Brief Definitive Report

FINE SPECIFICITY OF IDIOTOPE SUPPRESSION IN THE A/J ANTI-AZOPHENYLARSONATE RESPONSE*

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The serological response of strain A mice to the hapten, *p*-azophenylarsonate (Ar), is dominated by the presence of a cross-reactive idiotype (Id^{CR}) originally defined by a heterologous (rabbit) antiserum. Administration of this antiserum before Ar-immunization resulted in an anti-Ar response that lacked idiotype-positive (Id^+) antibodies (1). This suppression was shown to be associated with T suppressor cells capable of transferring the suppressed state to näive, lightly irradiated recipients (2). An apparent suppressed state could also be transferred by B cells, which suggests that relative inactivity or deletion of Id^+ B cell clones occurred in Id-suppressed mice (3). It has been reported that monoclonal antiidiotopes, as well as heterologous anti-Id, suppress Id production in the Ar system (4, 5). Further understanding of Id^{CR} has resulted from the construction of panels of Id^{CR+} and Id^{CR-} Ar-binding hybridoma proteins (HP). Amino acid sequence analysis of these proteins has shown that the Id^{CR} consists of closely related but nonidentical sequences (6, 7). Recently it has been reported that the amino acid sequence of the heavy chain of the Id^{CR+} HP 36-65 corresponds precisely to the germ line gene for the Id^{CR} family (8). Thus Id^{CR+} antibodies appear to represent a family of molecules derived from the HP 36-65 heavy chain variable (V_H) sequence through somatic diversification.

We have now studied the distribution of two idiotopes expressed by the heavy chain of HP 36-65 and the properties of the two murine monoclonal antibodies that define these idiotopes. Among Id^{CR+} HP and Ar-immune serum antibodies, the two idiotopes were not always expressed together. In view of the suggestion that Id^+ sequences are involved in immunoregulation, we evaluated the fine specificity of suppression induced by the two antiidiotopes. It seemed possible that (*a*) suppression induced by both reagents would be identical, because both of them react with the HP 36-65 germ line sequence; or (*b*) suppression induced by these reagents might be different and reflect the unlinked expression of the two idiotopes among diversified Id^{CR+} molecules.

Materials and Methods

Ar-binding HP. Monoclonal antibodies were produced and purified as previously described (4).

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Rabbit Antiidiotypic Antisera. Rabbit PP.2 was immunized with A/J Ar-immune sera and then boosted with HP 16-46 as described (4). Rabbit A was immunized with HP 36-65. Ammonium sulfate fractions of these antisera were absorbed to remove activity against normal mouse immunoglobulins (4), which yielded the antiidiotypic reagents, Rab-PP.2 and Rab-A, respectively.

Murine Monoclonal Antiidiotope Reagents. Hybrid cell lines resulted from fusion of SP2/O-Ag with spleen cells from BALB/c and A/J mice immunized with HP 36-65 (4). Secreted antibodies were designated MB (BALB/c fusion partner) and AI (A/J fusion partner). MB has been referred to as MB anti-Id^{CR} (4). Both AI and MB are IgG_1, κ antibodies.

Radioimmunoassays. Amounts of Id and anti-Ar antibodies were quantified as previously described (4). Ar-coupled tyrosine for the assay of hapten inhibition was obtained from Biosearch, San Rafael, CA.

Immunoadsorbents. AI and MB antiidiotopes were purified from ascites fluid by precipitation with 40% ammonium sulfate and coupled to cyanogen bromide-activated Sepharose 4B.

Preparation of HP 36-65 Heavy (H), Light (L), and Recombined (R) chains. H and L chains of HP 36-65 were reduced with dithiothreitol, alkylated with iodoacetamide (9), and separated on an ACA 44 column equilibrated in 5 M guanidine and 0.1 M sodium acetate, pH 5.5. Peak H and L fractions were dialyzed extensively against 0.01 M sodium acetate, pH 5.5, and then dialyzed against phosphate-buffered saline. Separated chains were recombined by mixing 1:1 molar ratios of H and L chains by optical density, in guanidine buffer, followed by dialysis as above.

Animals. 6–8-wk-old male A/J mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Rabbits were obtained from Margaret's Home Farm, Greenfield, MA.

Induction of Suppression. Adult A/J mice received either 0.2 cc 1:10 AI ascites, 0.1 cc 1:10 MB ascites, or 0.4 cc Rab-A intraperitoneally on day 1. Mice received five intraperitoneal immunizations with 0.1 mg Ar-keyhole limpet hemocyanin (KLH) on days 19, 33, 47, 61, and 76; the first two immunizations were emulsified in complete Freund's adjuvant, and the last three in incomplete Freund's adjuvant. Mice were bled on days 43 and 86.

Results

Hapten Inhibition of Antiidiotypic Binding. The concentrations of Ar-tyrosine required for 50% inhibition of the binding of ^{125}I-labeled HP 36-65 to polyvinyl wells coated with AI, MB, and Rab-A were 0.0166, 0.0647, and 0.188 mM, respectively. Both AI and MB appear to identify epitopes at or near the combining site of HP 36-65, since $<10^{-4}$ M hapten produced 50% inhibition of idiotope-antiidiotope binding.

Fine Specificity of Antiidiotypic Reagents. The fine-specificity profiles of AI, MB, and Rab-A with respect to seven different Id^{CR+} HP are shown in Table I, as indicated by the ability of each Id^{CR+} HP to inhibit the binding of ^{125}I-labeled HP 36-65 to polyvinyl wells coated with anti-Id. Rab-A recognized determinants on all of the HP identified as Id^{CR+} by Rab-PP.2. The monoclonal antiidiotopes displayed more restricted specificity. MB identified an epitope present on five Id^{CR+} HP (Id^{MB}). AI identified an epitope present on three Id^{CR+} HP (Id^{AI}), all of which also expressed the MB epitope. Neither monoclonal-defined epitope was present on five Id^{CR-} HP (data not shown).

Immunoadsorption of Ar-immune Sera. A pool of six Ar-immune sera was assayed for Id before and after depletion by AI and MB immunoadsorbent columns (Table II). Immunoadsorption by AI resulted in the loss of molecules bearing Id^{AI}; however, some molecules bearing Id^{MB} (in the absence of Id^{AI}) were still present. Immunoadsorption by MB resulted in the loss of molecules bearing Id^{MB} as well as those bearing Id^{AI}. As suggested by the fine specificity with Id^{CR+} HP, the AI idiotope appears to be more limited in its representation among anti-Ar antibodies from immune sera than

TABLE I

Specificity of Antiidiotypic Reagents

Antiidiotypic reagent	Inhibition by HP*						
	36-65	31-62	45-223	16-46	45-248	36-71	44-10
Rab-PP.2	96	89	85	96	88	88	75
Rab-A	94	59	34	73	36	46	50
MB	96	96	75	92	34	9	1
AI	89	79	51	11	0	0	0

* Percent inhibition by 10 ng/well unlabeled HP of radiolabeled HP 36-65 which bound to each antiidiotypic reagent in solid-phase radioimmunoassay.

TABLE II

Specific Depletion of Id-bearing Antibodies by MB- and AI-coupled Immunoadsorbents

	Id defined by various reagents*				Anti-Ar antibody*
	AI	MB	Rab-A	Rab-PP.2	
Immune serum pool	278	341	405	1710	1740
Depleted by MB	<2.0‡	<1.0	11.3	290	610
Depleted by AI	<2.0	7.3	17.7	304	888

* Microgram equivalents of HP 36-65.

‡ Below the limit of reliable detection; 1 λ/well produced <50% inhibition.

does the MB idiotope. Some immune antibodies bear Id^{MB} alone, without Id^{AI}, but there are no detectable antibodies that bear Id^{AI} without Id^{MB}.

Location of the AI and MB Idiotopes. L, H, and R chains (see Materials and Methods) were tested for their ability to compete with ^{125}I-HP 36-65 for binding to immobilized AI, MB, and Rab-A. The results of two experiments are shown in Table III and are expressed as a percent of unlabeled HP 36-65 activity determined simultaneously. The determinants on HP 36-65 that were recognized by all three anti-Id were shared by material in the 36-65 R fraction, whereas the 36-65 L chains failed to compete for binding to any of the anti-Id. HP 36-65 determinants recognized by AI and MB were shared by 36-65 H chains; for MB, 36-65 H chains appeared to compete for binding almost as well as R chains on a microgram for microgram basis. The determinant recognized by Rab-A was different in that it was not shared by 36-65 H chains. This latter result indicates that the binding of AI and MB to 36-65 H chains did not result from L chain contamination of the H chain preparation.

Fine Specificity of Suppression Induced by AI, MB, and Rab-A. Adult A/J male mice received anti-Id and Ar-KLH as described in Materials and Methods. Mice were bled after two and again after five immunizations and the sera were assayed for titers of idiotopic and idiotypic determinants. Results from the second bleeding are shown in Table IV and are reported as microgram equivalents of HP 36-65 per milliliter. Six of seven mice that received MB failed to express Id^{MB}, and all of these mice failed to express Id^{AI}. On the other hand, seven of seven mice that received AI failed to express Id^{AI} but six of them produced anti-Ar antibodies that expressed Id^{MB}. Thus, Id^{MB+} Id^{AI-} antibodies were not suppressed by prior administration of AI (nor were they depleted by immunoadsorption with AI; see Table II). Both groups of mice made antibodies that expressed the Rab-A and Rab-PP.2 determinants.

Five of five mice that received Rab-A failed to express the Rab-A determinant, and all of these mice failed to express the AI and MB idiotopes as well, which is consistent

TABLE III

Idiotypic Activity of Separated HP 36-65 H and L Chains

	Experiment 1			Experiment 2		
	AI	MB	Rab-A	AI	MB	Rab-A
HP 36-65	100*	100	100	100	100	100
36-65 L‡	1.5	0.68	0.95	0.94	0.56	0.22
36-65 H‡	38	54	3.3	21	61	1.3
36-65 R‡	97	63	64	49	69	35

* Micrograms per well required for 50% inhibition is expressed as percent HP 36-65 activity.

‡ L, H, and R chains were prepared as described in Materials and Methods.

TABLE IV

Specific Suppression of Id-bearing Antibodies by Prior Immunization with AI, MB, and Rab-A Antiidiotypic Reagents

Anti-Id	Id defined by various reagents*				Anti-AR antibody*
	AI	MB	Rab-A	Rab-PP.2	
AI	<1.0‡	24.6	25.3	722	1,200
	<1.0	17.4	176	1,040	2,470
	<1.0	40.3	370	2,230	7,420
	<1.0	4.2	232	1,880	2,930
	<1.0	7.0	15.0	303	2,640
	<1.0	7.6	6.8	517	4,280
	<1.0	<2.4	323	3,160	3,700
MB	<1.0	<1.0	336	1,200	3,180
	<2.5§	13.8	114	2,377	7,720
	<1.0	<2.4	226	1,910	3,240
	<1.0	<1.0	<1.0	30.4	6,700
	<1.0	<1.0	3.0	407	3,680
	<1.0	<1.0	170	1,830	5,170
	<1.0	<1.0	10.6	513	2,030
Rab-A	<1.0	<2.4	<1.0	3.6	4,470
	<1.0	<1.0	<1.0	<1.0	4,250
	<1.0	<1.0	<1.0	1.6	1,840
	<1.0	<1.0	<1.0	<1.0	3,890
	<1.0	<1.0	<1.0	102	1,230

* Microgram equivalents of HP 36-65 per milliliter; each line represents results on a single mouse.

‡ Below the limit of reliable detection; 1 λ/well failed to produce any significant inhibition and thus contained <1 ng equivalents of HP 36-65.

§ Below the limit of reliable detection; 1 λ/well produced <50% inhibition, and thus contained <2.5 ng equivalents of HP 36-65.

with the specificities of these reagents. Results from the first bleeding (not shown) were similar to those described above.

Discussion

We examined the fine specificity of Id suppression induced by two hapten-inhibitable monoclonal antiidiotopes that identify different epitopes on the H chain of HP 36-65, an Ar-binding protein whose sequence is found in the germ line of A/J mice (8). Reactivity of the antiidiotopes toward a panel of Ar-binding HP and immunoadsorption of Ar-immune sera by the antiidiotopes demonstrated that one idiotope (Id^{AI}) is found on a subset of molecules that bear the other (Id^{MB}), which indicates

that there are Id^{MB+} Id^{AI-} molecules among diversified Id^{CR+} HP and serum antibodies. In vivo administration of the antiidiotopes led to the deletion of nonidentical portions of the Ar-immune response; immune sera from mice suppressed with AI failed to express Id^{AI} but continued to express Id^{MB}, whereas sera from mice suppressed with MB were suppressed for both the AI and MB idiotopes. Thus, suppression was idiotope-specific and corresponded to the fine specificities of the antiidiotope antibodies.

Nelles et al. (5) reported that two murine monoclonal antiidiotopes suppressed most of the Id^{CR+} antibody response when administered before Ar-immunization. The present study extends these findings by examining the idiotope expression of anti-Ar antibodies that escaped such suppression. The present finding of idiotope specificity in antiidiotope-mediated suppression is consistent with earlier reports in the T15 (10) and NP^b (11) Id systems in which there was nonreciprocal variation in the expression of two idiotopes during antiidiotope-induced immunoregulation.

The most straightforward explanation for the present results is that homologous antiidiotope exerts its effect through direct binding to diversified B cell surface immunoglobulin. Mechanisms that involve receptor blockade (12) or tolerogenic signals (13) might initiate suppression that is subsequently perpetuated by clonal dominance of idiotope-negative B cells (3). The induction of a suppressive network via complexes with serum Id (as found in immune sera or in undiversified germ line sequences) seems unlikely because interaction with any intermediate that expresses the AI and MB idiotopes together would result in parallel effects upon both idiotopes, unless antiidiotope were able to "activate" only the occupied idiotope for network interactions in a molecule expressing both idiotopes. Because both the AI and MB idiotopes are represented in a single germ line gene that encodes Id, the mediation of suppression by regulatory T lymphocytes in which V_H is the T cell receptor would seem unlikely under the assumpiton that both idiotopes are represented within the V_H segment and are unaffected by D-region segments. Alternatively, sufficient diversity in T cell receptors may exist such that the AI and MB idiotopes are expressed separately, leading to activation of separate sets of idiotope-specific regulatory T lymphocytes. Regardless of the mechanism, the present results do not rule out the participation by T lymphocytes, assuming that suppressor cells, after activation by antiidiotope, retain idiotope specificity and induce suppression that does not spread to other closely related idiotopes over a relatively long period of time (3 mo).

Id^{CR+} antibodies appear to result from somatic diversification away from a germ line gene (8). Although the single germ line sequence for Id (defined by conventional rabbit antisera) contains both the AI and MB idiotopes, antiidiotope-induced suppression was not identical; moreover, Id^{CR+}, idiotope-negative sequences escaped suppression. This suggests that these sequences (as well as Id^{MB+} Id^{AI-} sequences) had diversified away from the germ line sequence before administration of the antigen. The possibility that the Id^{CR+}, idiotope-negative molecules correspond to the germ line V_H combined with a distinct D region cannot be ruled out. Examination of Id^{CR+}, idiotope-negative HP isolated from idiotope-suppressed mice may clarify this point.

Summary

Two hapten-inhibitable murine monoclonal antiidiotopic antibodies identified two idiotopes expressed by the heavy chain of hybridoma protein 36-65, whose amino

acid sequence is encoded in the germ line of A/J mice. Among cross-reactive idiotype-positive hybridoma proteins and *p*-azophenylarsonate-immune antibodies, the two idiotopes were not always expressed together; some diversified antibodies expressed one idiotope without the other. Suppression that was induced by the two antiidiotopes was idiotope specific and corresponded to the fine specificities of these two reagents.

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