QUANTITATIVE INVESTIGATIONS OF IDIOTYPIC ANTIBODIES

I. Analysis of Precipitating Antibody Populations*

By HARRY DAUGHARTY,‡ Ph.D., JOHN E. HOPPER, M.D., A. BRUCE MacDONALD, Ph.D., and ALFRED NISONOFF,§ Ph.D.,

(*From the Department of Microbiology, University of Illinois at the Medical Center, Chicago, Illinois 60680*)

(Received for publication 23 June 1969)

The production of antibodies specifically directed to other antibody molecules from an individual donor was discovered by Kunkel, Mannik, and Williams (1), who prepared such antibodies in rabbits against human antibodies to A substance, dextran, and levan, and, independently, by Oudin and Michel (2), who used *Salmonella typhi* coated with rabbit antibody to immunize recipient rabbits. The designation of the immunogenic portion of the donor population as "idiotypic antibody" (3) reflects the fact that the anti-antibodies are, with certain exceptions (4, 5), reactive only with the antibody injected, and not with antibody of the same specificity from another animal. Kelus and Gell (6) were able to produce anti-idiotypic antibodies against anti-*Proteus vulgaris* from a number of different rabbits in almost every recipient tested. Anti-*Proteus* from different animals, including members of a family group, were not cross-reactive with anti-idiotypic antisera. Individual antigenic specificity in specifically purified rabbit antibody to streptococcal carbohydrate was demonstrated by Braun and Krause (4), who utilized goat antisera to the rabbit antibodies. Idiotypic determinants have been localized to Fragment Fab of IgG donor molecules (1, 4, 6).

These findings demonstrate that antibodies of the same specificity from different animals differ sufficiently in their antigenic structure to be distinguishable by anti-antibody. Furthermore, they suggest that at least portions of the idiotypic, donor antibody populations are homogeneous; otherwise one might not expect to find concentrations of unique antigenic determinants sufficiently great to induce the formation of precipitating antibody in another animal. It is of interest in this connection that donor antibodies utilized by Braun and Krause exhibited limited electrophoretic heterogeneity (4), and that myeloma proteins possess individual antigenic specificities (7–9) which are localized in Fragment Fab.

Quantitative studies of idiotypic antibody populations have so far not been

* This work was supported by grants from the National Institutes of Health (AI-06281) and the National Science Foundation (GB-5424).

‡ Present address: Department of Microbiology, Baylor College of Medicine, Houston, Tex. 77025.

§ Recipient of a research career award from the National Institutes of Health (K06-2947).

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reported. We undertook such investigations in order to ascertain what fraction of a donor antibody population reacts with anti-idiotypic antibody; to explore cross-reactions in quantitative terms; to determine whether different recipients react with the same subpopulations of donor antibody molecules; and to study the onset and persistence of cell populations producing antibody molecules with a given idiotypic specificity. This paper presents results obtained with precipitating anti-idiotypic antisera. Subsequent reports will consider non-precipitating antisera and the question of persistence during prolonged immunization of molecules possessing a given idiotype. A preliminary report of some of these results has been presented (10).

Materials and Methods

Keyhole limpet hemocyanin was obtained from the Mann Research Laboratories (New York, N.Y.) and partially purified by passage through Sephadex G-200 at neutral pH. The material of high molecular weight, which passed through the column in the void volume, was used. Nonspecific IgG was prepared from the pooled sera of several rabbits by two precipitations with sodium sulfate followed by passage through DEAE-cellulose (11).

Recrystallized *p*-aminobenzoic acid was diazotized and coupled to proteins at pH 9–9.5, using a weight ratio of 40 mg/g of protein. Proteins conjugated in this way were hemocyanin, bovine γ-globulin (Pentex Co., Kankakee, Ill., Fraction II), ovalbumin (Pentex Co., five times crystallized), and rabbit IgG. Each hapten-protein conjugate was extensively dialyzed against cold neutral buffer.

Rabbits were immunized with the bovine γ-globulin–*p*-azobenzoate conjugate incorporated in complete Freund's adjuvant. After two injections of 3 mg of protein, spaced 3 wk apart, rabbits were given intravenous inoculations weekly or biweekly with the same amount of antigen. Sera were assayed by precipitin tests with the hemocyanin–*p*-azobenzoate test antigen. Sera of high titer collected from individual rabbits, over periods ranging from 3 wk to 6 months, were pooled.

Anti–*p*-azobenzoate antibodies were specifically purified from individual pools of serum. Precipitation was first carried out with an optimal amount of hemocyanin–*p*-azobenzoate antigen; ethylenediaminetetraacetate (0.01 M) was added to minimize uptake of complement. The washed precipitate was dissolved in 0.3 M *p*-nitrobenzoate, pH 8.5, and passed through DEAE-cellulose equilibrated with 0.0175 M phosphate buffer, pH 6.9. The column size was approximately 0.5 cm^3/mg of specific precipitate. Hemocyanin–*p*-azobenzoate, which is orange, was retained on the top of the column. The antibody eluted was dialyzed against several changes of neutral buffer over a period of at least 1 wk. When tested by immunoelectrophoresis, each of the isolated antibodies was found to be of the IgG class. Hapten-binding tests using ^{125}I–*p*-iodobenzoate were carried out by equilibrium dialysis (12) for 3 of the 12 purified anti–*p*-azobenzoate antibody preparations used as immunogens and for 6 of the 14 other purified anti–*p*-azobenzoate antibodies used for tests of cross-reactions. The number of combining sites per molecule of weight 150,000 varied from 1.5 to 2.1, and the average association constant, K_0, from 2×10^5 to 7.5×10^5. The purified antibodies used for immunization will be referred to as D (donor) antibodies (6) throughout this paper.

Purified antibodies or nonspecific IgG were labeled with ^{125}I by the method of MacFarlane (13), which utilizes ICl. A maximum of 1.5, and in nearly all instances less than 1, group of iodine per molecule of protein was incorporated. Iodinated samples were dialyzed until more than 98% of the radioactivity was precipitable by 5% trichloroacetic acid.

Fragments Fab and Fc were prepared from purified antibodies by digestion with papain

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(14) for 8 hr. Fragment Fab was isolated by passage through a column of carboxymethyl cellulose (14). Fragment Fc was crystallized at 5°C and washed several times with cold neutral buffer. Because of its limited solubility at neutral pH, Fragment Fc was dissolved in 0.03 M acetate buffer, pH 4.5, just prior to use. Fragment Fc was only used to test its capacity to inhibit reactions of D with anti-D serum. Since it failed to cause such inhibition, this cannot be attributed to the slight decrease in pH upon addition of Fragment Fc to antiserum.

Quantitative Assay of Idiotypic (D) Antibody.—For the quantitative estimation of antigenically active D molecules, a precipitin curve was first obtained with 0.4 ml portions of anti-antibody (anti-D) and increasing amounts of ^{125}I-labeled purified anti-*p*-azobenzoate antibody from the donor rabbit (D). Amounts of precipitate formed were estimated by dissolving the washed precipitate in 1 ml of 0.04 N NaOH and reading the optical density at 280 mμ. The extinction coefficient used, $E_{1cm}^{1\%}$, was 15.0. To determine the maximum percentage of D molecules precipitable by the antiserum, a mixture was then prepared in the region of antibody excess, as determined from the precipitin curve. In a typical experiment 75 μg of purified, ^{125}I-labeled D and 0.4 ml of anti-D serum were used. After standing for 1 hr at 37°C and 2 days in the cold, the precipitate was separated and washed, and the percentage of radioactivity precipitated was determined. Prior to counting, the precipitate was dissolved in 0.04 N NaOH. A portion of the supernatant (0.1 ml) was then added to a quantity of *unlabeled* D antigen equal to that used in the first precipitation (75 μg), 0.4 ml of anti-D serum was added, and the percentage of radioactivity precipitated was again determined. This process was repeated until the fraction of the total radioactivity brought down was 3% or less. As an example of the method of calculation, if 10% of the radioactivity was precipitated in the first stage and 5% of the remainder in the second stage, the total was 14.5% [10 + 0.05 (100 − 10)]. Nonspecific precipitation of labeled D due to entrapment or adherence to glass was estimated by measuring the coprecipitation of labeled D with 10 μg of ovalbumin and 0.4 ml of rabbit antiovalbumin serum. The precipitate formed was at least equal in weight to the precipitate of D with anti-D. The fraction of the labeled D precipitated in the control tube was always less than 2%.

Polymerization of Specifically Purified Antibodies.—Since polymerized protein molecules in some instances may be more immunogenic than the monomer (15), polymerized antibodies (D) were used in most of these experiments to elicit anti-idiotypic antibodies. Polymerization was carried out by adding a 20-fold molar excess of glutaraldehyde, in 0.1 M phosphate buffer, pH 7.0, to the purified anti-*p*-azobenzoate antibody (D) at a concentration of 5–10 mg/ml. The reaction was allowed to proceed at room temperature until definite turbidity developed, and was then stopped by the addition of 1 M L-lysine to a final concentration of 0.1 M. This was followed by dialysis in the cold against isotonic NaCl-borate buffer, pH 8. Small amounts of polymerized material that settled out were suspended prior to injection. It should be emphasized that polymerized D was used only for immunization; unpolymerized D was used as test antigen in all of the experiments described below.

Tests of Allotypic Specificities.—Allotypes of sera were determined by double diffusion analysis in agar gel, using monospecific anti-allotypic sera generously provided by Dr. Sheldon Dray.

RESULTS

Preparation of Anti-idiotypic (Anti-D) Antibodies.—Specifically purified anti-benzoate antibodies (D) from 12 individual rabbits were injected into recipient rabbits of matched allotype. Allotypes tested were a1, a2, a3, b4, b5, b6, b9, c7, and c21. The first group consisted of four unpolymerized D antibodies, with two recipients for each donor. Recipients were inoculated subcutaneously in the

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footpads and back with 1.5–3 mg of D in complete Freund's adjuvant; this was repeated at 2 or 3 wk intervals and followed by two intravenous inoculations spaced 2 wk apart. Three of the D preparations elicited no response in either recipient as determined by Ouchterlony analysis or by precipitin tests utilizing specifically purified antibenzoate antibody (D) at various concentrations as the test antigen. Precipitating antibodies against the fourth D preparation (from rabbit V15) were found in the sera of both recipients (rabbits E1 and E2). Intravenous inoculation of these recipients was continued at 2 wk intervals for 8 wk with weekly bleedings; at least 6 days were allowed to elapse between inoculation and bleeding. Precipitating anti-D sera from an individual recipient were pooled.

A second group consisted of four other D (purified antibody) preparations. Each of two of these was injected into two recipients, and each of the other two into one recipient rabbit. After two injections, with complete Freund's adjuvant, each recipient was given one intravenous inoculation. No precipitating antibodies were detected at this time. Polymerized D, prepared with glutaraldehyde as described above, was used as the antigen for subsequent inoculation of these rabbits. 3 mg portions in complete Freund's adjuvant were injected twice, 3 wk apart, followed after 2 wk by a single intravenous inoculation; test bleedings were taken 1 wk later. Sera of two of the six recipient rabbits, inoculated with antibodies from rabbits AZ5 and AZ11, respectively, contained antibodies that formed precipitates with unpolymerized D in agar gel and in solution. Specificity for the injected antigen was observed in all experiments and is discussed in detail below.

The third group of four specifically purified antibenzoate antibodies was inoculated only as the polymer. Either two or three recipients were used for each D antibody. The immunization schedule consisted of two subcutaneous inoculations in complete Freund's adjuvant, spaced 3 wk apart, followed by intravenous injections at 2–4 wk intervals. After three to five intravenous injections, all of the nine recipients responded with the formation of precipitating antibodies.

Table I summarizes the results of successful immunizations and the matched allotypic specificities among the rabbits used.

Specificity of Anti-D Antibodies.—All tests for activity were carried out with unpolymerized D as antigen. Tests for specificity were performed by Ouchterlony analysis and by direct precipitation in test tubes. Each of the anti-D sera was tested for specificity in agar gel against a panel of specifically purified anti-*p*-azobenzoate antibodies from 14 individual rabbits, whose allotypic specificities included all of those present on the D antibodies used as immunogens. These antibodies were used as antigens at concentrations of 4, 2, and 1 mg/ml. No cross-reactions were detected with this panel; each anti-D serum reacted only with its homologous D antibody. An example of the results is shown in Fig. 1.

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Evidence that the active D component is antibenzoate antibody, and not an impurity, was shown by the removal in every instance of activity upon absorption of purified D or D serum with a hemocyanin–*p*-azobenzoate conjugate. Examples are shown in Figs. 2 and 3. With D from rabbit V15, ovalbumin–*p*-azobenzoate was also tested and was found to remove D activity (Figs. 3 and 4).

TABLE I

*Formation of Precipitating Antibody upon Immunization with Specifically Purified Anti–p-azobenzoate Antibody from Individual Rabbits**

Purified donor (D) antibody from rabbit	Allotype of donor and recipient	Recipient (anti-D) rabbit‡	Ouchterlony test	Precipitin test	^{125}I-labeled antibody (D) precipitable
					%
AZ5	1, 3, 4, 7, 21	RD5	+	+	4
AZ11	1, 3, 4, 21	2X	+	+	7
V15	1, 4, 7	E1	+	+	32
		E2	+	+	29
A5	1, 3, 4, 7, 21	7A	+	+	4
		7C	+	+	10
		7D	+	+	11
A6A	1, 3, 4, 7	7O	+	+	14
		7E	+	+	13
A6B	1, 4, 7	7K	−	±	4
		7I	−	±	4
A7	1, 4, 7, 21	7B	+	+	56
		7M	+	+	41

* All tests for anti-D activity were carried out with monomeric purified anti–*p*-azobenzoate antibody as the test antigen.

‡ Anti-D was elicited by injection of polymerized anti–*p*-azobenzoate antibody, except for the antibody of rabbit V15, which was injected only as the monomer.

Further evidence that the reactive D component is the antibody was obtained by immunoelectrophoresis (Fig. 5). The mobility of the major D component in each instance was that of IgG. A slight amount of a faster moving D component appeared to be present in antibody from rabbit A6A, although it did not give a visible reaction with the sheep antiserum to rabbit serum.

The removal of D activity by the hemocyanin-azobenzoate conjugate provides strong additional evidence that the reaction with anti-D is idiotypic and not allotypic, since the allotype of the purified antibody would be represented in immunoglobulin components other than the antibenzoate antibody.

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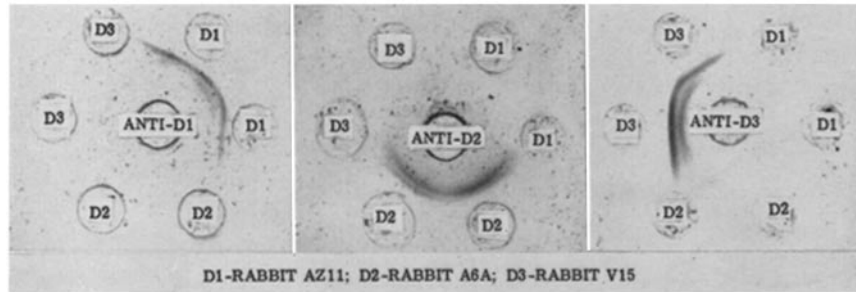

FIG. 1. Examples of tests for specificity by the Ouchterlony method. Each donor (D) antibody was tested at concentrations of 4 and 2 mg/ml (reading clockwise) against homologous and heterologous anti-D sera. D1, D2, and D3 represent specifically purified anti-*p*-azobenzoate antibodies from rabbits indicated on the figure.

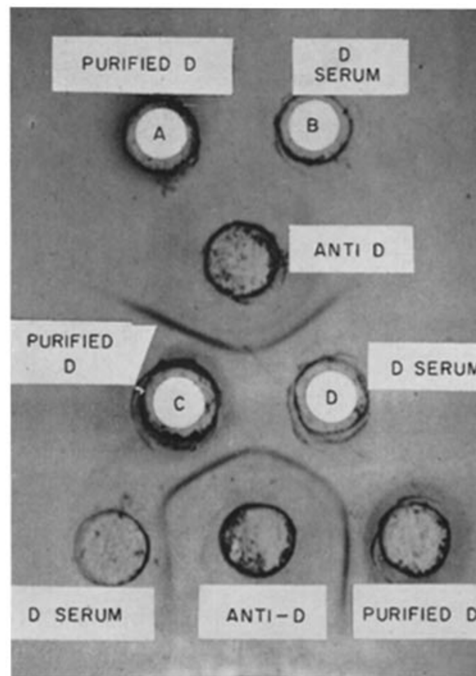

FIG. 2. Reactions in agar gel of purified anti-*p*-azobenzoate antibody (D), at 4 mg/ml, or whole D serum from rabbit AZ11, with anti-D serum from rabbit 2X. In wells A and B the purified antibody or serum was first absorbed with hemocyanin-*p*-azobenzoate (2 mg/ml of serum). In wells C and D the absorptions were carried out with the same weight of hemocyanin.

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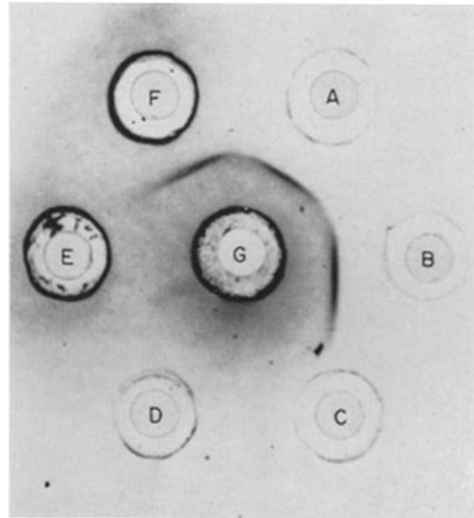

FIG. 3. Reactions with anti-D serum in agar gel of purified anti-*p*-azobenzoate antibody or whole donor serum from rabbit V15. The center well, G, contained anti-D serum from rabbit E1. A. Purified antibenzoate antibody at 1.8 mg/ml. B. Same as A but absorbed with ovalbumin (0.5 mg/ml). C. Same as A but absorbed with ovalbumin-*p*-azobenzoate (0.5 mg/ml). D. Donor serum absorbed with ovalbumin-*p*-azobenzoate (0.5 mg/ml). E. Donor serum absorbed with hemocyanin-*p*-azobenzoate (2 mg/ml). F. Donor serum.

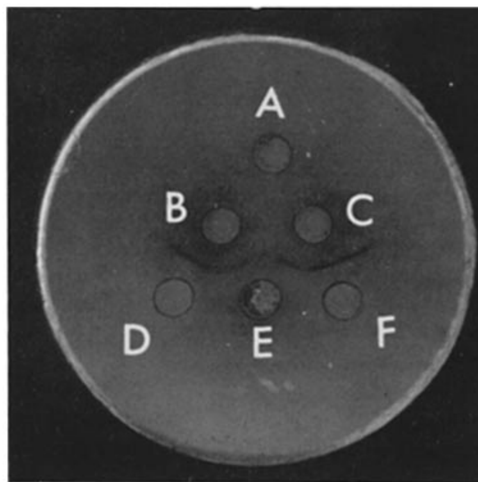

FIG. 4. Reactions in agar gel between purified anti-*p*-azobenzoate antibody from rabbit V15 (D) and two homologous anti-D sera. A. Donor serum absorbed with ovalbumin-*p*-azobenzoate (0.5 mg/ml). B. Anti-D serum from rabbit E1. C. Anti-D serum from rabbit E2. D and F. Antibenzoate antibody from the donor (1.8 mg/ml). E. Whole donor serum. Note the lines of identity between the two anti-D sera, and between purified D and D serum.

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Lines of identity were observed in each case when the whole serum containing D was placed in a well adjacent to the purified D (anti-*p*-azobenzoate antibody) and allowed to react with anti-D (Figs. 2–4).

Restricted Electrophoretic Mobility of the Reactive Portion of D Antigens.—The

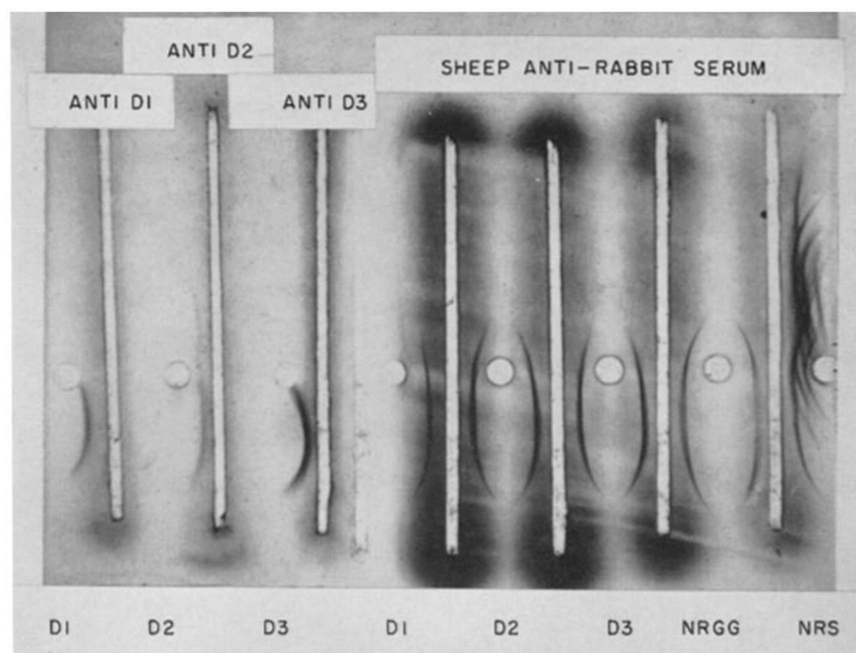

FIG. 5. Immunoelectrophoretic patterns of three purified antibenzoate antibodies from rabbits AZ11 (D1), A6A (D2), and V15 (D3). The donor antibodies (D) placed in the wells were reacted with homologous anti-D sera in the 3 troughs on the left. The troughs on the right contained sheep antiserum prepared against whole rabbit serum. The fourth trough from the left was empty. D antibodies were at a concentration of 5 mg/ml for the reactions with anti-D and at 2 mg/ml for the reactions with sheep antiserum. The two center wells on the far right contained normal rabbit IgG (NRGG, 2 mg/ml) and normal rabbit serum (NRS). Note that the purified antibody is similar in its range of electrophoretic mobility to normal IgG, but that the portion reactive with anti-D is restricted in mobility.

immunoelectrophoretic patterns shown in Fig. 5 indicate that the electrophoretic mobility of the reactive D population of the antigen is more restricted than that of the whole purified anti-*p*-azobenzoate antibody preparation; shorter lines were obtained with anti-D in the trough than those developed by the sheep anti-rabbit serum (Fig. 5). The fact that the reactive D population represents only a part of each purified anti-*p*-azobenzoate antibody is also indicated by quantitative results discussed below. The reduced electrophoretic heterogeneity of reactive D populations has been observed by others (1, 4, 6).

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Quantitative Precipitin Analysis.—The fraction of each D population reactive with anti-D was determined by exhaustive precipitation of ^{125}I-labeled D (purified antibenzoate antibody) with anti-D serum, as described under *Materials and Methods*. The maximal percentages of ^{125}I-D molecules precipitable by homologous anti-D sera are shown in Table I. Only a fraction of each D population (4–56%) was precipitable. Weights of precipitate formed by positive sera varied from 0.16 to 0.43 mg/ml of antiserum, as estimated from optical densities at 280 mμ of precipitates dissolved in 0.04 N NaOH.

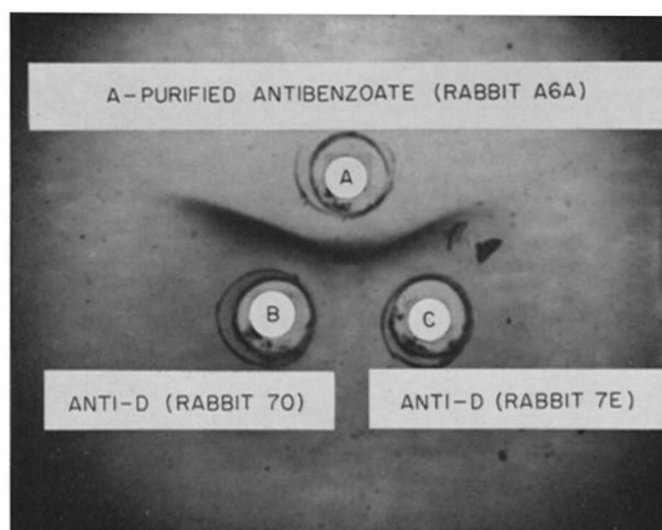

FIG. 6. Reaction in agar gel between purified antibody (D) from rabbit A6A (2 mg/ml) and anti-D sera from two different recipient rabbits.

These results demonstrate that the reactive D molecules constitute only a portion of each population of purified antibenzoate antibodies. They also show that a positive reaction can be observed in the Ouchterlony test when as little as 4% of the D population is reactive with anti-D.

Reactions of Anti-D Sera from Different Recipient Rabbits Immunized with the Same D Antigen.—The reactions in agar gel of purified anti-*p*-azobenzoate antibodies from rabbit A6A (D) with anti-D sera from two different recipient rabbits are shown in Fig. 6. The line of identity suggests that the two antisera are reactive with the same population of D molecules.

A similar result was obtained with D antibodies from rabbit V15, reacting with anti-D sera from two recipients (Fig. 7). With the relatively high concentration of purified D antigen used (4 mg/ml), double lines were observed with anti-D sera from each of two recipient rabbits. The stronger of the two lines shows a reaction of identity. Whether the weaker lines would also fuse was not

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ascertained, but the two patterns are virtually identical in their general appearance. That the two anti-D sera react with essentially the same subpopulation of D molecules is supported by quantitative data, described below.

A line of identity was similarly observed when D of rabbit A5 was allowed to react with two recipient sera (7A and 7C) placed in adjacent wells (pattern not shown). A third anti-D serum, from rabbit 7D, gave a faint line, and its relation to the other anti-D sera could not be clearly determined. The patterns

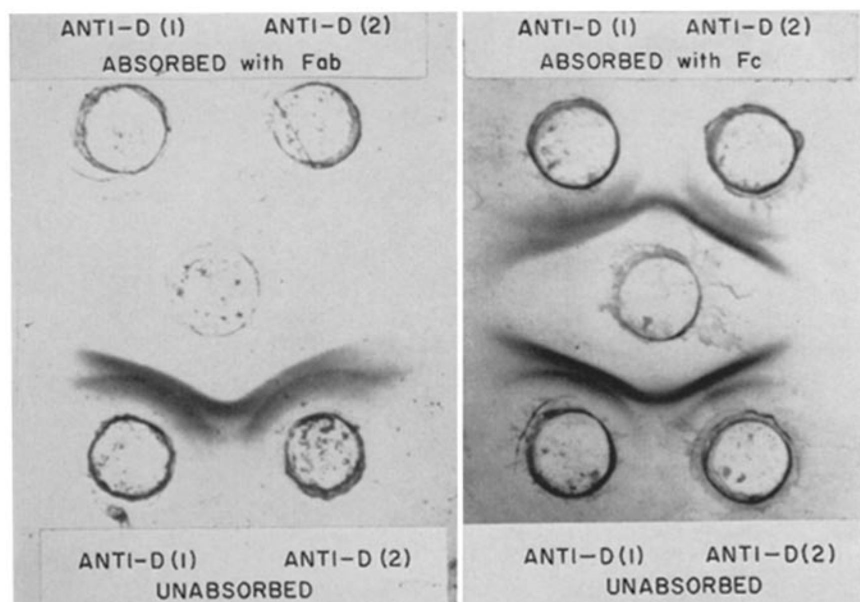

FIG. 7. Effect of absorption with Fab or Fc fragments of the donor antibody (D) on anti-D sera from two different recipient rabbits challenged with the same D preparation. The final concentration of Fab or Fc was 2.5 mg/ml. Anti-D (1) and anti-D (2) refer to the sera of the two recipients (rabbits E1 and E2). The concentration of donor antibody was 4 mg/ml.

of reaction of D antibody from rabbit A7 with anti-D from two recipients (7B and 7M) were more complex and consisted of multiple lines. Again, however, the strongest band showed a reaction of identity.

Thus, in all four systems studied so far, Ouchterlony analysis indicated that a large proportion of those D molecules which are immunogenic in one recipient are also immunogenic in a second recipient rabbit. Similar results have been reported by others (6).

This conclusion is substantiated by the quantitative data on three systems presented in Table II. For example, it is evident that approximately the same percentage of D molecules from rabbit V15 was precipitable by anti-D serum

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elicited in either of the two recipient rabbits (E1 or E2). Second, each anti-D serum failed to react with ^{125}I-D remaining in the supernatant after exhaustion with the other anti-D serum. It should be noted that unlabeled D was added as carrier at each stage so that the amounts of precipitate formed were approximately the same (about 110 μg from 0.4 ml of antiserum). Very similar results

TABLE II

Successive Precipitations of ^{125}I-Labeled Specifically Purified Anti-p-azobenzoate (D) with Two Anti-D Sera

^{125}I-labeled D from rabbit	Anti-D serum from rabbit	^{125}I-D precipitated: precipitation No.* 1	2	3	4	Cumulative ^{125}I-D precipitated
		%	%	%	%	%
V15	E1	23	6	3		32
	E2			1‡		
	E2	22	5	2		29
	E1			3‡		
A6A	7O	10	4	0		14
	7E			0‡		
	7E	9	4	0		13
	7O			1‡		
A7	7M	20	10	7	4	41
	7B				13‡	
	7B	38	8	6	4	56
	7M				4‡	

* Precipitation No. 1 was carried out with 0.4 ml of anti-D serum and 50–100 μg of homologous ^{125}I-labeled D. In succeeding precipitations the same weight of unlabeled D and 0.4 ml of anti-D serum were added to 0.1 ml of the preceding supernatant.

‡ Precipitation carried out with anti-D from the second recipient and the supernatant after two precipitations with the other recipient serum; or, in the case of D antibodies from rabbit A7, after three precipitations.

were obtained with the donor antibodies from rabbit A6A (Table II). In the case of D antibodies from rabbit A7, the data indicate that both anti-D sera reacted with the same subfraction (approximately 41% of the total D) but that the serum from rabbit 7B reacted with an additional 9–13% of the antibody molecules.

Measurement of the Extent of Cross-reactivity of Anti-D with Purified Antibenzoate Antibodies from Different Rabbits.—To estimate cross-reactivity quantitatively, anti-D was allowed to react with unlabeled D at equivalence in the presence of small amounts of ^{125}I-labeled antibenzoate antibodies from a number of rabbits, including the homologous ^{125}I-D antibody. Results obtained for

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the coprecipitation with purified V15 antibody are shown in Table III. It is evident that there was no appreciable cross-reaction of ^{125}I-labeled antibodies from heterologous rabbits. Similar data, indicating specificity of precipitation, were obtained with anti-D serum prepared against antibenzoate antibody of rabbits AZ11 and A6A (the latter with two different recipient antisera); however, a somewhat smaller panel of ^{125}I-labeled antibenzoate antibodies (from eight rabbits) was tested. Appreciable coprecipitation (7–11%) was observed only with homologous D preparations. All other values were less than 2%.

TABLE III

*Coprecipitation of ^{125}I-Labeled Specifically Purified Anti–p-azobenzoate Antibodies from Various Rabbits with Unlabeled Antibenzoate Antibody of Rabbit V15 (D) and Anti-D Serum**

^{125}I-labeled antibody from rabbit	Radioactivity coprecipitated
	%
V15 (homologous)	22.0
AZ1	0.5
AZ4	0.5
AZ5	0.4
AZ8	0.6
AZ10	0.5
AZ11	0.5
PG	1.1
PJ	0.1
HE18	0.9
CX	0.4
PM	1.3
Nonspecific IgG	1.1
^{125}I-V15 direct precipitation	24.0‡

* Each tube contained 75 μg of unlabeled D, 5 μg of ^{125}I-labeled anti–*p*-azobenzoate antibody from the rabbit specified, and 0.4 ml of anti-D serum (from rabbit E1).

‡ In this test 75 μg of labeled anti–*p*-azobenzoate antibody of rabbit V15 was added to 0.4 ml of anti-D serum.

Localization of Idiotypic Determinants.—Other investigators have shown that idiotypic determinants are found exclusively on the Fab fragment of IgG molecules (1, 4, 6, 8). The effect of absorption of anti-D by Fragment Fab or Fc of D antibody (from rabbit V15) is shown in Fig. 7. It is evident that anti-D activity is removed by absorption with Fragment Fab, but not with Fragment Fc. The same experiment was carried out with D from rabbit A6A and anti-D from rabbits 7O and 7E, with identical results.

DISCUSSION

Anti-idiotypic antibodies were regularly produced by injections of specifically purified rabbit anti–*p*-azobenzoate antibody into a recipient rabbit of an allo-

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type matched to that of the donor. When the antibenzoate antibody was polymerized with glutaraldehyde prior to injection, more than 80% of recipient animals responded with the formation of precipitating antibodies. This percentage includes a group of animals in which the initial inoculations were made with monomeric anti-*p*-azobenzoate antibody. Although our experience with monomeric purified antibody, used exclusively as the immunogen, is limited, the use of polymerized antigen appears to result in a higher percentage of responders that produce precipitating antibodies. All measurements of antibody activity were carried out with unpolymerized antibenzoate antibody (D) as the test antigen.

The use of donors and recipients of matched allotype resulted in the production of precipitating antibodies directed only to idiotypic determinants; there was no need for absorption of anti-D sera to render them specific. Evidence that allotypic specificities were not involved was the complete removal of activity from every D serum by absorption with hemocyanin-*p*-azobenzoate, which precipitates antibenzoate antibody (Figs. 2 and 3). Unconjugated hemocyanin had no effect. If allotypic specificities were responsible for the reactions, immunoglobulins other than antibenzoate antibodies in the donor's serum should have reacted with anti-D.

Additional evidence that the reactive D component was anti-*p*-azobenzoate antibody were the lines of identity obtained when D serum and purified D antibody were placed in adjacent wells in agar gel and allowed to react with anti-D serum (Figs. 2–4). Second, the percentages of the D antibody preparations that reacted with anti-D were frequently too high to be accounted for by impurities. Finally, immunoelectrophoresis indicated that the reactive D component was IgG (Fig. 5).

Precipitin tests carried out by the Ouchterlony method demonstrated complete specificity for the purified D antibody used as the immunogen. Tests made with a panel of anti-*p*-azobenzoate antibodies purified specifically from the sera of 14 rabbits failed to reveal any cross-reactions. With three systems, more sensitive tests for cross-reactions were carried out by measuring the incorporation of a trace of radiolabeled antibenzoate antibody from heterologous rabbits into a precipitate formed between unlabeled D antibody and its homologous anti-D serum. A significant degree of coprecipitation was observed only with labeled antibenzoate antibody from the homologous rabbit. The incorporation of other labeled antibenzoate antibodies was no greater than that observed with labeled nonspecific IgG, and was less than 2% in each case. Failure to detect any cross-reactions by this sensitive method emphasizes the remarkable specificity of the reactions of anti-idiotypic antisera.

The use of quantitative techniques indicated that reactive D populations constitute only a fraction of the purified antibenzoate antibody used as immunogen. The percentage of labeled D molecules precipitable varied from 4 to

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56. A possible interpretation of these results is that the reactive subpopulation of the D antibody is comprised of a limited number of groups of homogeneous antibody populations. It may be necessary for immunogenicity that a particular idiotype be present in appreciable concentration in order for it to elicit anti-antibodies. The nonimmunogenic portion of the D antibody may comprise such a large variety of different idiotypes that none is present in sufficiently high concentration.

Support for this view is derived from the observation that different recipient rabbits may recognize the same subpopulation of D antibodies. This was observed in two systems (D from rabbits V15 and A6A), in which anti-D sera were prepared against a single preparation in two recipients. By quantitative analysis (Table II) it was found that each anti-D serum failed to react with an appreciable fraction of the D molecules remaining after exhaustive precipitation with the other recipient serum. This finding cannot be completely generalized, since different recipient sera may sometimes react with different percentages of the D population (e.g. antisera against antibenzoate antibody of rabbit A5 or A7, Table I). In the case of D antibodies from rabbit A7, both recipient sera reacted with the same subpopulation (approximately 41%, Table II) of D molecules; however, one of the antisera reacted with an additional 9–13%. It appears significant that, in each case, certain subfractions of D populations were immunogenic in both recipients. Perhaps the simplest explanation for these results is that only those populations of antibody molecules with a particular structure, which are present in appreciable concentration, are able to elicit an immune response.

On the basis of these data one cannot decide how many different idiotypes are present among the molecules reactive with an anti-D serum. A suggestion that this number may be limited comes from Ouchterlony patterns (e.g. Figs. 1, 3, and 7). In each of the patterns one or two lines predominate, suggesting that the number of antigenic populations may be small. On the other hand, the possibility of cross-reactions among different idiotypes from the same rabbit cannot be excluded (4). Such cross-reactions might conceivably provide an alternative explanation for the very small number of discrete bands in an Ouchterlony pattern.

Additional evidence for the limited heterogeneity of the reactive idiotypic population is the shorter length of the band obtained upon immunoelectrophoresis with anti-D serum in the trough, as compared to that formed by the same D population reacting with sheep anti-rabbit serum (Fig. 5). This apparent limitation in electrophoretic heterogeneity has been observed by others (1, 4, 6) and has been interpreted to indicate that the reactive D population is less heterogeneous than the purified antibody used as immunogen. However, the fact that each of the bands occurs in the same region of the electrophoretic pattern raises the possibility of some other physical-chemical explanation, as

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an alternative to limited electrophoretic heterogeneity, for the shortness of the band.

It is of interest that percentages of D molecules as small as 4% are sufficient for visible precipitation. This finding may also be relevant to earlier investigations, in which precipitation methods were used to study the reactions of anti-idiotypic antibodies.

Results obtained with two of our systems confirm the finding of others (1, 4, 6) that the idiotypic determinants of antibodies are confined to Fragment Fab, and are not present in Fragment Fc. This is consistent with current evidence that differences in the Fc region would reflect differences in subclass or allotype. The multiplicity of sequences necessary to account for the great specificity of anti-idiotypic antibodies can best be accounted for on the assumption that idiotypic determinants occur in the variable region of the molecule, i.e., in the portion associated with antibody specificity.

If, as seems probable, antibodies with a given set of idiotypic specificities are products of a single clone of cells, quantitative analysis of idiotypic populations during the course of immunization of a rabbit should provide a method for determining the time of appearance of clones, their persistence, and replacement by different clones. Studies of this type have been carried out and will be described in a subsequent report.

SUMMARY

Specifically purified anti-*p*-azobenzoate antibodies of the IgG class from individual rabbits were used to elicit anti-idiotypic antibodies in recipient rabbits. Allotypes of each donor and recipient were matched. When polymerized antibodies were used for immunization, more than 80% of the recipients responded with the formation of antibodies that precipitated the monomeric donor antibody. Percentages of precipitable molecules in the donor antibody population (D) varied from 4 to 56. As little as 4% was readily detectable by the Ouchterlony method or precipitin test. Specificity of the reaction was tested by double diffusion in agar gel against a panel of purified antibenzoate antibodies from 14 heterologous rabbits and, quantitatively, in three systems by measurement of the extent of coprecipitation of heterologous, radiolabeled antibenzoate antibodies. No cross-reactions were observed. Reactions were shown to be attributable to antibenzoate antibodies in the donor serum, and contributions of allotypic reactions were excluded. In three systems investigated quantitatively, and in one studied qualitatively, two recipients of the same donor antibody produced anti-antibody that reacted with essentially the same subfraction of the donor antibody population. The findings that only a portion of the D population is immunogenic, and that the same subfraction is frequently immunogenic in different recipients, suggest that the immunogenic population comprises a limited number of homogeneous groups of antibody molecules.

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This is supported by the small number of bands usually observed by the Ouchterlony technique.

Quantitative methods of analysis should provide an approach to the study of cell populations producing antibodies of a particular idiotype.

The authors are grateful for the competent technical assistance of Miss Cynthia J. Subach and Miss Mary L. Bunchman.

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