

## REQUIREMENTS FOR PROLONGED SUPPRESSION OF AN IDIOTYPIC SPECIFICITY IN ADULT MICE\*

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All A/J mice immunized with keyhole limpet hemocyanin-*p*-azophenylarsonate (KLH-Ar)<sup>1</sup> produce anti-Ar antibodies with shared idiotypic specificity, as shown by the capacity of each immune serum to displace radiolabeled anti-Ar antibody of an individual A/J mouse from its rabbit antiidiotypic antibodies (1, 2). The IgG fraction of rabbit antiidiotypic antibody (anti-D), when inoculated intraperitoneally in saline solution into an adult or neonatal A/J mouse, suppresses the appearance of the cross-reactive idiotypic specificity, although substantial amounts of non-cross-reactive anti-Ar antibodies are produced in all mice (2, 3).<sup>2</sup>

In our previous experiments almost complete suppression persisted for up to 5 mo in the surviving mice. However, interpretation of these results in terms of the true duration of effective suppression is complicated because immunization was initiated only 2–9 wk after administration of the antiidiotypic antibody. Since antihapten antibody (not idiotypically cross-reactive) was produced as a result of such immunization, it is conceivable that members of the responsible clones of cells might capture antigen subsequently administered and in this way prevent the expression of the idio type by any appropriate precursor cells that might have arisen subsequent to the first challenge by antigen.

To investigate this possibility we have carried out a series of experiments with adult A/J mice, in which the initial inoculation of antigen was given at increasing intervals after immunosuppression, and have measured the duration of suppression under these conditions. The minimum quantity of antiidiotypic IgG required for suppression of idio type was measured and suppressive effects of Fab' and F(ab')<sub>2</sub> fragments of rabbit antiidiotypic IgG were investigated. The extent of suppression by antiidiotypic antibody, when administered together with or subsequent to antigen, was also determined.

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<sup>1</sup> *Abbreviations used in this paper:* anti-D, antiidiotypic antibody; Ar, *p*-azophenylarsonate; BGG, bovine gamma globulin; BSA, bovine serum albumin; CRI, cross-reactive idio type; KLH, keyhole limpet hemocyanin.

<sup>2</sup> Individual, suppressed A/J mice synthesize anti-Ar antibodies which do not share idio type specificity; i.e., unique specificities are present in each suppressed mouse (4).

### Materials and Methods

*Methods Previously Described.*—The following methods have been described (1, 2): hyperimmunization of mice with protein-*p*-azophenylarsonate conjugates; specific purification of mouse antihapten antibodies, employing a protein carrier other than that used for the immunization; preparation of rabbit antiidiotypic antiserum by immunization with a dissolved specific precipitate made with mouse anti-Ar antibody and (bovine gamma globulin) BGG-Ar; absorption of the antiserum with A/J serum and A/J serum globulins; adsorption of an IgG fraction of anti-D serum with Sepharose to which a crude globulin fraction (18% sodium sulfate precipitate) of A/J serum had been conjugated; labeling of purified anti-Ar antibodies with  $^{125}\text{I}$  by the chloramine-T method (5); removal of antihapten antibodies from antisera with an immunoadsorbent (Sepharose 4 B coupled to rabbit IgG-Ar); determination of the concentration of precipitable anti-*p*-azophenylarsonate antibodies in hyperimmune sera; quantification of the  $^{125}\text{I}$ -labeled anti-Ar antibody reactive with antiidiotypic antibody by a method of indirect precipitation; quantification of cross-reacting idiotypic antibodies present in various unlabeled sera by measurement of their capacity to inhibit the indirect precipitation of labeled ligand. In brief, the latter method consists in mixing 0.01  $\mu\text{g}$  of  $^{125}\text{I}$ -labeled specifically purified anti-Ar antibody of mouse 126 with slightly less than an optimal amount of antiidiotypic antiserum (5  $\mu\text{l}$  of an 1:10 dilution in a solution containing bovine serum albumin [BSA]) (1 mg/ml in NaCl-borate buffer, pH 8, ionic strength 0.15). To this mixture is added 25  $\mu\text{l}$  of a 1:10 dilution of rabbit antiovalbumin. After incubating for 1 h at 37°C, an excess (75  $\mu\text{l}$ ) of goat antiserum specific for rabbit Fc is added to precipitate rabbit IgG and complexes of the labeled ligand with rabbit antiidiotypic antibody. Unlabeled inhibitors, when present, are mixed with the antiidiotypic antiserum 15 min before the addition of the  $^{125}\text{I}$ -labeled purified anti-Ar antibody.

The BSA is used to provide a moderately high protein concentration and thus to retard denaturation of the highly dilute labeled anti-Ar antibody. Rabbit antiovalbumin is added so that the total concentration of rabbit IgG is sufficient to yield a heavy precipitate upon subsequent addition of goat anti-Fc.

Each set of experiments included controls in which additional rabbit antiovalbumin was substituted for the antiidiotypic antiserum. The percentage of radioactivity precipitated (8–12%) was subtracted from experimental values. In the absence of inhibitor 43–54% of the labeled ligand (net value) was precipitated. (Less than an optimal amount of anti-D is used to make inhibition tests more sensitive. With excess anti-D approximately 70% of the ligand is precipitated.) Another test of specificity was carried out by using  $^{125}\text{I}$ -labeled nonspecific A/J IgG in place of the labeled anti-Ar antibody; 10% of the radioactivity was precipitated.

*Inoculation of Mice with Antiidiotypic Antibody.*—In experiments designed to suppress idiotypic specificity, mice were inoculated with a saline solution of an IgG fraction of the antiidiotypic antiserum, adsorbed with Sepharose conjugated to a crude globulin fraction of A/J serum. The saline solution was sterilized by passage through a Millipore filter (Millipore Corp., Bedford, Mass.). Binding tests were carried out with anti-D antiserum that had been absorbed by addition of A/J serum and A/J IgG (1).

For those studies whose results are summarized in Tables I and II, the antiidiotypic IgG was administered intraperitoneally; in the remainder of the experiments it was given subcutaneously. That either route is effective in suppressing idiosyncrasy had been observed in preliminary experiments and is illustrated in data presented below.

An IgG fraction of the adsorbed antiidiotypic antiserum or of the normal rabbit antiserum was prepared by two precipitations with sodium sulfate followed by chromatography on DEAE-cellulose in 0.0175 M phosphate buffer, pH 6.9.  $\text{F(ab')}_2$  fragments were prepared by digestion with pepsin for 4 h at 37°C, followed by gel filtration (6).  $\text{Fab'}$  fragments were produced by reduction of  $\text{F(ab')}_2$  with 0.007 M dithiothreitol, followed by gel filtration. Both types of fragment failed to react in agar gel with goat antirabbit Fc. The absence of appreciable

contamination by undegraded IgG was demonstrated by trace-labeling each preparation with  $^{125}\text{I}$  and gel filtering in the presence of excess rabbit IgG. Nearly all of the radioactivity was eluted from the column after the peak of optical density corresponding to IgG. The degree of contamination by IgG was less than 2% for each of the four preparations [ $\text{F(ab')}_2$  and Fab' from antiidiotypic or normal serum].

#### RESULTS

*Evidence for Antiidiotypic Specificity of the Absorbed Rabbit Antiserum.*—Evidence that the absorbed rabbit antiserum directed to anti-Ar antibodies of mouse 126 is specific for idiotypic determinants has been reported elsewhere (3, 7). In brief, the indirect precipitation of 0.01  $\mu\text{g}$  of the  $^{125}\text{I}$ -labeled mouse anti-Ar antibody by antiidiotypic antibody was inhibitable by the hyperimmune donor serum but not by preimmune donor serum nor by hyperimmune donor serum from which the antihapten antibody had been specifically adsorbed. Antibodies to the carrier protein (KLH) or anti-KLH-*p*-azobenzoate antibodies prepared in other A/J mice were noninhibitory. Up to 2 mg of A/J anti-KLH antibodies and nonspecific A/J IgG caused less than 25% inhibition of binding. By comparison, 0.02  $\mu\text{g}$  of unlabeled autologous antibody was sufficient to cause 66% inhibition.

*Duration of Suppression of Idiotypic.*—Tables I and II present data relating to the duration of suppression of the cross-reactive idiotype (CRI). Each value in Tables I and II represents an individual mouse. Each mouse received 4 mg of an IgG fraction of the adsorbed rabbit antiidiotypic antiserum or, as a control, 4 mg of nonspecific IgG. At the end of the desired interval, mice were challenged with 500  $\mu\text{g}$  of KLH-Ar in Freund's complete adjuvant, injected intraperitoneally (i.p.); this was repeated 2 wk later. After another 2 wk interval, 500  $\mu\text{g}$  of the same antigen was inoculated i.p. in Freund's incomplete adjuvant. Bleedings were taken 1 wk after the second and third injections. The time periods specified in the tables (i.e. 2, 6, 12, and 20 wk) refer to the interval between the inoculation of the antiidiotypic or nonspecific IgG and the first challenge with antigen. CRI was quantified by determining the capacity of varying amounts of serum to inhibit the binding of 0.01  $\mu\text{g}$  of  $^{125}\text{I}$ -labeled anti-Ar antibody of mouse 126 to its antiidiotypic antibodies. Mice designated with the prefix C (control) were inoculated with nonspecific IgG; those with the prefix S (suppressed) were treated with antiidiotypic IgG.

In all experiments the control mice, inoculated with nonspecific IgG and challenged with KLH-Ar, produced high concentrations of idiotypically cross-reactive antibody, which was present in both the first and second bleedings. As little as 0.03  $\mu\text{l}$  of each antiserum was sufficient to cause 60% inhibition, with most values around 80%. Equal volumes of preimmune sera from the mice in each of the four control groups were then pooled. 10  $\mu\text{l}$  of pooled preimmune serum failed to cause significant inhibition of binding (less than 7%) in each case. This is in accord with previous results (2).

The sera of mice challenged with antiidiotypic IgG and then, beginning 2 wk later, with KLH-Ar, contained virtually no CRI in either bleeding. 10  $\mu\text{l}$  caused far less inhibition than 0.03  $\mu\text{l}$  of sera from control mice; thus, the ratio is greater than 300 to 1.

TABLE I  
Inhibition of Binding of  $^{125}\text{I}$ -Labeled Anti-Ar Antibody from Mouse 126 to its Rabbit  
Antiidiotypic Antibodies\*

Inhibitor (mouse no.)	Interval between suppression and first antigen challenge	<sup>125</sup> I-labeled purified anti-Ar antibody bound (% of control)						
		First bleeding			Second bleeding			
		μl of serum tested as inhibitor						
		0.1	3	10	0.03	0.1	3	10
C-100‡	2 wk	41			25	6	0	
C-101		35			22	24	0	
C-102		24			26	15	10	
C-103		14			28	7	1	
C-104		38			40	25	2	
C-105		37			35	23	12	
C-106		28			24	18	6	
C-107		24			23	18	13	
C-108		27			30	18	9	
C-109		20			22	15	8	
S-80‡	2 wk			98		98	96	94
S-81				98		97	96	94
S-82				97		98	94	93
S-83				96		99	98	93
S-84				101		99	98	94
S-85				97		99	97	93
S-87				98		98	98	96
S-89				102		99	99	94
S-121				92		98	96	92
S-122				77		94	81	75
C-201	6 wk	40			20	7		
C-202		15			24	18		
C-203		20			26	15		
C-204		24			35	22		
C-205		30			28	17		
C-206		37			22	12		
S-140	6 wk		93	97			94	77
S-141			97	90			95	90
S-142			95	90			96	90
S-143			83	82			82	75
S-144			98	98			97	93
S-145			86	79			78	74
S-147			100	96			98	96
S-148			98	92			98	90

\* In the absence of inhibitor, 46% (net value) of the labeled ligand was bound. All experiments were carried out in duplicate. The average deviation from the mean, expressed as percent of control, was 1.6%.

† The letter C indicates a control mouse, which received 4 mg of nonspecific IgG. The letter S designates a suppressed mouse, which had received 4 mg of antiidiotypic IgG. The protocol of immunization is given in the text. The nonspecific or antiidiotypic IgG was administered intraperitoneally.

TABLE II  
Inhibition of Binding of  $^{125}\text{I}$ -Labeled Anti-Ar Antibody from Mouse 126 to its Rabbit  
Antiidiotypic Antibodies\*

Inhibitor (mouse no.)	Interval between suppression and first antigen challenge	<sup>125</sup> I-labeled purified D antibody bound (% of control)							
		First bleeding				Second bleeding			
		μl of serum tested as inhibitor							
		0.03	0.1	3	10	0.03	0.1	3	10
C-11‡	12 wk	40	8	2		42	5	0	
C-12		32	0	0		30	0	0	
C-13		33	2	0		§	§	§	
C-14		37	9	1		36	4	0	
C-15		35	7	4		38	4	6	
S-150‡	12 wk		100	90	90		98	88	82
S-151			97	85	83		§	§	§
S-152			103	85	86		92	94	81
S-153			37	0	0		27	0	0
S-154			72	14	14		§	§	§
S-155			76	50	48		42	0	6
S-156			22	0	0		14	0	0
S-157			27	0	2				
S-158			78	45	36				
C-16	22 wk	49	18			§	§	§	
C-17		22	15			§	§	§	
C-18		44	9			§	§	§	
C-19		12	4			§	§	§	
C-20		27	7			§	§	§	
C-21		63	29			§	§	§	
C-22		0	0			35	19	3	
C-24		14	0			§	§	§	
C-25		6	0			§	§	§	
C-126		10	8			56	20	10	
C-127		20	0			§	§	§	
C-129		14	6			31	19	8	
S-161	22 wk			99	95			93	94
S-162				6	0			8	0
S-163				91	88			89	87
S-165				93	90			83	34
S-1				0	0			§	§
S-3				73	45			§	§
S-5				93	77			88	76
S-6				0	0			4	0
S-7				11	0			5	0
S-8				11	0			§	§
S-10				10	0			3	0

\* In the absence of inhibitor, 54% (net value) of the labeled ligand was bound. All experiments were carried out in duplicate. The average deviation from the mean, expressed as percent of control, was 1.4%.

‡ See second footnote, Table I.

§ Deceased.

6 wk after suppression, the amount of CRI produced upon challenge with KLH-Ar was similarly negligible in all mice (Table I).

Data on suppression of idiotypic, when the first challenge of antigen was delayed until 12 or 22 wk after suppression, are presented in Table II. As evidenced by the capacity of their antisera to inhibit the antiidiotypic antibody, all members of the control groups, which received nonspecific rabbit IgG, produced substantial quantities of the cross-reactive idiotypic. In contrast, 12 wk after administration of antiidiotypic antibody, seven of nine surviving mice were at least partially suppressed at the time of the first bleeding; (cf. data obtained in the control and experimental groups with 0.1  $\mu$ l of serum as inhibitor). In five of the nine mice in the experimental group, the degree of suppression was almost complete at this time, as indicated by the small degree of inhibition of binding caused by 3  $\mu$ l or 10  $\mu$ l of serum. By the time of the second bleeding, only five mice survived. Two of these were completely suppressed, one was partially suppressed, and two produced normal amounts of CRI. There was little change in the status of each of these five mice between the first and second bleedings.

Of the group of mice that were first immunized 22 wk after treatment with antiidiotypic IgG, 5 of the 10 were almost completely suppressed with respect to production of idiotypic at the time of the first bleeding, whereas 5 mice showed no indication of suppression. At the time of the second bleeding, four of the eight surviving mice failed to produce CRI; the other four did not differ from the controls. There was no change in the status of individual mice between the first and second bleedings.

*Levels of Antihapten Antibody in Control and Suppressed Mice.*—Quantitative precipitin analyses were carried out to determine the concentration of precipitable anti-Ar antibodies in the sera of control and suppressed mice subsequent to immunization with KLH-Ar. Sera of each group were pooled separately, using an equal amount of antiserum from each mouse. The mice that made up the "suppressed" group included only those mice which failed to produce CRI (not those which did produce CRI despite the administration of anti-D antibody). The test antigen used was BGG-Ar. Precipitin tests were carried out with five concentrations of antigen; the curve went through a maximum in each case. The methods used for quantitation and for correcting for the antigen content in the precipitate have been described (1).

The data are presented in Table III. It is evident that there were no significant differences between the control and suppressed groups at the time of the second bleeding. The average titers in both groups, however, decreased with time. This may possibly be a function of the age of the mice.

*Effect of Dosage of Antiidiotypic Antibody.*—Varying amounts of antiidiotypic IgG were administered to four groups of mice (10 per group). 6 wk later the mice were challenged intraperitoneally with 500  $\mu$ g of KLH-Ar in Freund's complete adjuvant. 2 wk and 4 wk later a second and third injection were given in the same manner, except that Freund's incomplete adjuvant was used for the third inoculation. Bleedings were taken 1 wk after the second and third injections.

TABLE III  
*Concentration of Anti-Ar Antibodies in Mice Treated with Nonspecific or Antiidiotypic IgG\**

Interval between suppression and first antigen challenge	Serum pool no. of mice	Immune status	Precipitable anti-Ar antibody
<i>wk</i>			<i>mg/ml</i>
2	9	Control	5.8
2	9	Suppressed	5.4
12	4	Control	4.1
12	2	Suppressed	4.6
22	3	Control	3.4
22	3	Suppressed	3.7

\* Serum samples were pooled from bleedings taken after two injections of KLH-Ar in Freund's complete adjuvant.

A control group of mice was treated identically, except that nonspecific IgG was injected in place of antiidiotypic IgG. The control mice in group 3, Table I, also served as controls for this experiment since they were inoculated with 4 mg of nonspecific IgG 6 wk before immunization, and immunization and bleeding of those mice were done in the same way.

Of the group of mice pretreated with 4 mg of antiidiotypic IgG, all seven survivors failed to produce a significant titer of antibody-bearing CRI (Table IV). This result is in agreement with the data obtained with the other group of mice, which also were inoculated with 4 mg of antiidiotypic antibody 6 wk before immunization with KLH-Ar (Table I).

With one exception, the mice which received 2 mg of antiidiotypic IgG were suppressed with respect to production of CRI; eight of the group were completely suppressed, one was partially suppressed, and one did not differ significantly from controls.

All mice which received 0.4 mg of antiidiotypic IgG were only partially suppressed or not suppressed at all at the end of the 6 wk period, as evidenced by the inhibitory capacity of serum from the first bleeding. Those mice receiving 0.04 mg showed little evidence of suppression (Table IV).

*Effect of Variation in the Time of Administration of Antiidiotypic Antibody Relative to that of Antigen.*—In the experiments described above, and in previous work (2, 3), anti-D was injected at least 2 wk before antigen. A series of experiments was carried out in which the anti-D was given on days -14, -7, -3, 0, or +3, with the time of injection of antigen denoted as day 0. The anti-D (4 mg of an IgG fraction) was inoculated subcutaneously in saline solution and the antigen (KLH-Ar) was given intraperitoneally in Freund's complete adjuvant. When the two reagents were given on the same day, antigen was injected immediately after the anti-D. A second inoculation of antigen was administered

TABLE IV  
*Variation of dose of Antiidiotypic IgG Used for Suppression of Idiotype\**

Mouse no.	Wt antiidiotypic or nonspecific IgG	<sup>125</sup> I-labeled purified D antibody bound (% of control)							
		Second bleeding				Third bleeding			
		μl of serum tested as inhibitor							
		0.03	0.1	3	10	0.03	0.1	3	10
7 mice	<i>mg</i> 4 (anti-D)			82-92†	78-86			61-94	51-82
S-11§	2-(anti-D)			30	6				
S-22				87	84			86	84
S-23				92	87			89	86
S-24			96	48	31				
S-25				96	96			101	99
S-26				86	79				
S-27				89	87			91	86
S-28				95	91			92	92
S-29				94	93			93	99
S-30				95	96			98	95
S-32	0.4 (anti-D)	95	28	11	0				
S-34			96	65	57	83	71	51	
S-35		94	85	78	73	85	78	73	
S-36				32	17	91	73	27	
S-37		93	32	16	0	61	29	14	
S-38		92	82	27	12	86	70	32	
S-40		42	6	0	0				
S-41	0.04 (anti-D)	44	24						
S-42		92	36			19	13	6	10
S-43		91	62	0	0	58	27	0	0
S-44		72	35						
S-47		76	31	10		36	22	8	3
S-48		91	84	9	0				
S-49		73	33			40	22	0	0
S-50		68	29			24	15	0	0
6 mice controls	4 (non-specific IgG)	19-86†	18-39			19-22	8-14		

\* In the absence of inhibitor, 53% (net value) of the labeled ligand was bound. All experiments were carried out in duplicate. The average deviation from the mean, expressed as percent of control was 4%.

† For mice injected with 4 mg of antiidiotypic or nonspecific IgG, a range of values is given.

§ Same as second footnote, Table I, except that nonspecific or antiidiotypic IgG was administered subcutaneously, rather than i. p.

|| Deceased.

in Freund's complete adjuvant 17 days after the first. Test bleedings were taken 10 days after the first inoculation of antigen and 7 days after the second. At the time of the second bleeding the sera of all mice gave strong precipitin lines in agar gel with the test antigen, BGG-Ar. No precipitin lines were observed with

TABLE V  
*Variation of the Time of Administration of Anti-D Relative to that of Antigen\**

Time of administration of anti-D	Inhibitor (mouse no.)	<sup>125</sup> I-labeled purified anti-Ar antibody bound (% of control)				
		First bleeding		Second bleeding		
		μl of serum tested as inhibitor				
		1	10	0.1	1	10
<i>day</i>						
(a)						
-7‡	Controls 12 mice	28-50§		6-20§		
-7	S-1		69		¶	¶
	S-2		74		86	74
	S-3		72		77	62
	S-4		89		94	90
	S-5	90	24		24	16
	S-6	99	40		20	6
	S-7		88		69	69
	S-8		94		75	65
	S-9		84		73	63
	S-10		84		¶	¶
	S-11	93	20		¶	¶
	S-12		81		75	73
	S-13	90	16		12	4
	S-14		83		¶	¶
-3	Controls 9 mice 1 mouse	20-40§ 74		14-19§ 20		
-3	S-31		89		0	0
	S-32	101	64		69	59
	S-33	102	34		49	17
	S-34		90		73	68
	S-35	99	37		11	6
	S-36		83		76	73
	S-37		86		55	14
	S-38	87	19		¶	¶
	S-39	94	75		56	22
	S-40		80		8	3
	S-41		82		¶	¶
	S-42		80		52	27
	S-43	96	76		¶	¶
	S-44	31	13		0	0

TABLE V—*Continued*

Time of administration of anti-D	Inhibitor (mouse no.)	<sup>125</sup> I-labeled purified anti-Ar antibody bound(% of control)				
		First bleeding		Second bleeding		
		1	$\mu$ l of serum tested as inhibitor 10	0.1	1	10
<i>day</i>						
(b)						
0†	Controls§ 14 mice	16-58§		5-18§		
0	S-61	103	88		21	14
	S-64	84	54		30	9
	S-66	75	16		15	7
	S-67	96	80		60	55
	S-68	98	81		65	48
	S-69	101	78		79	74
	S-70	100	83		41	20
	S-71	99	76		19	12
	S-72	98	68		37	17
	S-73	98	84		73	55
	S-74	98	68		30	9
+3	Controls 11 mice 1 mouse	16-49 93		8-37 53	0-25 24	
+3	S-91	93	67	62	40	8
	S-92	70	15	¶	¶	
	S-93	25	6	25	24	
	S-94	84	59	¶	¶	
	S-95	38	8	30	6	
	S-96	76	41	44	24	
	S-97	17	6	35	28	
	S-98	42	12	42	27	
	S-99	84	51	75	63	24
	S-100	23	7	¶	¶	
	S-101	58	12	54	23	
	S-102	69	30	51	34	
	S-103	50	8	37	26	
	S-104	66	11	¶	¶	

\* In the absence of inhibitor, 50% (net value) of the labeled ligand was bound. All experiments were carried out in duplicate. The average deviation from the mean, expressed as percent of control, was 3%.

† The time of the first inoculation of antigen is designated as day 0. Antigen was injected again on day +10. The number -7 indicates that anti-D was administered 7 days before antigen.

§ For the control mice, a range of values is given.

|| The letter S designates a suppressed mouse, which had received 4 mg of antiidiotypic IgG. Control mice received 4 mg of nonspecific rabbit IgG. The protocol of immunization is given in the text. The nonspecific or antiidiotypic IgG was administered subcutaneously.

¶ Deceased.

sera from the first bleeding although inhibition tests, which are more sensitive, showed the presence of cross-reactive idiotype in all nonsuppressed mice.

The results are shown in Tables V *a* and V *b*. Data on those mice treated with anti-D 14 days before antigen are not tabulated; the 10 mice in that group were all suppressed with respect to the formation of CRI, in confirmation of the results shown, for another similarly treated group, in Table I. As indicated in Table V *a*, 10 of 14 mice treated with anti-D 7 days before antigen did not possess significant titers of CRI at the time of the first bleeding (10 days after inoculation of antigen). Of these 10 mice, all that survived to the time of the second bleeding were still suppressed (Table V *a*). These results indicate that suppression may be somewhat less effective when anti-D is given at day -7, rather than day -14.

The anti-D was considerably less effective in suppression when given on day -3 (Table V *a*). Although 10 of the 14 mice had not produced CRI at the time of the first bleeding, all but 3 mice had escaped suppression, partially or entirely, by the time of the second bleeding.

The results were much the same when anti-D was given on day 0 (Table V *b*). Many mice lacked CRI at the time of the first bleeding; however, all but one mouse showed significant titers of CRI in the second bleeding. On the average the titers of CRI were somewhat lower than those of the control group, which had received 4 mg of nonspecific rabbit IgG in place of anti-D IgG. When the anti-D was given 3 days subsequent to antigen there was little evidence of suppression, even in sera of the first bleeding, and virtually none at the time of the second bleeding (Table V *b*).

Tests were carried out with sera of the latter group of mice to determine whether any circulating immunoglobulin having CRI was present on day 3 before the administration of anti-D. 10  $\mu$ l of each serum failed to inhibit significantly the binding of the labeled ligand (purified anti-Ar antibody of mouse 126) by its antiidiotypic antibodies.

Thus, for optimal suppression anti-D must be administered at least 2 wk before antigen. Its effectiveness is diminished at day -7 or -3. There is little suppressive effect when anti-D is given on day 0 and virtually no effect when it is administered 3 days after the antigen.

*Attempts to Suppress Idiotype with Fab' or F(ab')<sub>2</sub> Fragments of Antiidiotypic Antibody.*—Table VI summarizes the results of experiments in which mice were pretreated with Fab' or F(ab')<sub>2</sub> fragments of the IgG fraction of rabbit antiidiotypic antibody that was used in the experiments described above. Protocols were identical with those of the experiments summarized in Table IV. Thus, the first inoculation of antigen (500  $\mu$ g KLH-Ar) was given 6 wk after the antiidiotypic Fab' or F(ab')<sub>2</sub> fragments, and the second inoculation 2 wk later. Freund's complete adjuvant was used for both injections. The mice were bled 1 wk after the second inoculation.

The results in Table VI indicate that 1.3 mg or 2.7 mg of F(ab')<sub>2</sub> fragments,

TABLE VI  
*Effect of Administration of Fab' or F(ab')<sub>2</sub> Fragments of Antiidiotypic IgG on the Subsequent Production of the Cross-Reactive Anti-Ar Idiotypic*

No. of mice	Pretreatment	Volume of inhibitor* (serum)	<sup>125</sup> I-ligand bound	
			% of control†	
			Median	Range
		μl		
6	4 mg RIgG§	0.1	20	18-39
7	4 mg Anti-D (IgG)	10	81	79-86
7	2.7 mg F(ab') <sub>2</sub> (RIgG)	0.1	25	18-32
15	2.7 mg F(ab') <sub>2</sub> Anti-D	0.1	21	9-37
17				
2	2.7 mg F(ab') <sub>2</sub> Anti-D	0.1	72	68-76
		1.0	19	19-20
7	1.3 mg F(ab') <sub>2</sub> Anti-D	0.1	21	13-38
9				
2	1.3 mg F(ab') <sub>2</sub> Anti-D	0.1	78	67-88
		1.0	17	14-20
7	2.7 mg Fab' RIgG	0.1	27	13-38
14	2.7 mg Fab' Anti-D	0.1	21	9-44
5	1.3 mg Fab' Anti-D	0.1	21	18-28

\* IgG, Fab', or F(ab')<sub>2</sub> fragments were administered subcutaneously in saline 6 wk before challenge with KLH-Ar. Inhibition tests were carried out with antisera obtained from the second bleeding, 3 wk after the start of immunization. 10 μl of pooled normal A/J serum, tested as a control, caused less than 5% inhibition of binding.

† In the absence of inhibitor, 53% (net value) of the labeled ligand was bound. Experiments were carried out in duplicate. The average deviation from the mean, expressed as percent of control, was 4%.

§ RIgG, rabbit IgG.

(equivalent to 2 mg or 4 mg, respectively, of IgG with respect to number of combining sites) had very little if any suppressive effect on the appearance of the cross-reactive idiotype. 1 μl of antiserum from all mice receiving the Fab' or F(ab')<sub>2</sub> fragments strongly inhibited the binding reaction, whereas 10 μl of antiserum from mice receiving the whole IgG fraction of anti-D antibody was in each case not inhibitory.

#### DISCUSSION

When immunized with KLH-Ar all mice of the A/J strain investigated so far have produced substantial titers of anti-*p*-azophenylarsonate (anti-Ar) antibodies with cross-reactive idiotypic specificities (CRI); antiidiotypic antisera prepared in rabbits against specifically purified anti-Ar antibodies from seven individual mice have been used in these studies. The CRI is in each case present on a substantial fraction of the

total anti-Ar antibody population, as shown by the low concentration needed for inhibition of binding to its antiidiotypic antibodies of the labeled anti-Ar antibody used as the immunogen (2, 8). Including those in the present study, more than 250 mice have been investigated.

The presence of CRI in all immunized A/J mice made it possible to demonstrate suppression of the idiotypic specificity by inoculation of antiidiotypic antibody prepared against the anti-Ar antibodies of individual mice (2, 3). In those investigations the initial challenge with antigen was made 2 wk (in adult mice) or 9 wk (in neonatal mice) after the antiidiotypic antibody. In each mouse this regimen resulted in the subsequent production of anti-Ar antibodies lacking CRI. The suppression persisted, in all but one mouse, for the duration of the experiment (up to 5 mo). In each mouse, however, anti-Ar antibodies appeared in substantial concentrations. Thus, the initial challenge with antigen resulted in the stimulation of clones of cells which were not producing CRI. It is conceivable, therefore, that antigen injected later, to determine whether suppression had persisted, was captured by receptors on the cells of these stimulated clones. Thus, clones of cells capable of producing CRI, even if they had re-emerged, might not have been detected, owing to preferential capture of antigen by relatively large numbers of memory cells belonging to unrelated clones.

To circumvent this possibility, the duration of suppression was investigated in the present study by varying the time interval between the administration of antiidiotypic IgG and the first challenge with antigen. Adult A/J mice were used in all experiments.

When 4 mg of antiidiotypic IgG was injected, all mice were almost completely suppressed, with respect to the production of CRI, when challenged with antigen either 2 wk or 6 wk after inoculation of antiidiotypic IgG. When the first inoculation with antigen was given either 12 or 22 wk after the antiidiotypic IgG, roughly half of the mice in each group were strongly suppressed with respect to the production of CRI, a few other mice showed partial suppression, and some mice were not suppressed at all. These data, together with our previous results (2, 3), indicate that suppression of CRI is maintained more effectively if antigen is administered soon after anti-D. Nevertheless, a substantial proportion of the mice tested remained suppressed for 5 mo, even without intervening challenge with antigen. The greater suppression in previous experiments may have been due to stimulation by antigen of new clones of cells, producing anti-Ar antibody lacking CRI, which subsequently captured antigen and prevented the expression of reemerging clones capable of producing CRI.

Variations in the dose of antiidiotypic IgG (Table IV) indicated that at least 2 mg was required for substantial suppression of most mice and that 4 mg was somewhat more effective than 2 mg. Effective suppression was observed when the antiidiotypic antibody was administered either intraperitoneally (Tables I and II) or subcutaneously (Table IV), in each case without adjuvant.

The time of administration of anti-D, in relation to that of antigen, was

found to be critical. When anti-D was given 2 wk before antigen, each of the mice in the group failed to produce CRI. Suppression was somewhat less effective when anti-D was administered 7 days before the antigen; about one-fourth of the mice produced CRI. Still less suppression was noted when anti-D was given 3 days before, or on the same day as the antigen; nearly all mice produced CRI, although in many the serum concentration was less than that of control mice. Little, if any suppression was observed when anti-D was injected 3 days after the antigen. This is probably not attributable to simple absorption of the anti-D by circulating anti-Ar antibody since no such antibody was detectable by the very sensitive inhibition-of-binding test just before administration of anti-D.

The fact that anti-D is ineffective when given 3 days after the antigen suggests that it cannot inhibit production of antibody once precursor cells have been triggered by antigen and the process of differentiation has begun. A similar effect was noted by Cosenza and Kohler (9) in their studies carried out *in vitro*.

The fact that anti-D is ineffective when administered subsequent to antigen demonstrates that it does not suppress simply by absorbing D molecules, but rather exerts its effect on the antibody-producing system. We had reached a similar conclusion earlier (2) on the basis of the fact that control mice produce CRI in amounts far exceeding the capacity of the anti-D to absorb them, even if none of the anti-D were catabolized. It is of interest that X irradiation is also much less effective in suppressing an immune response when given subsequent to the antigen (10); this is attributable to rapid triggering of cellular differentiation upon contact with antigen, and relative insensitivity of the differentiated cells to irradiation.

Neither Fab' nor F(ab')<sub>2</sub> fragments of antiidiotypic IgG were effective in causing suppression. No significant differences could be detected among the responses of mice administered nonspecific IgG or either Fab' or F(ab')<sub>2</sub> fragments of antiidiotypic antibodies. The inactivity of Fab' or F(ab')<sub>2</sub> might be related to their inability to fix complement through the normal pathway. (We do not yet know, however, whether complement is required for inactivation of cells. This question is being investigated through experiments *in vitro*.) Alternatively, the fragments may be cleared so rapidly from the mouse that the concentration reaching the appropriate cell surfaces is too small to be effective. The half-life in the mouse of rabbit F(ab')<sub>2</sub> or Fab fragments, is less than 0.5 day, as compared to 5.7 days for rabbit IgG (11). (The half-life of rabbit Fab' in the mouse was not reported.)

A significant question is whether recovery from suppression is attributable to the reactivation of suppressed cells or to the generation of new cells capable of synthesizing molecules with CRI. One can rephrase the question by asking whether antiidiotypic antibodies kill, or merely inactivate, cells bearing receptors with CRI. In the case of immune suppression of one allotype of an allelic pair in rabbits, fluorescent staining has demonstrated the absence of cells containing molecules of the suppressed allotype (12). Because of this and

the following considerations we favor the possibility of elimination of cells, rather than reversible inactivation, as the basis of suppression. (a) It is known that rabbit antimouse Ig is capable of killing mouse B cells in the presence of guinea pig complement (13). (We could not find similar data for mouse complement.) (b) The prolonged presence of a heterologous immunoglobulin on a cell surface might induce phagocytosis of the cell even if it did not kill it directly. (c) Regeneration of surface determinants after cap formation and pinocytosis appears unlikely as an explanation for escape from suppression, since regeneration occurs within 24 h (14), whereas the mice remained suppressed for much longer periods of time. Repeated regeneration of idiotypic determinants followed by reaction with circulating antiidiotypic antibody seems conceivable but unlikely, especially since suppression persisted until levels of circulating rabbit IgG must have been extremely low (11).

One might inquire, then, as to why only half of the animals recovered in 12 or 22 wk. One possibility is that the regeneration of new precursors cells is a random process; the time required could vary greatly among individual mice. It is possible that all mice might recover in time. An alternative hypothesis is that the antiidiotypic antibodies are capable of attacking immature stem cells, responsible for the eventual regeneration (over a period of months) of clones producing CRI, and that these stem cells were eliminated in some but not all suppressed mice. (This assumes that the immediate precursor cells are removed or inactivated in all suppressed mice.)

The duration of suppression was less pronounced in these experiments than in previous work, in which antigen was first administered 2 wk or 9 wk after antiidiotypic IgG (2, 3). In those studies, except for one mouse which did not appear to be suppressed at any time (3), all mice failed to produce CRI throughout the period of observation (2, 3, and unpublished data). In those experiments 15 mice survived for 3 mo and 9 for 5 mo. The escape of some mice from suppression after 12 wk in the present experiments is probably related to the fact that antigen was not administered in the interim; the rationale for this was stated earlier in the Discussion.

Adoptive transfer experiments in which cells are treated with antiidiotypic antibody in vitro, in the presence or absence of complement and at different temperatures, should provide additional information as to mechanisms of suppression and recovery.

#### SUMMARY

The appearance of an idiotypic specificity, present in anti-*p*-azophenylarsenate (anti-Ar) antibodies of all immunized A/J mice, can be suppressed in adult mice by prior administration of an IgG fraction of rabbit antiidiotypic (anti-D) antiserum; anti-Ar antibodies arise but are of different idio type. Prolonged suppression was observed in earlier experiments, but antigen was first administered to adult mice only 2 wk or 9 wk after anti-D antibodies; subsequent escape from idiotypic suppression could have been masked by the capture of antigen by large numbers of memory cells having receptors of a different

idiotype. In the present experiments antigen was first administered at intervals up to 22 wk after the antiidiotypic antibody. Suppression was maintained for 6 wk in all mice and for 5 mo in about half the mice tested. It thus appears that suppression of idiotype is less reversible if antigen is administered soon after the antiidiotypic antibody. The data suggest that escape from suppression is attributable to the generation of new precursor cells rather than to reactivation of suppressed cells.

The minimum dosage of antiidiotypic IgG required for effective suppression was about 2 mg. The subcutaneous or intraperitoneal routes of inoculation of antiidiotypic IgG were equally effective. When antiidiotypic antibody was administered 3 days after antigen no suppressive effects were observed. There was partial suppression when antiidiotypic antibody was injected on the same day as the antigen. Fab' and F(ab')<sub>2</sub> fragments of antiidiotypic IgG had no suppressive effect. Quantitative measurements revealed no significant differences among control and suppressed mice with respect to total concentration of precipitable anti-Ar antibodies produced.

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#### REFERENCES

1. Kuettner, M. G., A. L. Wang, and A. Nisonoff. 1972. Quantitative investigations of idiotypic antibodies. VI. Idiotypic specificity as a potential genetic marker for the variable regions of mouse immunoglobulin polypeptide chains. *J. Exp. Med.* **135**:579.
2. Hart, D. A., A. L. Wang, L. L. Pawlak, and A. Nisonoff. 1972. Suppression of idiotypic specificities in adult mice by administration of antiidiotypic antibody. *J. Exp. Med.* **135**:1293.
3. Pawlak, L. L., D. A. Hart, A. Nisonoff, E. Mushinski, and M. Potter. 1972. Idiotypic specificity and the biosynthesis of antibodies. Proceedings of The Third International Convocation in Immunology. S. Karger, Basel. In press.
4. Hart, D. A., L. L. Pawlak, and A. Nisonoff. 1973. Nature of antihapten antibodies arising after immune suppression of a set of cross-reactive idiotypic specificities. *Eur. J. Immunol.* **1**:4.
5. Hunter, R. 1970. Standardization of the chloramine-T method of protein iodination. *Proc. Soc. Exp. Biol. Med.* **133**:989.
6. Nisonoff, A. 1964. *Methods Med. Res.* **10**:134.
7. Pawlak, L. L., A. L. Wang, and A. Nisonoff. 1972. Concentration of cross-reacting idiotypic specificities in unrelated mouse immunoglobulins. *J. Immunol.* **110**:597.
8. Pawlak, L. L., and A. Nisonoff. 1973. Distribution of a cross-reactive idiotypic specificity in inbred strains of mice. *J. Exp. Med.* **137**:855.
9. Cosenza, H., and H. Kohler. 1972. Specific suppression of the antibody response by antibodies to receptors. *Proc. Natl. Acad. Sci. U. S. A.* **69**:2701.
10. Taliaferro, W. H., and L. G. Taliaferro. 1954. Further studies on the radiosensitive stages in hemolysin formation. *J. Infect. Dis.* **95**:134.

11. Spiegelberg, H. L., and W. O. Weigle. 1965. The catabolism of homologous and heterologous 7S gamma globulin fragments. *J. Exp. Med.* **121**:323.
12. Harrison, M. R., R. G. Mage, and J. M. Davie. 1973. Deletion of b5 immunoglobulin-bearing lymphocytes in allotype-suppressed rabbits. *J. Exp. Med.* **137**: 254.
13. Takahashi, T., L. J. Old, K. R. McIntire, and E. A. Boyse. 1971. Immunoglobulin and other surface antigens of cells of the immune system. *J. Exp. Med.* **134**:815.
14. Loor, F., L. Forni, and B. Pernis. 1972. The dynamic state of the lymphocyte membrane. Factors affecting the distribution and turnover of surface immunoglobulins. *Eur. J. Immunol.* **2**:203.