QUANTITATIVE INVESTIGATIONS OF IDIOTYPIC ANTIBODIES

VI. IDIOTYPIC SPECIFICITY AS A POTENTIAL GENETIC MARKER FOR THE VARIABLE REGIONS OF MOUSE IMMUNOGLOBULIN POLYPEPTIDE CHAINS*

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Myeloma proteins (1) and antibodies of a given specificity (2, 3) produced in an individual animal possess individually specific or "idiotypic" (4) antigenic determinants.[1] Idiotypic cross-reactions among antibodies of the same specificity from different individual rabbits or humans are infrequent (2, 3, 5, 6) and are generally weak when they do occur (7). Eichmann and Kindt (8), however, recently reported strong cross-reactions among the antistreptococcal antibodies of some of the rabbits of a family group. These rabbits were descended from a brother-sister mating pair selected on the basis of production of antibodies of limited heterogeneity in response to streptococcal cell wall antigens.

There is evidence that idiotypic cross-reactions may be more frequent among mice of an inbred strain than among rabbits. Cohn et al. (9) prepared anti-idiotypic antibodies in an A/J mouse against a BALB/c myeloma protein with antibody-like activity against pneumococcal C carbohydrate. Antisera to C carbohydrate prepared in 6 of 10 normal BALB/c mice formed precipitates with the antiidiotypic antiserum in the Ouchterlony test. The intensities of the precipitin lines were weaker with the cross-reacting sera than with the myeloma protein used as immunogen and lines of identity were not demonstrated. In addition, idiotypic cross-reaction was noted with another myeloma protein with anti-C carbohydrate activity. Potter and Lieberman (10) examined eight BALB/c myeloma proteins which bind phosphorylcholine and found that five share idiotypic determinants, giving lines of identity in the Ouchterlony test.

The present investigation was designed to provide quantitative information

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[1] Because of its convenience, the term "idiotypic determinants" will be used in this paper to denote individually specific antigenic determinants recognized by an antiserum prepared in a heterologous species, in this case the rabbit. As originally defined (4) the term would apply only when homologous antiserum is used.

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on the extent of idiotypic cross-reactions within mice of a given strain and among different strains of mice.

Materials and Methods

Preparation of Antigens.—Hapten-protein conjugates were prepared by diazotizing *p*-aminobenzoic acid or *p*-aminophenylarsonic acid and coupling to protein in a ratio of 40 mg of hapten to 1 g of protein (11).

Immunization of Mice.—Mice were immunized with keyhole limpet hemocyanin (KLH)-*p*-azobenzoate or KLH-*p*-azophenylarsonate.[2] In all mice the method of Sommerville (12) was used initially to produce an ascites fluid. The antigen was incorporated in complete Freund's adjuvant for the initial intraperitoneal injection and incomplete Freund's adjuvant for the subsequent inoculations. *Mycobacterium smegmatis* was also present in the later injections of this series (12). The amount of antigen used for each inoculation was either 100 μg (KLH-*p*-azobenzoate) or 500 μg (KLH-*p*-azophenylarsonate). After the development of ascites fluid several milliliters of the fluid was removed. Inoculations were then continued at 2- to 3-wk intervals. The Ouchterlony method was used to determine the presence of precipitating antibodies in test bleedings, employing as antigen rabbit IgG to which the appropriate hapten was conjugated. Bleedings were initiated after the Ouchterlony test showed the presence of strongly precipitating antibodies. A booster inoculation was generally required every 2–3 wk. Mice were bled retroorbitally starting 5 days after an injection. Two groups of mice (A/J and BALB/c mice inoculated with KLH-*p*-azobenzoate) were bled as many as four times weekly. 1–2 ml of hyperimmune serum was obtained from most mice before they died. Nearly 4 ml of antiserum was obtained from each of two mice (one A/J antibenzoate and one BALB/c antibenzoate) and used as the basis for preparation of anti-idiotypic antibodies. The third antiidiotypic antiserum was prepared against anti-*p*-azophenylarsonate antibodies isolated from the ascites fluid of an A/J mouse. A total of about 200 mice were inoculated. A number of antisera of high titer were selected to test for cross-reactions with the three antiidiotypic antisera. All tests of inhibition were carried out with serum rather than with ascites fluid.

Quantitation of Antibody in Hyperimmune Antisera.—Quantitative precipitin tests were carried out with each serum used as a source of idiotypic antibody or as an inhibitor of idiotypic reactions. 50-μl portions of serum were mixed with 50 μl of the test antigen (rabbit IgG-hapten conjugate). Successive concentrations of antigen were varied by a factor of 1.7–2. Usually four concentrations were tested in order to determine the equivalence point. Amounts of precipitate were quantified by optical density measurements at 280 nm and 400 or 452 nm, after dissolving the precipitate at alkaline pH in a volume of 0.4 ml. The known ratio of absorbances of the antigen at the two wave lengths was used to correct readings at 280 nm for the contribution by antigen. The antibody concentration in serum was calculated from the net reading at 280 nm by using the extinction coefficient, $E_{1cm}^{1\%} = 15$. Optical densities were read in quartz microcells. The concentrations of antibody in the three donor preparations used for isolation of idiotypic antibody were: A/J antibenzoate serum, 3.0 mg/ml; BALB/c antibenzoate serum, 1.6 mg/ml; and A/J antiphenylarsonate ascites fluid, 1.2 mg/ml.

Immunization of Rabbits.—Specific precipitates were formed by adding an equivalent amount of rabbit IgG-hapten conjugate either to hyperimmune mouse serum (anti-azobenzoate) or ascites fluid (anti-azophenylarsonate). Precipitation was carried out in the presence of 0.01 M ethylenediaminetetraacetate (EDTA) to minimize uptake of complement components. The washed precipitate, containing approximately 1 mg of mouse antibody, was dissolved in 0.1 M sodium acetate buffer, pH 3.5, immediately emulsified in an equal volume of

[2] *Abbreviations used in this paper*: Ab, antibody; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin.

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complete Freund's adjuvant, and injected into several intradermal and intramuscular sites of a rabbit. This was repeated after 2 wk and again 1 or 2 wk later. The rabbit was bled 1 and 2 wk after the final injection. The serum of each recipient rabbit reacted strongly with mouse IgG in the Ouchterlony test.

Absorption of Antisera.—Rabbit antisera were rendered specific for idiotypic determinants by absorbing, first, with nonspecific IgG from the strain of mouse that provided the antibody used for immunization. Precipitation was carried out at equivalence and an additional 100–200 μg of mouse IgG/ml of rabbit antiserum was then added. Rabbit antiserum to mouse antiphenylarsonate antibody was further absorbed with 20 μl of mouse serum of the donor strain (A/J)/ml of antiserum. This absorption was found to be unnecessary to make the other rabbit antibodies specific for idiotypic determinants.

Specific Purification of Mouse Antibodies.—Three preparations, one from each mouse whose antibodies were used to elicit antiidiotypic antibodies, were purified specifically, labeled with ^{125}I, and used for quantitative testing. Mouse antibodies to KLH–*p*-azobenzoate or KLH–*p*-azophenylarsonate were precipitated at equivalence from hyperimmune serum with rabbit IgG conjugated to the corresponding hapten group; 0.01 M EDTA was present during the precipitation. The washed precipitate was dissolved in 0.3 M *p*-nitrobenzoate (antibenzoate antibody) or 0.3 M *p*-aminophenylarsonate (antiphenylarsonate antibody) and passed through diethylaminoethyl (DEAE)–cellulose equilibrated with 0.04 M phosphate buffer, pH 8. The antigen, which is colored, was retained near the top of the column. The eluted antibody was dialyzed extensively against borate-buffered saline, pH 8, then against 0.1 M sodium benzoate or sodium phenylarsonate, pH 8, and finally for a week against multiple changes of the borate buffer. Yields of antibody varied from 0.5 to 1.6 mg/ml of serum or ascites fluid.

The purified BALB/c antibenzoate antibody appeared to consist largely of γG_1 immunoglobulin. The A/J antibenzoate and the A/J antiphenylarsonate antibodies were mixtures of γG_1 and γG_2. The appropriate reagents were not available to distinguish between γG_{2a} and γG_{2b}.

Labeling of Purified Antibodies with ^{125}I.—Portions of the purified antibody (100–200 μg) were labeled with ^{125}I by the chloramine-T method (13). Less than 1.5 atoms of iodine per molecule of protein were incorporated. The specific activity of labeled proteins varied from 3000 to 7000 cpm/μg.

Quantitation of Antiidiotypic Antibodies.—This was done by an indirect method of precipitation. The ^{125}I-labeled specifically purified antibody (0.02 or 0.1 μg) was mixed with an excess of absorbed rabbit antiserum; the amount required varied from 1 to 6 μl in the three systems studied. When less than 3 μl was used, the rabbit antiserum was first diluted with rabbit antiovalbumin antiserum so that the total volume of rabbit serum present was 3–6 μl in each test. This provided an adequate precipitate upon subsequent addition of excess goat antiserum (40–50 μl) specific for fragment Fc of rabbit IgG. The goat antiserum was first absorbed with mouse serum of the strain which provided the purified antibody.

After standing overnight at 5°C the precipitate was washed three times with neutral buffer and dissolved in dilute NaOH. The dissolved precipitate and the combined supernatants were adjusted to the same volume and counted separately to determine the fraction of radioactivity precipitated. A minimum of 3000 counts above background was recorded for each precipitate-supernatant pair. Various unlabeled antisera to be tested as inhibitors were preincubated for 30 min with the absorbed rabbit antiserum. Radiolabeled specifically purified antibody was then added to the mixture, followed by goat antirabbit fragment Fc.

Removal of Antibodies from Antisera by Immunoabsorption.—In each series of experiments antibodies were removed from hyperimmune sera by immunoabsorption to determine how this affected the capacity to inhibit the reactions of antiidiotypic antibodies. Immunoabsorbents were prepared by the method of Axen et al. (14) using Sepharose 4B (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) and cyanogen bromide. The proteins coupled to Sepharose were bovine serum albumin–*p*-azophenylarsonate or rabbit IgG–*p*-azobenzoate.

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The initial reaction mixture contained 5–10 mg of protein/g of packed Sepharose. After coupling, the absorbent was treated with dilute ammonia and with a 1% solution of bovine serum albumin and was then washed extensively. To carry out an absorption, hyperimmune serum was mixed with the Sepharose absorbent in a ratio of 50–100 μl/200 mg of packed absorbent. After agitating gently for 4 hr the liquid phase was eluted with buffer. Appropriate corrections for dilution were made when the eluate was subsequently tested. In all such experiments controls were carried out in which the hyperimmune serum was treated in an identical manner with Sepharose 4B to which no antigen had been coupled.

RESULTS

Specificity of Antiidiotypic Antisera.—Tables I, II, and III present data relevant to the specificity of the absorbed rabbit antibodies directed to mouse antihapten antibody in the three systems studied. In each case, as expected, the autologous (donor) antiserum was a strong inhibitor of the binding of labeled purified mouse antihapten antibody to its rabbit antiidiotypic antiserum. Idiotypic specificity was shown by the absence of inhibitory capacity in preimmune serum of the donor or in normal serum of other mice of the same strain or of two other strains. Removal of antihapten antibody from the donor serum by specific immune absorption also completely removed its inhibitory capacity. Control absorptions, carried out with unconjugated Sepharose, had no significant effect on the observed inhibition. Strongly precipitating antiserum to the carrier protein raised in the same strain of mouse was tested in one system (Table I) and failed to inhibit the reaction of antiidiotypic antibody directed to the antihapten antibody. Finally, antiserum to a different hapten elicited in the same strain of mouse was noninhibitory in each system (Tables I, II, and III).

Idiotypic Cross-Reactions of Antihapten Antisera Prepared in Individual Mice of the Same Strain.—Data on cross-reactions within a strain are shown in Figs. 1–4. Figs. 1, 2, and 4 also present data for antibodies of strains other than that of the donor mouse; these data will be discussed later. Fig. 1 shows the results obtained with antiidiotypic antibodies directed to anti-*p*-azophenylarsonate antibody of A/J mouse No. 413. Varying quantities of antiserum were tested for inhibition of binding of the ^{125}I-labeled antibody. The data are plotted in terms of the weight of precipitable antihapten antibody in the volume of antiserum used. It is apparent that the antiphenylarsonate antiserum of each of the 10 A/J mice investigated cross-reacted strongly with the antiidiotypic antiserum directed to the donor (No. 413) antibodies. At the highest concentration tested each A/J antiserum caused at least 80% inhibition of binding of ^{125}I-labeled antibodies of mouse No. 413. This result indicated that most of the specificities identified by the antiidiotypic antiserum were present in the immune serum of each A/J mouse.

A quantitative indication of the relative effectiveness as inhibitors of the antibodies of different mice can be obtained by comparing the weights of precipitable serum antibody necessary to give 50% displacement of the labeled

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reference antibody. These values, obtained by interpolation of the data in Fig. 1, are shown in Table IV.

The antibodies of the donor mouse, No. 413, were the most effective on a weight basis. From 4 to 38 times as much antibody from the sera of the other mice

TABLE I

*Reactions of an Antiidiotypic Rabbit Antiserum Directed to Anti-p-Azobenzoate Antibody from an A/J Mouse (No. 63)**

Inhibitor	Amount of inhibitor	^{125}I-labeled antibody precipitated
		% of control
Normal A/J serum (pool)‡	10 μl	94 ± 1
Normal BALB/c serum (pool)	10 μl	94 ± 0
Normal C57/BL serum (pool)	10 μl	92 ± 0
Preimmune donor serum§	10 μl	90 ± 3
Hyperimmune donor serum‖	10 μl	0 ± 1
Hyperimmune donor serum¶ (absorbed)	10 μl (equiv.)	101 ± 0
Hyperimmune donor serum** (control absorption)	10 μl (equiv.)	0 ± 3
Spec. purified donor antibody	30 μg	2.3 ± 5
Anti-KLH antisera (four A/J mice)	10 μl	93–108 (mean, 103)
Antiphenylarsonate antisera (nine A/J mice)‡‡	10 μl	95–108 (mean, 100)

* Precipitations were carried out by the indirect procedure using 0.1 μg of the labeled specifically purified antibenzoate antibody. Values are corrected by subtracting the percentage of radioactivity precipitated (9 ± 3% in three series of experiments) when rabbit antiovalbumin serum was substituted for the rabbit antiserum directed to mouse antibenzoate antibody. In the absence of inhibitor 45% (corrected value) of the labeled antibody was precipitated. When ^{125}I-labeled IgG from an A/J mouse was substituted for the labeled anti-azophenylarsonate antibody, 8% of the radioactivity was precipitated. Experiments were run in duplicate with the average deviations shown. When a group of individual sera was tested the range of values and the mean are given.

‡ Pooled from the sera of several nonimmunized mice.

§ From the A/J mouse which provided the antibody used for immunization and testing.

‖ Concentration of precipitable antibenzoate antibody, 3.0 mg/ml.

¶ Absorbed with Sepharose conjugated to rabbit IgG-*p*-azobenzoate (see Materials and Methods). The volume used for the inhibition test was equivalent to 10 μl of undiluted antiserum.

** Absorbed with unconjugated Sepharose.

‡‡ Containing 2.0–4.5 mg/ml of precipitable antibody.

tested was required to give 50% inhibition. The ratio was 6:1 or less in a majority of the mice. These extensive cross-reactions contrast with those observed in noninbred rabbits.

Results of similar experiments with antiidiotypic antibodies directed to anti-

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benzoate (rather than antiphenylarsonate) antibodies of an A/J mouse, No. 63, are shown in Figs. 2 and 3. In Fig. 2 data for individual mice are plotted with separate symbols. Again, a strong cross-reaction is seen with the hyperimmune antiserum of each of the nine A/J mice tested. The maximum degree of inhibition of binding by individual antisera ranges from 50 to 98%. All antisera but one caused at least 75% inhibition, indicating that a large fraction of the idio-

TABLE II

*Reactions of an Antiidiotypic Rabbit Antiserum Directed to Anti-p-Azophenylarsonate Antibody from an A/J Mouse (No. 413)**

Inhibitor	Amount of inhibitor	I^{125}-labeled antibody precipitated
		% of control
Normal A/J sera (11 mice)‡	10 μl	85–102 (mean 94)
Normal C57/BL sera (10 mice)	10 μl	90–98 (mean 93)
Normal DBA sera (10 mice)	10 μl	90–99 (mean 96)
Preimmune donor serum§	10 μl	98±2
Hyperimmune donor serum‖	10 μl	7±0
Hyperimmune donor serum¶ (absorbed)	10 μl (equiv.)	95±3
Hyperimmune donor serum** (control absorption)	10 μl (equiv.)	0±1
Spec. purified donor Ab	10 μg	3±1
Anti-*p*-azobenzoate antisera‡‡ (10 A/J mice)	10 μl	80–109 (mean 95)

* As in Table 1 except as follows: the amount of labeled, specifically purified antibody used per test was 0.02 μg. The percentage of radioactivity precipitated when rabbit anti-ovalbumin was substituted for antiidiotypic antiserum was 17±2%. In the absence of inhibitor 36% (corrected value) of the radioactivity was precipitated. The antiidiotypic antiserum precipitated 5% of ^{125}I-labeled normal mouse IgG. The amount of antiidiotypic antiserum used was about 2/3 of the quantity needed for maximal precipitation.

‡ Pooled from the sera of several nonimmunized mice.

§ From the A/J mouse which provided the antibody used for immunization and testing.

‖ Concentration of precipitable antibody, 1.8 mg/ml.

¶ Absorbed with Sepharose conjugated to BSA-*p*-azophenylarsonate.

** Absorbed with unconjugated Sepharose.

‡‡ Containing 0.9–6.5 mg/ml of precipitable antibenzoate antibody.

typic specificities in the donor antibodies were represented in the antiserum of each hyperimmunized A/J mouse.

To obtain a more quantitative comparison of the inhibitory capacity of antibodies of individual A/J mice, a series of tests was run with lower concentrations of inhibitory antisera with the results shown in Fig. 3. In this figure one symbol is used for all mice other than the donor (No. 63) but the data were again obtained with individual sera. It is apparent that, on a weight basis, the unlabeled antibody of the donor mouse was once again the most effective inhibitor. A

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quantitative comparison is shown in Table V, which was compiled by interpolation of data plotted in Figs. 2 and 3. Taking the amount of donor antibodies required for 50% inhibition as 1.0, the relative weights of antibenzoate antibodies required for 50% inhibition ranged from approximately 2 to 10 for the other A/J mice. The cross-reactions are quite similar in their pattern to those in the antiphenylarsonate system already discussed. The fact that more than

TABLE III

*Reactions of an Antiidiotypic Rabbit Antiserum Directed to Anti-p-Azobenzoate Antibody from a BALB/c Mouse (No. 401)**

Inhibitor	Amount of inhibitor	I^{125}-labeled antibody precipitated
		% of control
Normal BALB/c serum‡	10 μl	103 ± 2
Normal A/J serum	10 μl	104 ± 4
Normal C57/BL serum	10 μl	104 ± 1
Preimmune donor serum§	10 μl	113 ± 3
Hyperimmune donor serum‖	10 μl	0 ± 0
Hyperimmune donor serum¶ (absorbed)	10 μl (equiv.)	97 ± 4
Hyperimmune donor serum** (control absorption)	10 μl (equiv.)	1 ± 1
Spec. purified donor antibody	20 μg	0 ± 1
Antiphenylarsonate antisera from each of nine A/J mice‡‡	10 μl	95–105 (mean 100)

* As in Table I, except as follows: the percentage of radioactivity precipitated by rabbit antiovalbumin was 10 ± 2%. In the absence of inhibitor 38% (corrected value) of the radioactivity was precipitated. The antiidiotypic antiserum precipitated 13% of ^{125}I-labeled normal mouse IgG.

‡ Pooled from the sera of several nonimmunized mice.

§ From the A/J mouse which provided the antibody used for immunization and testing.

‖ Concentration of precipitable antibenzoate antibody, 1.6 mg/ml.

¶ Absorbed with Sepharose conjugated to rabbit IgG-*p*-azobenzoate (see Materials and Methods). The volume used for the inhibition test was equivalent to 10 μl of undiluted antiserum.

** Absorbed with unconjugated Sepharose.

‡‡ Containing 2.0–4.5 mg/ml of precipitable antibody.

an equal weight of unlabeled, as compared to labeled, reference antibody was required for 50% inhibition reflects the use of an excess of antiidiotypic antiserum.

In the third system studied, antibenzoate antibodies from a BALB/c mouse were used to elicit antiidiotypic antibodies. Cross-reactions in this system are shown in Fig. 4. The hyperimmune antiserum of the donor mouse, No. 401, completely displaced the reference-labeled antibodies from their antiidiotypic antibodies, as would be expected. The antisera of four of the other five BALB/c

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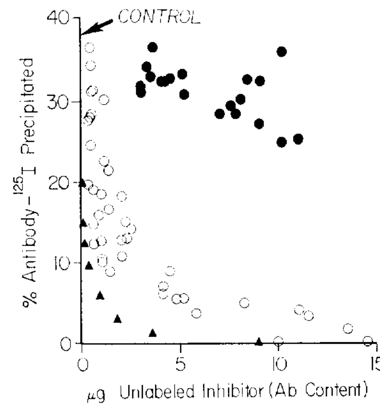

Fig. 1. Inhibition of binding to its antiidiotypic antibodies of 0.02 μg of ^{125}I-labeled specifically purified anti-*p*-azophenylarsonate antibody from A/J mouse No. 413. Inhibitors are unlabeled anti-azophenylarsonate antisera from individual mice. Data are plotted in terms of the weight of precipitable antibody in the serum. ▲, autologous antiserum (from A/J mouse No. 413); ○, antisera from 10 individual A/J mice, tested at varying concentrations; ●, antisera from 10 individual C57/BL mice.

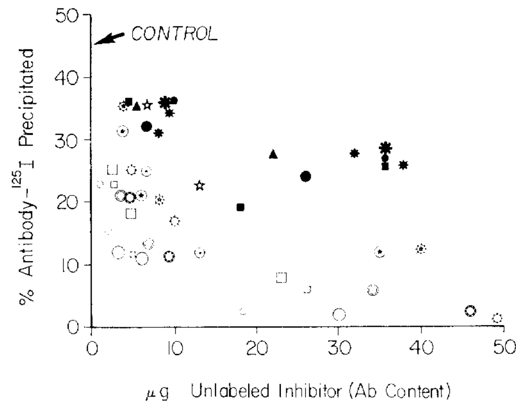

Fig. 2. Inhibition of binding to antiidiotypic antibodies of 0.1 μg of ^{125}I-labeled specifically purified anti-*p*-azobenzoate antibody from A/J mouse No. 63. Inhibitors are anti-*p*-azobenzoate antisera from individual hyperimmunized mice. Data are plotted in terms of the precipitable antibody content of the serum. Each different symbol represents the serum of a different mouse. ○, autologous antiserum (A/J mouse No. 63). The open symbols represent A/J mice, and the filled symbols, C57/BL mice.

mice tested caused about 85% inhibition, and that of a fifth BALB/c mouse inhibited to the extent of 70% at the highest concentration tested. Another set of experiments (not shown) was carried out in which lower concentrations of the BALB/c sera were used in order to determine the point of 50% inhibition for

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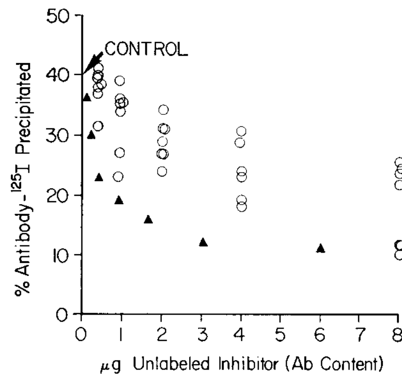

FIG. 3. This is the same system as described in Fig. 2. It shows results obtained with lower concentrations of individual A/J antibenzoate antisera. ▲, autologous antiserum (mouse No. 63); ○, data for nine other individual A/J mice, tested at varying concentrations.

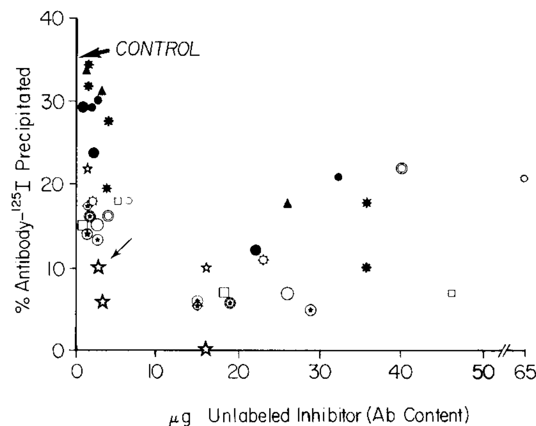

FIG. 4. Inhibition of binding to antiidiotypic antibodies of 0.1 μg of ^{125}I-labeled specifically purified anti-*p*-azobenzoate antibodies from BALB/c mouse No. 401. Inhibitors are hyperimmune antibenzoate antisera from individual mice. Each symbol represents a different mouse. Filled symbols indicate C57/BL mice; open symbols containing a star refer to BALB/c mice and the other open symbols to A/J mice. The symbol representing the donor mouse (No. 401) is indicated by a small arrow in the lower left portion of the graph.

each antibenzoate antiserum. The results, obtained by interpolation of the data, are presented in Table VI. On a weight basis the unlabeled antibodies of the donor mouse again were the most effective inhibitors; two to five times as much antibody of the other BALB/c mice was required for 50% inhibition. The data for A/J and C57/BL antisera, also listed in Table VI, will be considered later.

Each of the 23 cross-reacting antisera used in the three sets of experiments described above was treated with the appropriate immunoabsorbent in which

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TABLE IV

*Comparison of Weights of Antiphenylarsonate Antibodies (Ab) from Different A/J Mice Required for 50% Displacement of ^{125}I-Labeled Antiphenylarsonate Antibodies of A/J Mouse No. 413 from Its Antiidiotypic Antibodies**

A/J Mouse	Wt Ab required for 50% displacement	Relative wt required‡
No.	*μg*	
413	0.05	1
623	0.2	4
021	0.2	4
222	0.3	6
311	0.3	6
513	0.3	6
921	0.3	6
822	0.5	10
722	0.6	12
411	1.3	26
111	1.9	38

* Results were obtained by interpolation of data plotted in Fig. 1.
‡ Relative to the weight of antibody of the donor mouse, No. 413.

TABLE V

Comparison of Weights of Antibenzoate Antibodies from Different A/J and BALB/c Mice Required for 50% Displacement of ^{125}I-Labeled Antibenzoate Antibodies of A/J Mouse No. 63 from Its Antiidiotypic Antibodies

Strain of mouse	Mouse No.	Wt Ab required for 50% displacement	Relative wt required*
		μg	
A/J	63	0.8	1
	224	1.3	2
	61	3.5	4
	241	3.6	5
	205	5.0	6
	85	5.4	7
	135	6.1	8
	154	7.6	10
	184	7.6	10
BALB/c	441	11	14
	431	12	15
	465	13	16
	314	17	21
	455	>29	>36

* Relative to the weight of antibody from the donor mouse, No. 63.

the hapten, coupled to a protein other than that used for immunization, was conjugated to Sepharose (see Materials and Methods). In each instance at least 89% of the inhibitory capacity was removed by the absorption. More than 95% was removed in most cases. After similar treatment of each antiserum with un-

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conjugated Sepharose the inhibitory capacity remained the same within experimental error.

Variations in Idiotypic Specificities Among Inbred Strains of Mice.—The results in Fig. 1 show that antiphenylarsonate antibodies produced in C57/BL mice, in contrast to those of A/J mice, cross-react poorly with antiidiotypic antibodies directed to antiphenylarsonate antibodies of A/J mouse No. 413. Although some inhibition of binding occurred, relatively high concentrations of C57 antibodies were required. Whereas the amount of antiphenylarsonate antibody from most A/J mice required for 50% inhibition was less than 0.6 μg (Table IV), the largest amounts of C57 antibodies tested (7–10 μg) failed to give 50% inhibition (Fig. 1).

TABLE VI

Comparison of Weights of Antibenzoate Antibodies from Different BALB/c, A/J, and C57/BL Mice Required for 50% Displacement of ^{125}I-Labeled Antibenzoate Antibodies of BALB/c Mouse No. 401 from Its Antiidiotypic Antibodies

Strain of mouse	Mouse No.	Wt Ab required for 50% displacement	Relative wt required*
		μg	
BALB/c	401	0.3	1
	431	0.7	2
	455	0.8	3
	465	1.0	3
	314	1.0	3
	441	1.5	5
A/J	224	0.8	3
	61	1.8	6
	114	1.8	6
	205	6.3	21
	154	7.7	26
	111	>6.5	>22
C57/BL	663	13.2	43
	531	23	77
	624	>26	>87
	681	>32	>107
	545	>36	>120

* Relative to the weight of antibody from the donor mouse, No. 401.

The results in Fig. 5 indicate that antiphenylarsonate antibodies elicited in DBA mice similarly cross-react very poorly with antiidiotypic antiserum directed to the antiphenylarsonate antibodies of A/J mouse No. 413.

Although the cross-reactions of C57/BL and DBA antisera are relatively weak they are nevertheless due to the presence of antiphenylarsonate antibodies. Preimmune sera of each strain were noninhibitory (Table II). Also four of the C57/BL hyperimmune antisera and four of the DBA antisera which gave significant inhibition were treated with the Sepharose–bovine serum albumin (BSA)–azophenylarsonate immunoabsorbent. In each case, no significant in-

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hibitory capacity remained after the absorption. After control absorptions with unconjugated Sepharose the degree of inhibition observed remained the same within experimental error.

Cross-reactions of a second antiidiotypic antiserum with antibodies from other strains of mice are shown in Fig. 2 and Table V. The antiserum was directed to antibenzoate antibodies of A/J mouse No. 63. The data in Fig. 2 show that antibenzoate antibodies elicited in C57/BL mice cross-react poorly with this antiserum. 4–8 μg of the antibodies of most A/J mice was sufficient to cause 50% inhibition of binding; the C57/BL antibenzoate antibodies gave maximum inhibition approaching 50% in each case, but 20–40 μg was required.

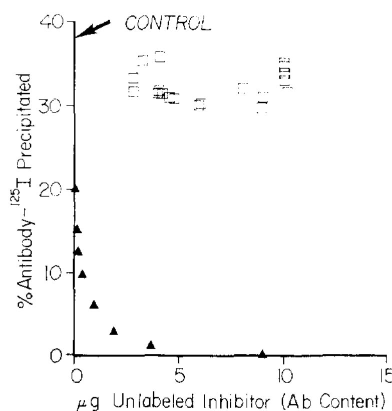

FIG. 5. The legend to this figure is identical to that of Fig. 1. ▲, inhibitory antiphenylarsonate antiserum from the autologous, donor A/J mouse (No. 413); □, hyperimmune antiphenylarsonate antisera from 10 individual DBA mice.

Antisera from individual BALB/c mice hyperimmunized with KLH-*p*-azobenzoate were also tested for their ability to inhibit the same antiidiotypic antiserum directed to A/J antibenzoate antibodies (Table V). Four of the five antisera tested gave 50–60% inhibition with the largest amounts tested (containing 15–20 μg of precipitable antibody). It is apparent that the BALB/c antibodies are consistently less effective as inhibitors than those of A/J mice but cross-react more strongly than C57/BL antibodies, which failed to give 50% inhibition. The weights of BALB/c antibodies required for 50% inhibition were on the average about three to five times as great as the weights of antibody from A/J mice other than the donor, and about 20 times as great as that of the donor A/J mouse, No. 63.

Idiotypic cross-reactions among different strains were also investigated with the third antiidiotypic antiserum, directed to antibenzoate antibodies of BALB/c mouse No. 401. The results are shown in Fig. 4 and Table VI. The data in Table VI are interpolated from values shown in Fig. 4 and from other results

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(not shown) obtained by using lower concentrations of A/J and BALB/c antisera.

Antibenzoate antibodies from C57/BL mice are seen to cross-react poorly (Table IV). Antibodies from one of the A/J mice tested was slightly more effective on a weight basis than those of one of the BALB/c mice, but otherwise the BALB/c antibodies were the strongest inhibitors. Antibodies of the donor mouse, No. 401, were the most effective on a weight basis. The results (Table VI) strengthen the inference, derived from results obtained with A/J antibenzoate antibodies, that the idiotypes of BALB/c and A/J antihapten antibodies are more closely related to one another than to those of C57/BL antibodies.

DISCUSSION

The conlusions to be drawn are based on rabbit antiidiotypic antisera prepared against purified anti-*p*-azophenylarsonate antibodies elicited in an A/J mouse, or against anti-*p*-azobenzoate antibodies from an A/J and a BALB/c mouse. The absorbed rabbit antisera reacted with 35–45% of the radiolabeled purified mouse antihapten antibody, when tested by indirect (antiglobulin) precipitation. Evidence that reactions exclusively involved idiotypic determinants included: (*a*) the failure of preimmune serum of the donor mouse and of normal sera from the same strain or from two other strains of mouse to inhibit binding, (*b*) the loss of inhibitory capacity of the hyperimmune antiserum of the donor mouse and of cross-reacting antisera after treatment with an immunoabsorbent specific for the antihapten antibody, and (*c*) the absence of cross-reactions against antibodies prepared in the same strain of mouse to the carrier-protein or to a different hapten group.

Each antiidiotypic antiserum cross-reacted strongly with antihapten antibody of the same specificity prepared in other mice of the same strain. When sufficiently high concentrations were tested, nearly all individual isologous antisera were able to displace at least 75% of the labeled antibody of the donor mouse from its antiidiotypic antiserum. On a weight basis, however, the unlabeled antibody of the donor mouse was the most effective inhibitor in each of the three systems studied. The weights of antibody required for 50% inhibition of binding were 2–38 times as great for isologous as compared to the autologous antibodies. In the BALB/c antibenzoate system, however, the maximum ratio was only 5 to 1 and in each system the ratio was generally 7 to 1 or less.

It appears then that most idiotypic specificities of A/J or BALB/c antihapten antibodies are shared by antibodies of the same specificity within a strain but that their distribution varies considerably among individual mice. Any significant alteration in distribution of specificities, as compared to that in the donor mouse, would be expected to increase the amount of antibody necessary to displace a given weight of the labeled donor antibody from its antiidiotypic antibodies.

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In addition to the likelihood of differences in distribution there is the possibility that some of the cross-reactions are attributable to molecules with idiotypic determinants that are similar but not identical to those of the antibody used as the immunogen. This is suggested by the partial cross-specificity sometimes observed among antistreptococcal antibodies within a family of inbred rabbits (8).

Marked differences among strains were noted in the cross-reactions of their antibodies with antiidiotypic antisera. Antibenzoate antibodies from most BALB/c mice were able to displace more than 50% of the ^{125}I-labeled A/J antibenzoate antibody from its antiidiotypic antibodies; however, the weights of BALB/c antibenzoate antibodies required were higher than those of A/J mice (Table V). Antibenzoate antibodies from C57/BL mice showed definite but weak cross-reactions and failed to cause 50% displacement, even when present in very large excess.

That BALB/c and A/J antibodies are more closely related to one another than to C57/BL antibodies was also suggested by results obtained with the antiidiotypic antiserum directed to BALB/c antibenzoate antibody (Fig. 4, Table VI). Most A/J antisera were able to displace more than 50% of the reference, ^{125}I-labeled BALB/c antibodies but larger amounts were required (with one exception) as compared to the isologous BALB/c antibodies. Antibodies from C57/BL mice were, in comparison, very poor inhibitors.

In the case of A/J anti-*p*-azophenylarsonate antibodies, the corresponding BALB/c antisera were not available for testing. Very weak idiotypic cross-reactions were observed with antibodies of the same specificity from C57/BL or DBA mice (Figs. 1 and 5).

Even when cross-reactions were weak they were found to be attributable to the antihapten antibodies present in the hyperimmune antiserum. Treatment with a Sephrose-hapten immunoabsorbent removed the inhibitory capacity in each of many such sera tested, whereas unconjugated Sepharose had no significant effect.

We cannot rule out the possibility that apparent differences among strains may be related to the elicitation of different immunoglobulin classes, although immunoelectrophoresis indicated that only precipitable γG_1 or γG_2 antibodies were present in significant amounts in each antiserum. Since only a portion of each antibody population represented idiotypic antibody it is difficult to ascertain the class responsible for inhibition, although attempts will be made to do this in future work. In view of the fact, however, that different immunoglobulin classes can share the same idiotypic specificities (15–17), it seems somewhat unlikely that the observed differences among strains will prove to be related to the class of immunoglobulin produced.

These results, obtained with donor antibodies of two specificities, each in only one or two strains of mouse, can of course not yet be generalized. Nevertheless, there is a striking difference between A/J or BALB/c mice and rabbits since

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strong idiotypic cross-reactions are rarely observed in the latter species, even among members of the same family (5). The only notable exception so far is the investigation of Eichmann and Kindt (8), who demonstrated numerous idiotypic cross-reactions among antistreptococcal antibodies produced by rabbits in a family group. The family comprised a brother-sister mating pair, both capable of producing antistreptococcal antibodies of limited heterogeneity, and their F_1 and F_2 descendants.

Two examples of idiotypic cross-reactions among BALB/c mice were mentioned in the introduction. On the basis of amino acid sequence studies, showing remarkably little variation among the lambda chains of BALB/c mice (18), one would predict extensive idiotypic cross-reactions among immunoglobulins with lambda chains, which constitute a few per cent of the normal population.

It is difficult to decide at this point whether the results of Eichmann and Kindt (8) with inbred rabbits are entirely analogous to our observations with inbred mice. It seems possible that in rabbits producing antibodies of restricted heterogeneity some inherited trait might cause preferential activation of a particular precursor cell among many cells with antistreptococcal specificity. If such were the case, inheritance of this trait (rather than a limited germ line, for example) could account for the genetic transmission of idiotype, which apparently does not occur to an appreciable extent in normal rabbit families (5). Such a characteristic might conceivably be a cell-surface receptor of exceptionally high affinity, or a tendency of cells bearing a particular receptor to proliferate rapidly with minimal antigenic stimulation.

Eichmann and Kindt propose that there are multiple germ-line genes controlling V regions which are inherited as a group, but which differ among rabbits and thus act as pseudoalleles. Inbreeding results in homozygosity and thus a greater tendency to transmit idiotypic specificity.

Our data obviously are also consistent with the hypothesis that multiple germ-line genes control the variable regions of immunoglobulin polypeptide chains. Idiotypic differences among mouse strains could simply be explained by differences in the germ line. The failure to observe frequent idiotypic cross-reactions among rabbit antibodies could be interpreted on the assumption that rabbits possess a more extensive germ line controlling V regions than do mice, or, as Eichmann and Kindt propose (8), that the arrays of germ-line genes vary among individual rabbits.

Although the germ-line theory provides the simplest explanation for these observations, the alternative hypothesis of somatic mutation is not ruled out. If a gene which encodes a polypeptide chain of a particular anti-hapten antibody can be derived from a germ-line gene by a small number of mutations in hypervariable regions, it might occur in nearly all mice of a given strain, despite the necessity for random mutation to arrive at the required polynucleotide sequence. Mice of another strain, possessing different germ-line genes might, through mutation, attain an appropriate but different polynucleotide sequence

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by a similar mechanism, thus accounting for the observed variations in idiotype among strains.

Perhaps the most interesting aspect of the present findings is that they may provide an additional phenotypic marker for the variable regions of mouse immunoglobulin chains. That such markers exist in mouse light chains has already been demonstrated by Edelman and Gottlieb, using the method of peptide mapping (19). Idiotypic specificity is almost certainly dependent on amino acid sequences in the variable regions of the polypeptide chains, and our data indicate that idiotype can be related to the strain of mouse. Investigations are in progress to determine the pattern of inheritance of idiotypic specificity and to localize strain differences to light or heavy chains. Studies of the linkage of V-region idiotype to other genetically inherited traits should then be possible.

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SUMMARY

Antisera were prepared in rabbits against anti-*p*-azobenzoate antibodies of an A/J and a BALB/c mouse and anti-*p*-azophenylarsonate antibodies of an A/J mouse. After appropriate absorption the antisera reacted with the antihapten antibody of the donor mouse but, by sensitive quantitative tests, not at all with other components of the hyperimmune serum or with preimmune serum of the donor mouse. The absorbed antiserum therefore appeared to be specific for idiotypic determinants. Nearly all idiotypic specificities identified in the serum of the donor were also present in the serum of other mice of the same strain, immunized against the same hapten group, but not in mice immunized with a different hapten. In each case the antibodies of the donor mouse reacted most effectively on a weight basis with antiidiotypic antiserum. Cross-reactions were observed among different strains of mice but homologous antibodies reacted most effectively with antiidiotypic antisera. C57/BL and DBA antisera contained very low concentrations of specificities present in the A/J and BALB/c antibody populations; antibodies of A/J and BALB/c antisera are more closely related to one another. The results indicate that idiotypic specificity may provide a genetic marker for the variable regions of immunoglobulin polypeptide chains.

Note Added in Proof.—The finding of extensive idiotypic cross-reactions within a strain of mouse does not apply in all instances. Using two antiidiotypic antisera, we have failed to observe strong idiotypic cross-reactions among C57/BL mice immunized to the *p*-azophenylarsonate group. Thus, C57/BL mice contrast with A/J or BALB/c mice in this respect.

REFERENCES

1. Slater, R. J., S. M. Ward, and H. G. Kunkel. 1955. Immunologic relationships among the myeloma proteins. *J. Exp. Med.* **101**:85.
2. Oudin, J., and M. Michel. 1963. Une nouvelle forme d'allotypie des globulines γ du sérum de lapin apparemment liée à la jonction et à la specificité anticorps. *C. R. Seances Acad. Agr. Fr.* **257**:805.

3. Kunkel, H. G., M. Mannik, and R. C. Williams. 1963. Individual antigenic specificities of isolated antibodies. *Science (Washington)*. **140**:1218.
4. Oudin, J. 1966. The genetic control of immunoglobulin synthesis. *Proc. Roy. Soc. Ser. B Biol. Sci.* **166**:207.
5. Kelus, A. S., and P. G. H. Gell. 1968. Immunologic analysis of rabbit anti-antibody systems. *J. Exp. Med.* **127**:215.
6. Daugharty, H., J. E. Hopper, A. B. MacDonald, and A. Nisonoff. 1969. Quantitative investigations of idiotypic antibodies. I. Analysis of precipitating antibody populations. *J. Exp. Med.* **130**:1047.
7. Hopper, J. E., A. B. MacDonald, and A. Nisonoff. 1970. Quantitative investigations of idiotypic antibodies. II. Nonprecipitating antibodies. *J. Exp. Med.* **131**:41.
8. Eichmann, K., and T. J. Kindt. 1971. The inheritance of individual antigenic specificities of rabbit antibodies to streptococcal carbohydrates. *J. Exp. Med.* **134**:532.
9. Cohn, M., G. Notani, and S. Rice. 1969. Characterization of the antibody to the C-carbohydrate produced by a transplantable mouse plasmacytoma. *Immunochemistry*. **6**:111.
10. Potter, M., and R. Lieberman. 1970. Common individual antigenic determinants in five of eight BALB/c IgA myeloma proteins that bind phosphoryl choline. *J. Exp. Med.* **132**:737.
11. Nisonoff, A. 1967. Coupling of diazonium compounds to proteins. *In* Methods in Immunology and Immunochemistry. C. A. Williams and M. W. Chase, editors. Academic Press, Inc., New York. **1**:120.
12. Sommerville, R. G. 1967. The production of fluorescent antibody reagents for virus diagnosis in the albino mouse. *Arch. Virusforsch.* **20**:445.
13. Hunter, R. 1970. Standardization of the chloramine-T method of protein iodination. *Proc. Soc. Exp. Biol. Med.* **133**:989.
14. Axen, R., J. Porath, and S. Ernback. 1969. Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. *Nature (London)*. **214**:1302.
15. Oudin, J., and M. Michel. 1969. Idiotypy of rabbit antibodies. II. Comparison of idiotypy of various kinds of antibodies formed in the rabbits against *Salmonella typhi*. *J. Exp. Med.* **130**:619.
16. Wang, A. C., S. K. Wilson, J. E. Hopper, H. H. Fudenberg, and A. Nisonoff. 1970. Evidence for control of synthesis of the variable regions of the heavy chains of immunoglobulins G and M by the same gene. *Proc. Nat. Acad. Sci. U.S.A.* **66**:337.
17. Penn, G. M., H. G. Kunkel, and H. M. Grey. 1970. Sharing of individual antigenic determinants between a gamma G and a gamma M protein in the same myeloma serum. *Proc. Soc. Exp. Biol. Med.* **135**:660.
18. Weigert, M. G., I. M. Cesari, S. J. Yonkovich, and M. Cohn. 1970. Variability in the lambda light chain sequences of mouse antibody. *Nature (London)*. **228**:1045.
19. Edelman, G. M., and P. D. Gottlieb. 1970. A genetic marker in the variable region of light chains of mouse immunoglobulins. *Proc. Nat. Acad. Sci. U.S.A.* **67**:1192.

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