the 5′ end of the V_H gene locus on chromosome 12 in the mouse (6).

In this paper we demonstrate the existence of a hierarchy in the expression of idiotopes by a panel of monoclonal antibodies. Although they derive from the same V_H germline gene, they exhibit various unexpected antigen-binding specificities.

Materials and Methods

Animals. New Zealand White rabbits of either sex, weighing 2 kg, were obtained from Pocono Farms, Canadensis, PA.

Antigens. Bacterial levan (BL) from *Aerobacter levanicum* (ATCC1552) was obtained as previously described (1). Grass (rye) levan (GL) was the kind gift of Dr. Franco Celada (Genoa University, Milan, Italy). Insulin–bovine serum albumin (BSA) was coupled according to the method of Chien et al. (7). Galactan-BSA conjugate, arabinogalactan, and gum ghatti were kindly donated by Dr. M. Potter (National Cancer Institute, NIH, Bethesda, MD).

Monoclonal Antibodies. (*a*) Monoclonal Ab_1 and Ab_3 antibodies: All antibodies used in this study have been previously described (4) and were purified by chromatography on Sephacryl G-300 (Pharmacia, Inc., Uppsala, Sweden) in 10 mM Tris-HCl, pH 8, 0.9% NaCl, and 0.3% sodium azide. A summary of their origins and isotype is presented in Table I. Ab_3 antibodies of the AIDA series were kindly donated by Dr. Pierre Legrain (Pasteur Institute, Paris).

(*b*) Monoclonal anti-Idiotype antibodies: IDA10, a monoclonal, syngeneic (BALB/c) anti-Id antibody specific for A48 and UPC-10 was kindly donated by Dr. Pierre Legrain (Pasteur Institute), and is described in detail elsewhere (8). 10-1 is a monoclonal, semisyngeneic anti–UPC-10-Id antibody obtained from a (BALB/c × A/J)F_1 mouse, as described (8). Monoclonal rat anti–kappa chain antibody from the hybridoma 187.1 was the kind gift of Dr. M. Scharf (Albert Einstein College of Medicine, New York).

Anti-V_κ10 Antibodies. Purified light chains were prepared from partially reduced and alkylated UPC-10 antibodies according to the method of Jaton et al. (9). The purity of the light chain preparation was assessed by electrophoresis through a 5% sodium dodecyl sulfate (SDS)-polyacrylamide gel using a sodium phosphate buffer system (10). The purified light chain protein was covalently coupled to keyhole limpet hemocyanin (KLH), after which this conjugate was used to immunize rabbits as previously described (11). Affinity-purified anti-V_κ10 antibodies were prepared by elution from an UPC-10–Sepharose column and then adsorbed on other V_κ immunoadsorbents. The specificity of the eluted antibody was confirmed by radioimmunoassay (RIA) against a panel of monoclonal proteins expressing a variety of heavy chain isotypes and other V_κ subgroups.

Immunochemical Techniques. (*a*) Determination of Id expression was carried out using a previously described, sensitive RIA sandwich technique (4) or an enzyme-linked immunosorbent assay (ELISA) inhibition assay. Briefly, for the RIA technique, microtiter plates were coated with the monoclonal test antibodies at 5 μg/ml and then incubated with monoclonal anti–A48-Id or anti–UPC-10-Id antibodies. After extensive washing the plates were incubated with ^{125}I-labeled A48 or UPC-10 protein, respectively. In the ELISA, the degree to which the test monoclonal antibodies inhibited the binding of alkaline phosphatase–labeled monoclonal or polyclonal anti–A48-Id or anti–UPC-10-Id antibodies to A48

or UPC-10, respectively, was quantitated by incubating the alkaline phosphatase–labeled anti-Id antibodies, first, with the test antibodies (1 μg/ml) for 1 h at 37°C, and then applying them to a microtiter plate previously coated with A48 or UPC-10 (10 μg/ml) before incubation overnight at 4°C. After extensive washing with phosphate-buffered saline (PBS)–0.5% Tween, *p*-nitrophenylphosphate substrate in diethanolamine buffer (0.4 mg/ml) was added to the plate. The reaction was allowed to develop at 37°C for 30 min to 1 h before it was stopped by the addition of 25 μl of 3 N NaOH to each well. The optical density at 405 nm was read on an ELISA microreader (Dynatech Laboratories, Inc., Alexandria, VA).

(*b*) The antigen binding specificity of the monoclonal antibodies was determined in RIA by incubating them in microtiter plates coated with GL, BL, arabinogalactan, or gum ghatti at 30 μg/ml, and inulin-BSA or galactan-BSA conjugates at 3 μg/ml. Antibodies binding to these antigens were measured with ^{125}I–rat anti–mouse C_κ (kappa chain constant region) monoclonal antibody (187.1) according to a previously described technique (4).

(*c*) Determination of V_κ10 expression was carried out in an ELISA. Microtiter plates were coated with 10 μg/ml of affinity-purified monoclonal 187.1 anti-C_κ or polyclonal rabbit anti-V_κ10 antibodies, washed three times with PBS–0.5% Tween, and incubated overnight with alkaline phosphatase–labeled antibodies (1:5,000) as previously described (4). The reaction was developed and quantitated as described above.

Molecular Techniques. (*a*) Cytoplasmic RNA extraction: 5×10^7 to 10^8 cells obtained from logarithmically growing cultures or ascitic fluid were washed twice in cold PBS and resuspended in 3.0 ml of cold buffer containing 20 mM Tris-HCl, 2 mM $CaCl_2$, 3 mM $MgCl_2$, pH 8.3. Cells were lysed by adding Nonidet P-40 to a final concentration of 1%, and the nuclei were immediately pelleted at 4°C. To the supernatant, in the following order, were added EDTA to a final concentration of 10 mM, NaCl to a final concentration of 0.1 M, and SDS to a final concentration of 0.5%. This cytoplasmic lysate was phenol extracted two to three times, followed by the same number of extractions with 98% chloroform/2% isoamyl-alcohol. The RNA was then ethanol precipitated and stored at −20°C.

(*b*) Oligo(dT) selection: Poly(A)$^+$ mRNA was isolated after one cycle of selection over a type 3 oligo(dT)-cellulose column (Collaborative Research Inc., Lexington, MA), according to the method of Aviv and Leder (12).

(*c*) Genomic DNA preparation: 5×10^7 to 10^8 cells were washed twice in PBS and resuspended in 10 ml 10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 8 buffer. SDS was added to a final concentration of 0.5% to lyse the cells. The lysate was digested with proteinase K at 200 μg/ml (EM Reagents, Federal Republic of Germany) for 3 h at 37°C with constant rotation. The lysate was then phenol extracted three times, followed by an equivalent number of extractions with 98% chloroform/2% isoamyl alcohol. The DNA was ethanol precipitated, spooled out of the solution, and resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 8, where it was stored at 4°C.

(*d*) ^{32}P-Nick-translation of probes: This technique was carried out as described by Weinstock et al. (13). The presence of $V_H^{441\text{-}4}$ sequences was determined by hybridization with the appropriate enzyme fragment of a plasmid containing the V_H^{Gal39} germline V gene (kindly given to us by Dr. S. Rudikoff, NIH, Bethesda, MD), which has been shown to be identical in sequence to the $V_H^{441\text{-}4}$ germline V gene (25). The plasmid containing the J606 V_H gene kindly given to us by Dr. R. Perlmutter, California Institute of Technology, Pasadena, CA) was nick-translated in its entirety.

(*e*) Northern blotting analysis: 200 ng of poly(A)$^+$ mRNA were resuspended in 50% deionized formamide, 2.2 M formaldehyde, 20 mM 3-(4-morpholino)propanesulfonic acid (MOPS)/5 mM sodium acetate buffer, pH 7.0, and denatured by heating at 65°C for 10 min. After quick chilling on ice for 5 min, Ficoll and bromphenol blue were added and the RNA was fractionated on a 0.8% agarose gel containing 2.2 M formaldehyde in a running buffer of 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7. The electrophoresis was carried out in a wick-type apparatus at 4°C, 115 V, while continuously circulating the buffer between the anodic and cathodic buffer compartments. The RNA

was blotted onto BA85 nitrocellulose paper (Schleicher and Schuell, Inc., Keene, NH) and hybridized as described by Thomas (15).

(*f*) RNA slot blotting: Cytoplasmic RNA was applied to BA85 nitrocellulose filters with a Minifold II apparatus (Schleicher and Schuell, Inc.) and hybridized to ^{32}P-labeled probes according to White et al. (16).

(*g*) Southern blotting: 10 μg of genomic DNA were digested with 50 U Eco RI (New England Biolabs, Beverly, MA) at 37°C for 3 h, and applied to a 40 mM Tris-HCl, 20 mM acetate, 2 mM EDTA, 0.8% agarose gel, containing 1 μg/ml ethidium bromide. The DNA was transferred to BA85 nitrocellulose paper (17) and the blots were hybridized to ^{32}P-nick-translated probes according to standard procedures (18).

Results

In previous studies (19, 20), we showed that the normally silent A48-Id is expressed by a large proportion of anti-BL antibodies in BALB/c mice immunized at birth with either the A48 monoclonal protein or anti-A48-Id antibodies. While the expansion of the A48-Id$^+$ clones in the first system was mediated by Id-specific helper T cells (19), in the second system it was related to a direct expansion of the A48-Id$^+$ precursor B cells (20). Based on these results, we devised the following three protocols to generate the monoclonal antibodies used in this study. Series 1 antibodies were derived from BALB/c mice immunized at birth with A48 protein and challenged 1 mo later with BL. Series 2 antibodies are from BALB/c mice immunized at birth with minute amounts of syngeneic anti–A48-Id antibodies and challenged 1 mo later with BL. Series 3 antibodies were obtained from adult BALB/c mice who were first hyperimmunized with an anti–A48-Id/KLH conjugate and then challenged with BL after completion of immunization. The monoclonal antibodies generated from each of these series were screened for their ability to inhibit the binding of ^{3}H-A48 to polyclonal anti-A48-Id–coated plates (4). Those that demonstrated this ability (Table I) were selected for this study. The AIDA series are monoclonal antibodies obtained from mice immunized with IDA10 or IDA23 monoclonal anti–A48-Id/KLH conjugates whose antigen specificity and V_H origin have been previously described (21).

Characterization of the Idiotypes Recognized by the Monoclonal Antiidiotopes Used in this Study. In our previous work (4), we observed that polyclonal anti–A48-Id and anti–UPC-10-Id antibodies recognized antigen-inhibitable as well as antigen-noninhibitable idiotypes (4). Therefore, it was important to determine the nature of the idiotope that was recognized by the two monoclonal antiidiotopes, IDA10 and 10-1, used in this study. IDA10 is a syngeneic anti–A48-Id antibody, and 10-1 is a semisyngeneic anti–UPC-10-Id antibody. The data in Fig. 1 show that IDA10 recognizes antigen-inhibitable sites on both A48 and UPC-10. 10-1 recognizes an antigen-inhibitable idiotope on UPC-10 but not on A48, because, in the latter case, 50% inhibition was achieved only at 1 μg/ml BL, while the same degree of inhibition with UPC-10 was reached at <0.01 μg/ml.

Characterization of the Idiotopes Displayed by the Monoclonal Antibodies. An RIA was used to determine the reactivity of the monoclonal antibodies with IDA10 and 10-1. The data in Table II show that, except for 2-9-17, all monoclonal antibodies bound IDA-10. However, the binding by the majority of the series 1

TABLE I
Origin of Monoclonal Antibodies

Designation	Treatment of BALB/c donor mice
ABPC-48, UPC-10, MOPC-173	BALB/c myeloma
1-5-1 (μ, κ)	BALB/c mice injected at birth with 10 μg A48 and challenged 1 mo later with 20 μg levan
2-1-3 (γ3, κ) 2-1-10 (γ3, κ) 2-11-1 (μ, κ) 2-11-3 (μ, κ) 2-8-2 (μ, κ) 2-28-9 (γ3, κ) 2-9-17 (μ, κ) 2-12-10 (μ, κ) 2-12-19 (μ, κ)	BALB/c mice injected at birth with 10 ng anti-A48-Id antibodies and challenged 1 mo later with 20 μg levan
3-76-4 (μ, κ) 3-76-42 (μ, κ) 3-14-9 (μ, κ) 3-27-6 (μ1, κ) 3-101-14 (γ1, κ) 3-9-9 (μ1, κ)	BALB/c mice hyperimmunized with polyclonal anti-A48-Id/KLH and challenged 1 mo later with 20 μg levan
AIDA 10-16 (γ1, κ) AIDA 10-21 (γ1, κ)	BALB/c mice immunized with IDA10-KLH conjugate
AIDA 23-2 (γ1, κ) AIDA 23-3 (γ1, κ)	BALB/c mice immunized with IDA23-KLH conjugate

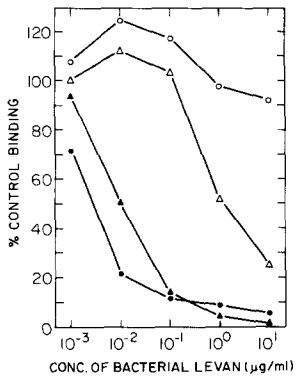

FIGURE 1. Inhibition by BL of the binding of monoclonal anti-Id antibodies to the A48 and UPC-10 myeloma as determined in a solid phase RIA. Data points represent the mean of three measurements. Values of control (100%) cpm are: (○) 10-1 with A48, 789; (△) 10-1 with UPC-10, 8,522; (●) IDA10 with A48, 8,797; and (▲) IDA10 with UPC-10, 17,711. See Materials and Methods for experimental details.

TABLE II

Binding of Monoclonal Anti-Id Antibodies to Antibodies With and Without Fructosan Binding Activity

Monoclonal antibodies	Anti-Id antibodies	
	IDA10	10-1
MOPC-460*	184 ± 17‡	51 ± 13
A48	5,183 ± 289	456 ± 20
UPC-10	1,492 ± 141	2,213 ± 278
MOPC-173	519 ± 233	681 ± 212
1-5-1	991 ± 301	215 ± 184
2-1-3	679 ± 228	319 ± 128
2-8-2	804 ± 276	404 ± 15
2-11-1	728 ± 313	454 ± 93
2-12-10	1,140 ± 136	532 ± 288
2-9-17	356 ± 257	246 ± 43
2-28-9	795 ± 181	932 ± 222
3-76-4	1,548 ± 128	217 ± 86
3-14-9	3,745 ± 152	554 ± 175
3-27-6	6,030 ± 369	640 ± 186
3-9-9	3,078 ± 111	349 ± 144
3-101-14	2,093 ± 41	204 ± 92
AIDA 10-16	1,616 ± 457	531 ± 216
AIDA 10-21	3,197 ± 674	667 ± 148
AIDA 23-2	9,619 ± 107	747 ± 258
AIDA 23-3	9,578 ± 553	678 ± 185

IDA10 is a syngeneic monoclonal anti-A48-Id antibody; 10-1 is a monoclonal anti-UPC-10-Id antibody. In a different experiment, using another preparation of ^{125}I-A48 and ^{125}I-UPC-10 of different specific activity, we found that 2.1.10 bound IDA10, whereas 2-11-3, 2-12-19, and 3-76-42 bound both IDA10 and 10-1.

* MOPC-460 is an IgA_κ with dinitrophenyl binding specificity.

‡ Average cpm ± SE after the subtraction of background (3% BSA instead of monoclonal antibodies).

and 2 antibodies was lower than the binding of A48, UPC-10, or any of the series 3 antibodies. The anti–UPC-10-Id (10-1) was bound by several series 2 antibodies, i.e., 2-11-3 and 2-12-19, as well as series 3 and AIDA antibodies (i.e., 3-76-42, AIDA 10-21).

Characterization of V_κ10 Light Chain Expression. The differences observed in the magnitude of binding of monoclonal anti-Id antibodies IDA10 and 10-1 to the antibodies expressing A48 and UPC-10 idiotopes can be related to heterogeneity in the V_H or V_L regions. From protein sequence data, it is known that UPC-10 and MOPC-173 use a V_κ10 light chain (22). Therefore, it appeared important to determine whether or not the idiotopes recognized by anti-Id antibodies required the association of the appropriate V_H with a V_κ10 light chain.

In an ELISA, we studied the expression of V_κ10 light chain by several A48-Id^+, UPC-10-Id^+ antibodies using an affinity-purified rabbit anti-V_κ10 antibody obtained from an anti-UPC-10 light chain serum. Since the various antibodies have been purified from ascites on Sepharose columns, their binding to anti-

TABLE III
Binding of Alkaline Phosphatase–labeled Monoclonal Antibodies to Rabbit Anti-$V_\kappa 10$ Antibodies

Labeled antibodies	Microtiter plates coated with:		
	BSA	Anti-κ	Anti-$V_\kappa 10$
A48	0.08 ± 0.01*	0.84 ± 0.02	0.29 ± 0.01
UPC-10	0.18 ± 0.02	1.34 ± 0.03	0.91 ± 0.05
MOPC-173	0.10 ± 0.01	1.44 ± 0.01	0.96 ± 0.04
PY211	0.10 ± 0	0.62 ± 0.01	0.09 ± 0
PY207	0.09 ± 0.01	0.66 ± 0.01	0.01 ± 0.01
XY102	0.09 ± 0.01	1.06 ± 0.03	0.10 ± 0.01
1-5-1	0.19	0.62 ± 0.03	1.47 ± 0.06
2-11-1	0.17 ± 0.02	1.47 ± 0.01	0.20 ± 0.02
2-17-2	0.19 ± 0.04	1.23 ± 0.04	0.46 ± 0.03
3-9-9	0.19 ± 0.05	1.59 ± 0.01	0.25 ± 0.03
3-27-6	0.13 ± 0.01	0.94 ± 0.16	0.42 ± 0.07
3-101-14	0.22 ± 0.06	1.58 ± 0.01	0.34 ± 0.05
AIDA 10-16	0.10 ± 0.02	0.49 ± 0.01	0.20 ± 0.01
AIDA 10-21	0.10 ± 0.01	0.83 ± 0.02	0.28 ± 0.01
AIDA 23-2	0.17 ± 0.03	1.57 ± 0.01	0.34 ± 0.05
AIDA 23-3	0.10 ± 0.01	0.96 ± 0.01	0.10 ± 0.01

PY211, PY207, and XY102 are monoclonal antibodies specific for influenza virus hemagglutinin using $V_\kappa 21$ light chain. Background binding to microtiter plates coated with BSA varied between 0.09 and 0.22 and was subtracted.
* Optical density at 405 nm (mean of triplicate determinations ± SE).

$V_\kappa 10$ antibodies was compared with a monoclonal rat anti–mouse C_κ antibody at a constant protein dilution.

The data in Table III show that three influenza virus–specific antibodies known to use $V_\kappa 21$[2] bound to only anti-C_κ antibody, whereas UPC-10 and MOPC-173, known to use $V_\kappa 10$ (22) bound well to both the anti-C_κ and anti-$V_\kappa 10$ reagents. The monoclonal 1-5-1 exhibited a higher binding to anti-$V_\kappa 10$ than to anti-C_κ, whereas A48, 2-17-2, and 3-27-6 showed significant binding to anti-$V_\kappa 10$ antibodies and a much stronger binding to the anti-C_κ antibodies.

RNA Slot Blotting Analysis of the Hybridomas. Usage by the hybridomas of V_H sequences related to the 441-4 or J606 V_H genes, which encode the V_H regions of A48 and J606, respectively, was rapidly surveyed by slot blotting various amounts of their cytoplasmic RNA and hybridizing the filters with ^{32}P-nick-translated $V_H^{441\text{-}4}$ or V_H^{J606} probes. RNA from all the hybridomas except 2-8-2, 3-76-42, and 3-76-4 hybridized with the $V_H^{441\text{-}4}$ probe. Hybridization with the J606 V_H probe was obained only with J606 RNA (data not shown).

Northern Blotting Analysis of the Hybridomas. To confirm that the hybridization observed by slot blotting was due to full-length, polyadenylated heavy chain mRNA and not to incomplete immunoglobulin transcripts often seen in B cell hybridomas, we performed Northern blotting experiments.

Cytoplasmic poly(A)$^+$ RNA from each series 1, 2, 3, and AIDA hybridoma was electrophoresed, Northern blotted, and the filters hybridized under highly

[2] Moran, T. M., M. Reale, J. Schulman, M. Monestier, R. Mayer, M. A. Thompson, R. Riblet, and C. A. Bona. Monoclonal antibodies that define a shared idiotope on antibodies to different influenza viruses. Manuscript in preparation.

stringent conditions with the ^{32}P-nick-translated $V_H^{441\text{-}4}$ probe. As shown in Figs. 2 and 3, in all instances where we observed hybridization of a hybridoma's RNA in slot blotting, we observed, by Northern blotting, hybridization of the $V_H^{441\text{-}4}$ probe. The size of this mRNA species is consistent with the isotype of the antibody produced by the hybridoma. RNA from hybridomas not hybridizing with this probe, namely, series 2 antibodies 2-8-2, 2-11-3, 2-9-17, and 2-12-9, and series 3 antibodies 3-76-4 and 3-76-42, was again slot blotted and hybridized to a μ constant region probe to verify that this lack of $V_H^{441\text{-}4}$ hybridization was not due to a lack of μ mRNA in these IgM-producing hybridomas. Hybridization was observed in all cases except 3-76-4 and 3-76-42. These two hybridomas are currently being recloned.

Southern Blotting Analysis. The molecular configuration of the two genes belonging to the X24 V_H family, 441-4 and X24, were studied in those hybrids in which the H chain mRNA hybridized to the $V_H^{441\text{-}4}$ probe. Genomic DNA was digested with Eco RI and Southern blotted. Duplicate blots were hybridized with the $V_H^{441\text{-}4}$ probe, as well as a J_3-J_4 probe, to confirm the identity of the band representing rearranged 441-4 or X24 V_H gene sequences.

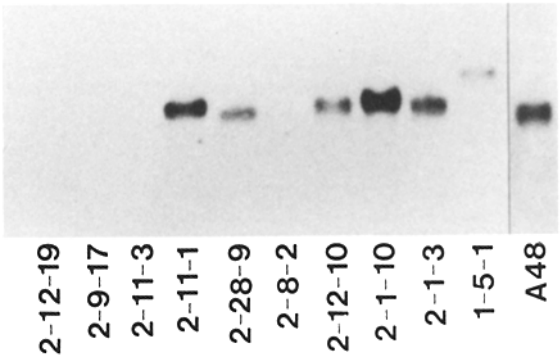

FIGURE 2. Northern blotting analysis of series 1 and 2 hybridomas: 200 ng of oligo(dT)-selected RNA was electrophoresed through a 0.8% formaldehyde-containing agarose gel, Northern blotted, and hybridized to the ^{32}P-nick-translated $V_H^{441\text{-}4}$ probe.

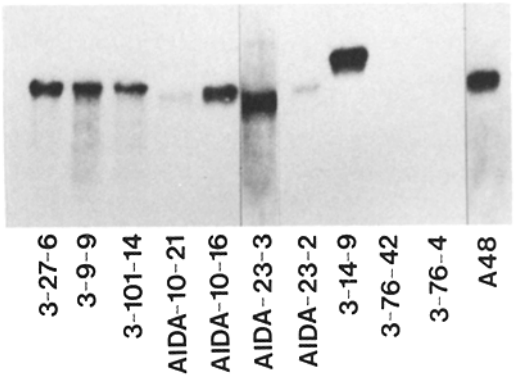

FIGURE 3. Northern blotting analyses of series 3 hybridomas: 200 ng of oligo(dT)-selected RNA was electrophoresed through a 0.8% formaldehyde–containing agarose gel, Northern blotted, and hybridized to ^{32}P-nick-translated $V_H^{441\text{-}4}$ probe.

As can be seen (Fig. 4*A*) in BALB/c liver, both unrearranged genes belonging to this family migrate as a single 500 basepair (bp) Eco RI fragment, which agrees with previously published observations (6). All the series 1 and 2 hybridomas show hybridization with the 441-4 V_H probe to the same 2.91 kb band, which represents a rearrangement of these V sequences to the J_3-J_4 cluster (Fig. 4, *A* and *B*). This fragment is distinct from the A48 rearrangement. By contrast, among the series 3 hybridomas, we see five different types of rearrangements, including the one observed in A48. Bands of 2.95, 2.55, 2.4, 2.2, and 1.65 kb are seen, consistent with the usage of four different J_H sequences (Fig. 5, *A* and *B*).

Antigen Binding Specificity of the Monoclonal Antibodies. The antigen binding specificities of monoclonal antibodies were examined in an RIA using GL (β2-6 fructosan linkages), BL (β2-6 and β2-1 fructosan linkages), and inulin (β2-1 fructosan linkages) (Table IV). As previously reported (23), A48 and UPC-10 bound to GL and BL but not to inulin, demonstrating their specificity for the β2-6 fructosan linkage. On the other hand, J606 bound to BL and inulin but not to GL, indicating the specificity for the β2-1 fructosan linkage. 1-5-1, a series 1 antibody, bound only to BL. Of the 10 antibodies of series 2, 2 displayed the β2-6 fructosan–specific binding of A48, 4 bound only to BL, and 1 bound to BL and inulin, as does β2-1 fructosan–specific J606 protein. Surprisingly, three antibodies bound to all three polysaccharides.

However, the binding of these three antibodies to BL was not inhibited by either inulin or GL (data now shown). These data suggest that these antibodies have a very low affinity for isolated β2-6 or β2-1 fructosan linkages and recognize some conformational determinant present in the BL polysaccharide.

Of the 10 antibodies of series 3 and the AIDA series, 4 had no demonstrable binding activity to any of the polysaccharides, 1 bound to all of them, and the

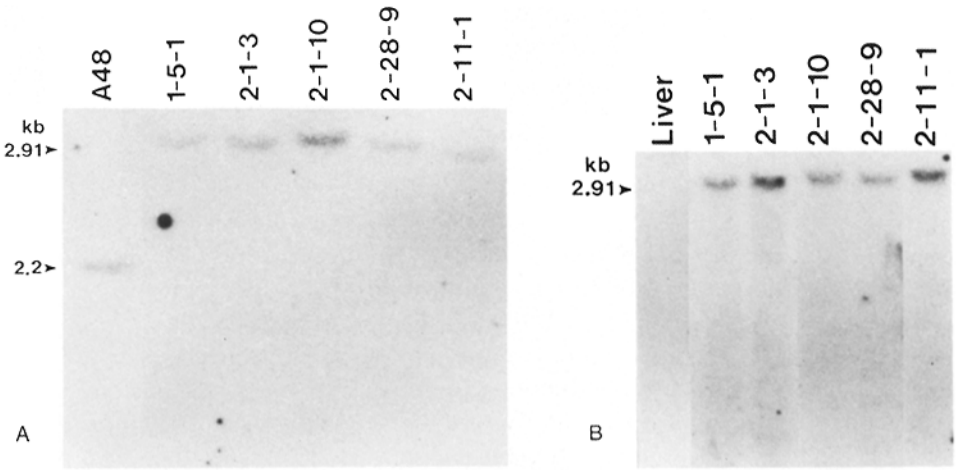

FIGURE 4. Southern blotting analysis of series 1 and 2 hybridomas: 10 μg of Eco RI–digested genomic DNA was electrophoresed through a 0.7% agarose gel and Southern blotted. The filters were hybridized to a ^{32}P-nick-translated $V_H^{441\text{-}4}$ (*A*) and J3-J4 probes (*B*), respectively. The 500 bp fragment containing the germline $V_H^{441\text{-}4}$ and V_H^{X24} genes, respectively, was run off the gel on this blot but has been observed in all these hybridomas in other blots.

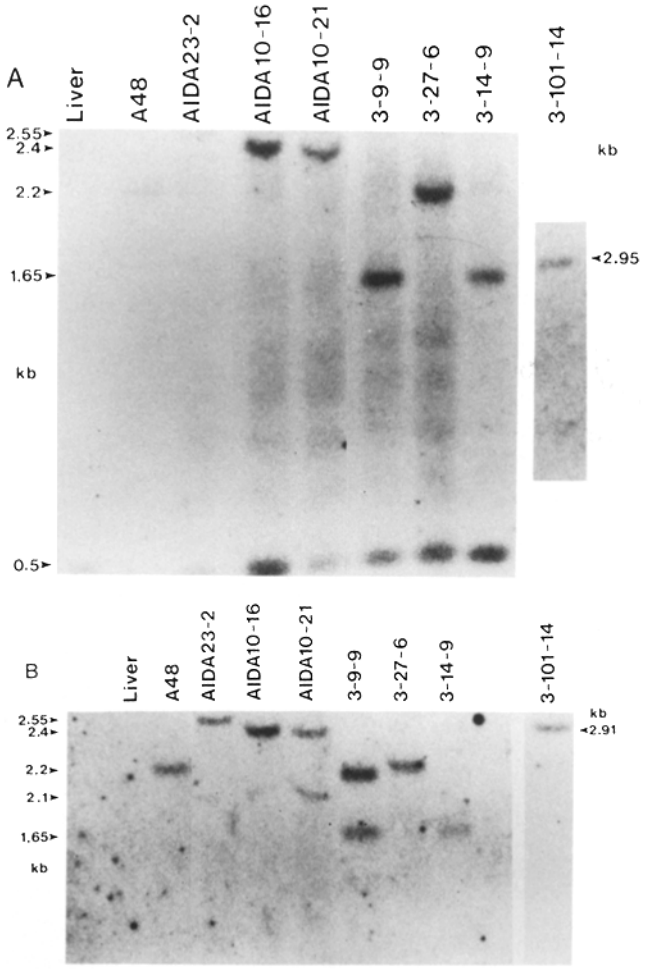

FIGURE 5. Southern blotting analysis of series 3 hybridomas: 10 μg of Eco RI–digested genomic DNA was electrophoresed through a 0.7% agarose gel and Southern blotted. The filters were hybridized to a ^{32}P-nick-translated $V_H^{441\text{-}4}$ (*A*) or J3-J4 probe (*B*). The 3-10-4 lane was taken from another Southern blot carried out under identical conditions and aligned here according to fragment size computed by λ Hind III marker migration.

remaining 5 bound, respectively, to GL and inulin; GL only; BL only; inulin only; and to inulin only, with very low affinity.

Galactan Binding Abilities of Antibodies Lacking Fructosan Specificity. The V_H^{Gal39} and V_H^{X24} germline V genes are used to derive the active V_H region gene encoding the β1-6-D-galactan–binding properties of myeloma proteins (24). While V_H^{Gal39} is identical with $V_H^{441\text{-}4}$, V_H^{X24} differs by seven substitutions from the 441-4 genes. The homology is great enough to suggest that these two genes may have arisen via a gene duplicate process (25). The extreme relatedness of these two V genes prompted us to investigate whether or not those series 3 monoclonal antibodies that displayed the A48-Id but exhibited no specificity for fructosan determinants could, in fact, bind galactan determinants. An RIA was

TABLE IV
Antigen Binding Specificity of Monoclonal Antibodies

Designation	Rye levan	Bacterial levan	Inulin
MOPC-460	225 ± 79*	376 ± 116	44 ± 2
A48	5,659 ± 275	12,819 ± 945	91 ± 5
UPC-10	1,353 ± 95	19,233 ± 723	188 ± 30
MOPC-173	194 ± 32	234 ± 45	67 ± 21
J606	360 ± 87	4,265 ± 833	5,875 ± 419
1-5-1	819 ± 272	12,576 ± 1,867	223 ± 53
2-1-3	4,013 ± 328	12,570 ± 903	8,347 ± 1,024
2-1-10	802 ± 222	8,503 ± 1,516	249 ± 147
2-8-2	1,057 ± 161	16,232 ± 1,418	98 ± 47
2-11-1	1,592 ± 206	13,286 ± 805	186 ± 40
2-11-3	102 ± 3	12,354 ± 697	151 ± 26
2-12-10	113 ± 76	11,230 ± 1,650	40 ± 13
2-12-19	2,528 ± 408	15,028 ± 1,095	1,453 ± 527
2-9-17	62 ± 15	13,434 ± 2,915	2,228 ± 645
2-28-9	666 ± 294	5,232 ± 1,421	61 ± 21
3-76-4	550 ± 69	127 ± 1	77 ± 12
3-76-42	1,544 ± 352	190 ± 41	1,171 ± 271
3-14-9	5,623 ± 947	1,413 ± 263	3,583 ± 733
3-27-6	1,693 ± 293	96 ± 11	177 ± 88
3-9-9	118 ± 30	126 ± 14	105 ± 19
3-101-14	103 ± 19	152 ± 12	1,482 ± 275
AIDA 10-16	179 ± 119	2,888 ± 291	125 ± 89
AIDA 10-21	143 ± 76	294 ± 145	486 ± 235
AIDA 23-2	311 ± 86	151 ± 125	149 ± 34
AIDA 23-3	164 ± 44	405 ± 127	851 ± 180

* Counts per minute ± SE of triplicate determinations.

TABLE V
Binding to Galactan of Monoclonal Antibodies Devoid of β2,6 Fructosan Binding Activity

Monoclonal antibodies (5 μg/ml)	Arabinogalactan (20 μg/ml)	Galactan-BSA (5 μg/ml)	Gum ghatti (20 μg/ml)
BSA	65 ± 3*	86 ± 13	119 ± 18
A48	299 ± 199	357 ± 105	191 ± 07
XRPC-24	872 ± 13	5,272 ± 161	3,537 ± 51
MOPC-173	694 ± 61	981 ± 242	919 ± 268
3-9-9	273 ± 45	183 ± 21	189 ± 15
3-101-4	249 ± 42	301 ± 63	215 ± 30
AIDA 10-21	730 ± 352	575 ± 64	465 ± 107
AIDA 23-2	1,709 ± 324	1,832 ± 300	1,376 ± 54
AIDA 23-3	296 ± 112	709 ± 110	483 ± 96

* Average cpm of triplicate determinations ± SE.

used to examine the ability of five such monoclonal antibodies and the MOPC-173 myeloma to bind β1-6-D-galactan determinants found in galactan-BSA and in two naturally occurring polysaccharides, arabinogalactan and gum ghatti.

The data in Table V show that the monoclonal AIDA 23-2 is specific for β1-

TABLE VI

Inhibition of Binding of Alkaline Phosphatase–labeled Anti–A48-Id and Anti–UPC-10-Id Antibodies to A48 and UPC-10 by β1-6-D-Galactan-binding Myeloma Proteins

Inhibitors (1 μg/ml)	Idiotypic systems		
	Anti-A48*/A48	Anti-UPC-10*/UPC-10	IDA10*/A48
Nil	0.84 ± 0.03‡	1.26 ± 0.02	1.38 ± 0.03
A48	0.10 ± 0.01	1.14 ± 0.04	0.03 ± 0.01
UPC-10	0.80 ± 0.02	0.10 ± 0.01	0.91 ± 0.04
XRPC-24	1.08 ± 0.01	1.28 ± 0.03	1.38 ± 0.03
XRPC-44	0.96 ± 0.02	0.63 ± 0.03	1.31 ± 0.03
TEPC-601	1.01 ± 0.03	1.23 ± 0.03	1.29 ± 0.03
J539	1.02 ± 0.06	1.02 ± 0.01	1.32 ± 0.02

* Labeled antibody.
‡ OD_{405}; average of triplicate determinations ± SE.

6-D-galactans since it bound to all the galactan polysaccharides in this RIA. AIDA 10-21, AIDA 23-2, and the MOPC-173 myeloma exhibited a lower galactan-binding activity but were clearly above background values.

Because the ability of A48-Id–bearing antibodies to bind galactan was so unexpected, we decided to investigate whether galactan-binding myeloma proteins express A48 and UPC-10 idiotopes. This was examined by studying in an ELISA the ability of four galactan-binding myeloma proteins to inhibit the binding of either polyclonal or monoclonal anti-Id antibodies to A48 or UPC-10. The galactan-binding myeloma protein XRPC44 inhibited by 50% (Table VI) the binding of syngeneic polyclonal anti–UPC-10-Id antibodies to UPC-10. The results clearly demonstrate that one of four galactan-binding myeloma proteins shares an idiotope with UPC-10.

Discussion

The data in this paper demonstrate that low doses of antiidiotypic antibodies can expand B cell precursors bearing the corresponding Id. Consistent with our earlier observations (19, 20), anti-Id antibodies administered to neonatal or adult mice preferentially expanded Ab_1 or Ab_1-like clones, rather than a true Ab_3 population. The syngeneic origin of the antiidiotypic antibodies makes it highly likely that these clones were activated through regulatory idiotopes. Analysis with seven monoclonal anti–A48-Id and two anti–UPC-10-Id antibodies identified two monoclonals, IDA10 (anti–A48-Id) and 10-1 (anti–UPC-10-Id) that recognize determinants on most of the antibodies secreted by our hybridomas. The differences in the binding of the IDA10 and 10-1 to various monoclonal antibodies appeared to be independent of V_K10 light chain utilization. However, our data do not preclude the possibility that these antibodies use V_K10 subgroups related to the UPC-10 V_K10 light chain. In the case of V_K21, several subgroups have been identified (14) that are probably markers of active V_K genes originating from six V_K21 germline genes composing the V_K21 germline gene family. Thus, our data suggest that A48 and UPC-10 regulatory idiotopes are mainly markers of V_H regions. Furthermore, the idiotopes recognized by IDA10 and UPC-10 may not be identical to the one displayed by A48, due to a degree of heteroge-

neity in V_H region itself. The series 3 antibodies bound to IDA10 and 10-1 better than did those from series 1 and 2. Whether this reflects greater homology between $V_H^{441\text{-}4}$ and series 3 V_H sequences will be determined by nucleotide sequence analysis currently in progress.

The existence of regulatory idiotopes on antibodies produced by cells expanded by anti-Id had suggested that such idiotopes were V_H germline gene markers. Our data strongly support this idea. Poly(A)$^+$-enriched H chain mRNA from most of the hybridomas that were derived from clones stimulated with anti–A48-Id antibodies, hybridized to the $V_H^{441\text{-}4}$ probe under highly stringent conditions. Furthermore, we demonstrated in Southern blotting experiments that the hybridomas containing mRNA hybridizing to $V_H^{441\text{-}4}$ also had DNA sequences hybridizing with the $V_H^{441\text{-}4}$ probe, rearranged to the J_H (heavy chain joining segment) cluster. This analysis further supports the notion that V_H genes derived from the $V_H^{441\text{-}4}$ germline gene code for Id-positive heavy chains.

Interesting molecular differences exist between hybridomas derived from clones expanded in neonatal vs. adult animals. While the 441-4 probe hybridized to only 6 of 10 H chain mRNAs from series 1 and 2 hybridomas, these 6 hybridomas in Southern blots all demonstrated the same rearrangement to the J_H cluster of sequences complementary to the $V_H^{441\text{-}4}$ probe. This rearrangement was different from that seen in A48 DNA. By contrast, H chain mRNA from all the series 3 hybridomas hybridized to the $V_H^{441\text{-}4}$ probe, but the Southern blots revealed four patterns of rearrangement, which may reflect random rearrangement of $V_H^{441\text{-}4}$ complementary sequences to only one of the four J_H sequences. Nucleotide sequence analysis of cDNA complementary to the V_H region of the antibodies should provide further information on this point.

Collectively, these results show that regulatory idiotopes are probably V_H germline gene markers and that the majority of antibodies produced by clones activated by anti-Id antibodies are encoded by V_H genes deriving from a common V_H germline gene.

A key prediction of the regulatory idiotope concept is that clones exhibiting different antigenic specificities can be coordinately activated by virtue of shared regulatory idiotopes (2). The diverse antigen binding patterns manifested by our hybridomas are in complete agreement with this prediction. All the hybridomas derived from newborn mice treated at birth with minute amounts of anti–A48-Id antibodies showed an affinity for a fructosan determinant that, in most cases, was different from the β2-6 linkage specificity of A48 and UPC-10. Furthermore, several of these clones represent a new type of fructosan-specific antibody, as they display a specificity for a conformational determinant within the BL polysaccharide that has never been observed among the numerous fructosan-binding myelomas studied by Lieberman et al. (23). In contrast, among the series 3 antibodies derived from adult mice treated with anti–A48-Id antibodies, only about half displayed an affinity for a fructosan determinant, which again was not the A48 or UPC-10 specificity. Of the remaining series 3 hybridomas, several, in addition, surprisingly, to the MOPC-173 myeloma, showed a clear specificity for the β1-6-D-galactan determinant. The significance of this observation was reinforced by finding expression of an UPC-10 idiotope on one of the four galactan-binding myeloma proteins studied, i.e., XRPC44. Only one of the series

3 hybridomas, 3-9-9, which did not show any affinity for either galactan or fructosan determinants, can be considered a true anti(anti–A48-Id) antibody (i.e., Ab_3).

If the A48 regulatory idiotope is a marker of the $V_H^{441\text{-}4}$ gene, one may ask how we can explain the diverse array of antigen specificities among these A48 regulatory idiotope–bearing hybridomas. The expression of this same germline V gene in the context of different D (diversity) and J_H segments, or of different light chains, might subtly alter the combining site such that, although these antibodies still recognize fructosan determinants, their precise specificity differs from that of A48 and UPC-10. The finding of antibodies binding the antigenically unrelated galactan determinant was not completely unexpected, since $V_H^{441\text{-}4}$ is identical to the V_H^{Gal39} germline gene and differs by only 7 bp from V_H X24 (25). This finding does, however, emphasize a major point of the regulatory idiotope concept. Anti-Id antibodies will certainly activate clones bearing idiotope, but, if the regulatory idiotope is a germline V gene marker, clones with various antigen specificities may be activated through a crossreactive regulation process.

We define "cross-regulation" as a humoral and cellular mechanism controlling discrete sets of clones via recognition of self-regulatory idiotopes. This concept emphasizes that there is a major difference between the expression of clones expanded via recognition of foreign antigen and the clonal proliferation that occurs after recognition of self idiotope.

Antigenic stimulation should invoke a low degree of cross-regulation since the activated clones have a very restricted antigenic specificity and can express a multitude of idiotopes depending on the number of distinct epitopes. The activation of clones induced by anti-Id would be Id restricted but would exhibit a broader spectrum of specificities. This spectrum is probably limited to a group of antigens reacting with antibodies encoded by the active V genes of a single V_H gene family. Cross-regulation also implies a competition between clones, leading to the dominance of those that express regulatory idiotopes and the suppression of other clones. Our data strongly support this idea since we observed that A48-Id and UPC-10-Id, minor idiotopes of a conventional anti-levan response, became dominant idiotypes in animals receiving minute amounts of Id-bearing or anti-Id antibodies either parenterally at birth or in utero by crossing the placenta (26). The ability to expand clones with various antigen specificities by anti-Id antibodies, particularly in ontogeny, demonstrates that cross-regulation mediated by the recognition of self-regulatory idiotypes can shape the immune repertoire. This also shows that the equilibrium established between clones in the immune system, which is based on idiotypic links, is weak, since minute amounts of Id or anti-Id could upset it and lead to the clonal dominance of a minor Id.

Summary

Hybridomas secreting antibodies bearing the ABPC48 (A48) regulatory idiotype (Id) were generated from BALB/c mice treated at birth or as adults with minute amounts of anti-A48-Id antibodies. The majority of these antibodies were recognized by the syngeneic monoclonal anti–A48-Id and anti–UPC-10-Id

antibodies, IDA10 and 10-1, respectively. In Northern blotting experiments, most of these hybridomas were shown to use V_H (heavy chain variable region) genes related to the 441-4 germline V_H gene that encodes the A48 V_H region. Hybridization was detected between polyadenylated H chain mRNA, isolated from the majority of the hybridomas, and the $V_H^{441\text{-}4}$ probe. Southern blots confirmed these results by showing a rearrangement of $V_H^{441\text{-}4}$-related sequences to the J_H (H chain joining segment) clusters on these same hybridomas. The antibodies from all of the hybridomas that derived from neonatal mice and half of those derived from adult mice showed specificity for fructosan determinants that, in most cases, was different from the β2-6 fructosan linkage specificity of A48. Surprisingly, several of the non–fructosan-binding hybridomas generated from the adult mice and the MOPC-173 myeloma demonstrated a clear specificity for the β1-6-D-galactan determinant. Of four galactan-binding myeloma proteins studied, XRPC 44 alone shared idiotypy with the UPC-10 myeloma. These findings suggest a possible clonal crossreactive regulation mediated by regulatory idiotopes. The crossreactive regulation concept is discussed.

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