QUANTITATIVE INVESTIGATIONS OF IDIOTYPIC ANTIBODIES

II. Nonprecipitating Antibodies*

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The studies of Kunkel, Mannik and Williams (1) and of Oudin and Michel (2) first demonstrated that a population of antibody molecules of a given specificity from a single donor may possess unique antigenic determinants—"idiotypic specificity" (3)—which are unrelated to allotypic specificities. Anti-antibodies prepared against such populations of molecules generally will not react with antibody of the same specificity from other donors nor with other immunoglobulin molecules of the donor (1, 2, 4), although certain exceptions have been reported (5, 6). Kelus and Gell (4) were able to elicit anti-idiotypic antibodies to anti-proteus antibody with nearly all of a large group of donor and recipient rabbits. We will refer to the donor (idiotypic) antibody population as D (4) and to the anti-antibody as anti-D.

We have undertaken investigations of idiotypic antibodies in order to characterize such populations quantitatively and with the ultimate aim of applying these methods to an analysis of the onset and persistence of clones of cells producing antibody of a given idiotypic specificity. In a recent report we presented data on precipitating anti-idiotypic antibodies (7). Such antibodies could be elicited in most recipient rabbits by inoculation of polymerized, specifically purified rabbit anti-*p*-azobenzoate antibody. Quantitative measurements, using unpolymerized anti-*p*-azobenzoate antibody as test antigen, indicated that 4–56% of the donor antibody populations (D) were precipitable by anti-D antisera. As little as 4% was sufficient for visible precipitation in aqueous solution or agar gel. In three instances anti-D sera from different recipients reacted with essentially the same subpopulation of D molecules from a single donor. Sensitive quantitative tests failed to reveal any cross-reactions of anti-D sera with antibenzoate antibodies from rabbits other than the donor.

The present report describes quantitative investigations of the reactions of idiotypic antibenzoate antibodies by a method of indirect precipitation; i.e., precipitation of antigen-antibody complexes with an antiglobulin reagent. For this purpose ^{125}I-labeled F(ab')$_2$ fragments of specifically purified donor antibody were used as the test antigen. (Previous investigations have localized

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idiotypic determinants to fragment Fab (1, 4, 6, 7)). It was found that in nearly all instances larger amounts of D molecules were precipitable by the indirect method than by direct precipitation. Substantial percentages of molecules were precipitable indirectly from two D preparations which failed to form precipitates with homologous anti-D sera. Extensive quantitative tests of specificity revealed only a few weak cross-reactions. Tests by the indirect method confirmed and extended the observation (7) that antisera prepared against a given D preparation in different recipients generally react with the same, or overlapping subpopulations of D molecules.

The methods described here have been applied to the analysis of the appearance and disappearance of antibodies of a given idiotype during prolonged immunization; the results will be reported separately. Some of these data have been presented in a preliminary report (8).

Materials and Methods

The following materials and methods were described previously (7): conjugates of *p*-azobenzoate with bovine γ-globulin and hemocyanin; rabbit IgG; isolation of specifically purified anti-*p*-azobenzoate antibodies of the IgG class; polymerization of such antibodies with glutaraldehyde for use in immunization; iodination of proteins with ^{125}ICl (less than 1 atom per molecule of protein); tests of allotypic specificities; quantification of percentages of idiotypic antibenzoate antibodies (D) directly precipitable by anti-D sera.

To estimate total amounts of labeled D antibodies directly precipitable, additional unlabeled D and anti-D serum were added to a small portion of the supernatant from the first precipitation, and the percentage of radioactivity precipitated was again determined. This procedure was repeated until the percentage of total radioactivity brought down was 4% or less. The method of calculation has been described (7).

Allotypic specificities for which tests were carried out were: a1, a2, a3, at the locus controlling heavy chains; b4, b5, b6, and b9 (kappa light chains); c7, c21 (lambda light chains). Each recipient rabbit was matched with its donor with respect to the above allotypes. Additional evidence that allotypic specificity was not responsible for reactions observed is discussed below.

Although most anti-idiotypic antibodies were elicited by inoculation of polymerized, specifically purified antibenzoate antibodies (D), monomeric D or its fragments were used exclusively for tests for activity with anti-D antibodies.

Immunization of Donor Rabbits.—Anti-*p*-azobenzoate antibodies were elicited by immunization with a bovine γ-globulin-*p*-azobenzoate conjugate. A total of 3 mg in complete Freund's adjuvant was injected subcutaneously into the footpads and back. This was repeated after 3 wk and was followed by weekly intravenous inoculation of 2–3 mg. Sera were tested for antibenzoate antibodies by precipitin analysis with the hemocyanin-*p*-azobenzoate conjugate. Sera of high titer from rabbits V15, A5, A6A, A6B, and A7 were collected over periods ranging from 4 wk–6 months; sera from each rabbit were pooled individually. In the case of rabbits AZ1, AZ5, and AZ11, each pool of serum comprised bleedings taken 5–9 wk after the start of immunization.

Anti-Idiotypic (Anti-D) Antibodies.—Preparations of the anti-D sera used in this investigation have been described previously (7). In each case the immunogen was specifically purified anti-*p*-azobenzoate antibody. Antibody from rabbit V15 was injected only as the monomer. Antibodies from rabbits A5, A6A, A6B, and A7 were injected exclusively as the polymer.

Antibodies from rabbits AZ1, AZ5, and AZ11 were injected first as the monomer and later as the polymer (7).

Fragments Fab and Fc of Rabbit IgG.—Proteolysis of rabbit IgG by papain was carried out by Porter's method (9), but the time of digestion was limited to 4 hr. Fragment Fc was isolated by dialysis of the digest against cold isotonic NaCl-borate buffer, pH 8. Crystals of fragment Fc that formed were washed, dissolved in water at 52°C, and allowed to recrystallize.

Fragment Fab was isolated from the supernatant, after removal of the crystals, by passage through carboxymethyl (CM) cellulose equilibrated with 0.01 M sodium acetate adjusted to pH 6.0. Undigested IgG and Fragment Fc are retained on the column under these conditions, while most of fragment Fab is eluted in the void volume.

Goat Antisera to Rabbit Fragments Fab and Fc.—Goats were inoculated subcutaneously with 1.5–2 mg of fragment Fab or Fc, incorporated in complete Freund's adjuvant. This was repeated at 2-wk intervals. Sera were tested periodically, and immunization was continued until a strongly precipitating antiserum was obtained.

Antiserum to fragment Fc also contained small amounts of antibody directed to fragment Fab. Such sera were absorbed by addition of fragment $F(ab')_2$ at equivalence. After removal of the precipitate an additional 50–100 μg of $F(ab')_2$ per ml of antiserum was added. Antiserum to fragment Fab did not contain demonstrable anti-Fc activity.

Preparation of $F(ab')_2$ Fragments.—Quantitation by the technique of indirect precipitation is described below; the method utilized $F(ab')_2$ fragments of the donor (D) specifically purified antibenzoate antibody. $F(ab')_2$, rather than fragment Fab was utilized because the method for isolation of Fab requires chromatography on CM-cellulose. Complete separation from fragment Fc is difficult to accomplish by this method, and elimination of Fc involves a selective loss of the more positively charged subfraction of the Fab population. D antibody was first trace-labeled with ^{125}I to give a specific activity between 1×10^6 and 3×10^6 cpm/mg. After dialysis, the antibody was mixed with 20 times its own weight of unlabeled, nonspecific IgG prepared from a large pool of normal rabbit serum. This was done to conserve D antibodies, which were available in limited amounts. It also provided a large excess of unlabeled, nonspecific $F(ab')_2$ fragments which would act as an absorbent for antibodies in anti-D sera not directed to idiotypic determinants. Allotypes detected in the nonspecific IgG used as carrier were: a1, a3, b4, c7, c21. The mixture of proteins was treated (10), in 0.1 M sodium acetate buffer, pH 4.3, with 2% by weight of pepsin (Worthington Biochemical Corp., Freehold, N. J., 2 × crystallized). The reaction was allowed to proceed for 6 hr at 37°C and was terminated by neutralization. After dialysis against isotonic NaCl-borate buffer, pH 8, the mixture was gel-filtered at room temperature through Sephadex G-150; the column volume for 20 mg of protein was 200 cm^3. That protein in the major peak which reacted in agar gel with goat anti-rabbit Fab but failed to react with goat anti-Fc serum was utilized for further work. The trailing portion of the peak usually contained material reactive with anti-Fc and was discarded. Seven of the ^{125}I-$F(ab')_2$ preparations were tested for precipitability by a goat antiserum specific for fragment Fab. More than 85% of the radioactivity in each preparation was precipitated. The supernatant was not subjected to a second precipitation.

Quantitation of Idiotypic Antibody by Indirect Precipitation.—Indirect precipitation was carried out with ^{125}I-$F(ab')_2$ fragments derived from D antibodies and prepared as described above. The labeled fragments were mixed with anti-D serum and incubated for 1 hr at 37°C. An excess of goat anti-Fc was then added to precipitate IgG and complexes of anti-D of the IgG class with ^{125}I-$F(ab')_2$. After standing overnight at 5°C, precipitates were washed 3 times with 0.5 ml of cold neutral buffer, then dissolved in 1.5 ml of 0.03 N NaOH. Radioactivities of the dissolved precipitate and of the combined supernatant and washes were determined and the percentage of radioactivity precipitated was calculated. Experiments were carried out in which the amounts of anti-D and of anti-Fc were varied separately to ensure that both reagents

were present in excess, as shown by percentages of radioactivity precipitated. With every set of experiments, controls were included in which antiovalbumin serum was substituted for the anti-D serum. This was essential to ensure that the ^{125}I-F(ab')$_2$ was not precipitated nonspecifically by the goat anti-Fc. Although the anti-Fc had been absorbed exhaustively with unlabeled F(ab')$_2$ fragments, a small percentage of radioactivity was always precipitated in the control tubes. It was found that this value decreased significantly if 50 μg of crystallized bovine serum albumin was dissolved and added to the test mixture prior to the ^{125}I-F(ab')$_2$. This was shown to be attributable to decreased adherence of the labeled F(ab')$_2$ to glass. Bovine serum albumin (50 μg) was therefore used in all experiments. Nevertheless, a small percentage of radioactivity was always found in precipitates in the control tubes containing antiovalbumin. The average value in all of these experiments was 5.3%. Data obtained with anti-D sera were corrected by subtracting the percentage of radioactivity precipitated nonspecifically.

A typical reaction mixture contained 50 μg bovine serum albumin; 10 μg F(ab')$_2$, of which 0.5 μg was ^{125}I-F(ab')$_2$ derived from D antibody, and the remainder was unlabeled, nonspecific F(ab')$_2$; 10 μl of anti-D serum; and 0.6 ml of anti-Fc. The combined volume of the F(ab')$_2$ and albumin solutions was 0.15 ml.

In many instances a second indirect precipitation was carried out with the supernatant of the first precipitation. This was done by adding 0.1 ml or 0.2 ml of the supernatant to additional anti-D serum; after incubating for 1 hr at 37°C the goat anti-Fc was added. The percentage of radioactivity precipitated was determined and the cumulative percentage precipitable was calculated (7).

RESULTS

Precipitability of D Molecules by Direct and Indirect Methods.—Table I presents data on percentages of ^{125}I-anti-p-azobenzoate antibody (D) molecules directly precipitable by homologous anti-D sera and percentages of ^{125}I-F(ab')$_2$ fragments of D precipitable by the indirect method. Most, although not all of the data on direct precipitation were reported previously (7), and are included in Table I for the purpose of comparison. As indicated in the table, two or three different recipients were utilized for several of the individual D antibody preparations. In the direct method, data were obtained by exhaustive precipitations utilizing portions of the radioactive supernatants and unlabeled carrier D antibody, as described above. Results by the indirect method were obtained from two successive precipitations utilizing anti-D serum and goat anti-rabbit Fc, except for the D antibodies of rabbits AZ5 and AZ11, for which a single precipitation was carried out. Relatively small amounts of radioactivity were brought down by the second indirect precipitation.

With the exception of D antibodies from rabbit V15, larger percentages of molecules were precipitable by the indirect, than by the direct method. In two instances (Table I) substantial fractions of populations which failed to form precipitates when tested by the Ouchterlony technique were precipitable by the indirect method; in each case the same result was observed with antiserum from each of two recipients. It is apparent that part or all of an idiotypic antibody population may escape detection by methods of direct precipitation. A possible explanation for differences in results of the direct and indirect methods is given

in the Discussion. The question as to whether anti-D sera from different recipients react with the same, or overlapping populations of D molecules was investigated and is discussed below.

Inhibition of Indirect Precipitation by Homologous Preparations.—The results in Table II indicate, first, that the reaction of anti-D sera with ^{125}I-labeled F(ab')$_2$

TABLE I

*Comparison of Percentages of Donor Antibody Populations Precipitable by Direct and Indirect Methods**

Rabbit donor antibody (D)	Rabbit recipient antiserum (anti-D)	Allotype of donor and recipient	Ouchterlony test	Precipitable	
				Direct precipitation	Indirect precipitation
				%	%
AZ5	RD5	1, 3, 4, 7, 21	+	2	23
AZ11	2X	1, 3, 4, 21	+	7	13
AZ1	1I	1, 3, 4, 7, 21	0	1	34
	RD8		0	1	42
A5	7A	1, 3, 4, 7, 21	+	4	29
	7C		+	10	43
	7D		+	11	43
A6A	7O	1, 3, 4, 7	+	14	58
	7E		+	13	41
A6B	7I	1, 4, 7	0	4	28
	7K		0	4	32
A7	7B	1, 4, 7, 21	+	56	69
	7M		+	41	74
V15	E1	1, 4, 7	+	32	35
	E2		+	29	31

* ^{125}I-labeled specifically purified anti-*p*-azobenzoate antibody (D) was used as antigen in the direct precipitations; ^{125}I-F(ab')$_2$ fragments of D were used in the indirect method.

fragments of specifically purified anti-*p*-azobenzoate (D) antibodies can be inhibited by the homologous unlabeled purified antibody (D) or by whole serum containing the D antibody.

The inhibitory capacity of each homologous D serum was greatly decreased by prior absorption with an optimal quantity of the hemocyanin-*p*-azobenzoate conjugate; absorption with the same weight of hemocyanin had little effect on the degree of inhibition (Table II). Since hemocyanin-*p*-azobenzoate should

remove only antibenzoate antibodies, the large decrease in inhibitory capacity indicates that antibenzoate antibodies in each serum are largely or entirely responsible for the inhibition. This also provides one type of evidence that the observed reactions are not against allotypic determinants; if they were, immunoglobulins other than antibenzoate antibodies would react with anti-D. In this context it is relevant that the antibenzoate antibody titer rarely exceeded 2

TABLE II

*Inhibition of Indirect Precipitation of ^{125}I-F(ab')$_2$ from Donor Antibenzoate Antibodies by Unlabeled, Homologous Donor Preparations**

Rabbit donor F(ab')$_2$	Rabbit recipient antiserum (anti-D)	Inhibitors (homologous)			
		Purified antibody‡	D serum§	D serum absorbed with hemocyanin-azobenzoate‖	D serum absorbed with hemocyanin¶
		^{125}I-F(ab')$_2$ bound, % of control			
AZ1	RD8	14 (2)**	17 (1)	78 (3)	7 (4)
AZ5	RD5	27 (1)	26 (4)	98 (3)	7 (5)
AZ11	2X	13 (1)	23 (5)	96 (8)	13 (4)
A5	7D	15 (1)	21 (3)	85 (3)	33 (3)
A6A	7O	20 (1)	9 (4)	83 (4)	13 (3)
A6B	7I	17 (2)	25 (1)	83 (4)	36 (4)
A7	7B	9 (2)	4 (2)	73 (3)	10 (3)
V15	E1	17 (4)	17 (2)	67 (4)	15 (8)

* In each test 0.5 μg ^{125}I-F(ab')$_2$, excess anti-D serum, and excess goat anti-rabbit Fc were used; 9.5 μg unlabeled nonspecific F(ab')$_2$ were also present.

‡ Unlabeled purified antibenzoate antibody, from the homologous (donor) rabbit, in 60-fold excess by weight (30 μg) over the ^{125}I-F(ab')$_2$.

§ Whole serum containing the D antibody; 10 μl of serum was mixed with ^{125}I-F(ab')$_2$ prior to the addition of anti-D serum.

‖ Hemocyanin-*p*-azobenzoate was added in optimal proportions and in equal volume to the D serum to remove antibenzoate antibodies. After centrifugation, 20 μl of the supernatant was tested as the inhibitor.

¶ Hemocyanin was added in a weight and volume equal to that of the hemocyanin-*p*-azobenzoate used (preceding column).

** Values in parentheses are average deviations of triplicate determinations.

mg/ml, which would ordinarily represent less than 20% of the IgG in a hyperimmunized rabbit. Therefore, removal of antibenzoate antibody did not exhaust the IgG, and allotypic determinants could still have been expressed.

In three systems (the first three listed in Table II), serum taken from the donor rabbit prior to immunization was tested as an inhibitor of binding of homologous ^{125}I-F(ab')$_2$ to anti-D. No significant degree of inhibition was observed. This provides additional evidence against a role of allotypic determinants. Further evidence is the specificity of the reactions, discussed below (Tables III, IV, and V); i.e., the failure of anti-D sera to cross-react strongly

with immunoglobulins from a large number of heterologous rabbits. Similar results excluding allotypic determinants as the basis of such reactions have been obtained by others (1,2,4,6), and in our previous work using the Ouchterlony method of analysis and quantitative, direct precipitations (7). The failure of

TABLE III

*Inhibition of the Indirect Precipitation of ^{125}I-F(ab')$_2$ Fragments Derived from Donor Antibody. Inhibitors: Unlabeled Specifically Purified Antibenzoate Antibodies from Individual Rabbits**

Donor rabbit...	AZ1	AZ1	AZ5	AZ11	A5	A6A
Recipient rabbit (Anti D)...	RD8	1I	RD5	2X	7D	7O
Inhibitor (rabbit)	^{125}I-F(ab')$_2$ bound, % of control‡					
AZ1	*14* (2)§	*21* (<1)	110 (8)	92 (1)	92 (1)	110 (3)
AZ5	90 (4)	81 (3)	*27* (1)	91 (3)	99 (1)	95 (2)
AZ11	101 (2)	102 (3)	111 (10)	*13* (1)	80 (4)	80 (1)
A5	98 (2)	93 (1)	110 (6)	103 (1)	*15* (1)	92 (1)
A6A	102 (4)	97 (7)	95 (4)	97 (6)	84 (1)	*20* (1)
AZ3	103 (4)	99 (4)	101 (3)	92 (8)	92 (8)	101 (3)
AZ4	101 (2)	99 (4)	96 (2)	89 (7)	102 (6)	97 (2)
AZ8	100 (1)	100 (2)	98 (5)	91 (3)	93 (<1)	103 (3)
AZ9	101 (1)	97 (2)	104 (8)	105 (8)	98 (7)	100 (3)
AZ14	99 (1)	92 (5)	102 (1)	79 (7)	95 (3)	109 (3)
AZ15	101 (1)	97 (4)	97 (2)	96 (7)	91 (2)	99 (1)
A6B	104 (1)	99 (3)	100 (8)	91 (1)	95 (4)	82 (2)
A7	102 (2)	104 (4)	99 (2)	92 (5)	103 (<1)	93 (3)
V15	101 (1)	99 (6)	100 (8)	99 (2)	90 (3)	106 (2)
IgG‖	101 (1)	103 (4)	94 (2)	84 (1)	96 (2)	95 (3)

* Each test utilized 10 μg F(ab')$_2$, of which 0.5 μg was ^{125}I-F(ab')$_2$ derived from the donor antibenzoate antibody (see text), and excess homologous anti-D serum. Unlabeled inhibitors were added in 60-fold excess (30 μg). Inhibitors: specifically purified anti-*p*-azobenzoate antibodies from the rabbits specified.

‡ Control refers to amount of ^{125}I-F(ab')$_2$ bound in the absence of inhibitor. Data for inhibition by the homologous donor antibody are italicized.

§ Numbers in parentheses are average deviations of triplicate determinations.

‖ Nonspecific IgG was obtained from a pool of sera of several rabbits. Allotypes present were a1, a3, b4, c7, and c21.

hemocyanin-*p*-azobenzoate to remove all inhibitory capacity of D serum is considered in the Discussion.

Specificity of Reactions of Anti-D Sera.—Data on the inhibition of indirect precipitation of ^{125}I-F(ab')$_2$ fragments of purified antibenzoate antibodies (D) are shown in Table III. Homologous anti-D serum was used in each test. Unlabeled, specifically purified anti-*p*-azobenzoate antibodies from individual rabbits, or IgG prepared from the pooled serum of a number of nonimmunized

TABLE IV

*Reactions by the Indirect Method of Anti-D Sera with ^{125}I-Labeled F(ab')$_2$ Fragments Derived from Specifically Purified Antibenzoate Antibodies of the Homologous (D) or Heterologous Rabbits**

Anti-D serum Rabbit...	RD8	RD5	2X	7O	7B	7D
Source of ^{125}I-F (ab')$_2$ Rabbit	% ^{125}I-F (ab')$_2$ precipitated					
AZ1	32 (2)	2 (<1)	0 (<1)	1 (<1)	0 (<1)	1 (<1)
AZ5	0 (<1)	*18* (<1)	0 (<1)	0 (<1)	0 (<1)	0 (<1)
AZ11	0 (<1)	1 (1)	*10* (1)	3 (2)	4 (1)	12 (1)
A6A	1 (<1)	1 (<1)	0 (<1)	*42* (1)	0 (<1)	0 (<1)
A7	1 (<1)	0 (<1)	0 (<1)	2 (1)	*60* (1)	1 (<1)
A5	2 (<1)	0 (<1)	0 (<1)	4 (1)	3 (1)	*35* (<1)
A6B	2 (<1)	3 (2)	0 (<1)	3 (2)	5 (1)	3 (<1)
V15	1 (<1)	0 (<1)	0 (1)	1 (1)	0 (<1)	1 (<1)

* Data in this table were obtained by a single indirect precipitation of ^{125}I-F(ab')$_2$. Data for the homologous reactions are italicized. Values in parentheses are average deviations of triplicate determinations. Each test utilized 0.5 μg ^{125}I-F(ab')$_2$ and an amount of anti-D serum (10 μl) that provided an excess over the ^{125}I-F(ab')$_2$ fragments derived from the homologous donor antibody.

TABLE V

*Inhibition of Indirect Precipitation by Immune Sera From the Donor or Heterologous Rabbits**

^{125}I-F(ab')$_2$ from donor rabbit...	AZ1	AZ5
Recipient rabbit (anti-D)...	RD8	RD5
Inhibitor (serum)	^{125}I-F(ab')$_2$ bound, % of control‡	
AZ1	*17* (1)	81 (1)
AZ5	86 (<1)	*26* (4)
AZ11	81 (<1)	94 (3)
AZ3	100 (2)	91 (<1)
AZ4	99 (1)	91 (2)
AZ6	99 (<1)	92 (2)
AZ8	96 (<1)	105 (3)
AZ9	96 (1)	104 (5)
AZ14	93 (<1)	88 (1)
A7	94 (<1)	102 (1)
V15	100 (<1)	97 (<1)
A6A	86 (<1)	100 (6)

* In each test 0.5 μg ^{125}I-F(ab')$_2$, prepared from specifically purified antibenzoate antibody (D), excess homologous anti-D serum and excess goat anti-rabbit Fc were used. Inhibitors were 10 μl of whole serum from the homologous (donor) or heterologous rabbits hyperimmunized with bovine γ-globulin-*p*-azobenzoate. Data for inhibition by homologous sera are italicized. Values in parentheses are average deviations of triplicate determinations.

‡ The control tubes contained 10 μl of antiovalbumin serum in place of the inhibitor.

rabbits, were tested as inhibitors. Data in the table, expressed as a percentage, compare the amounts of radioactivity bound in the presence and absence of inhibitor. In each test the weight ratio of unlabeled inhibitor to ^{125}I-F(ab')$_2$ was 60:1 (in addition to the 20:1 excess of unlabeled, nonspecific F(ab')$_2$ already present; see Methods).

Experiments similar to those in Table III were also carried out with D antibodies from rabbit A5 (recipients 7A and 7C); A6B (recipient 7K); A7 (recipients 7B and 7M); and V15 (recipient E1). A panel of 8 inhibitors (specifically purified antibenzoate antibodies from individual rabbits) was tested with each system. The maximum degree of inhibition by a heterologous antibody was 17%; in 43 of the 48 tests inhibition was less than 10%. Inhibition by the homologous preparation exceeded 80% in each case. The failure to observe complete inhibition with a 60-fold excess of homologous inhibitor is attributable to the use of a large excess of anti-D antibody.

Thus, inhibition by heterologous antibodies or nonspecific IgG in these experiments did not exceed 21%, and in 125 of 138 tests the inhibition was less than 10%. A large degree of inhibition was caused only by homologous unlabeled D antibody. It is probable, in view of the precision of the data as indicated by the average deviations, that inhibitions greater than 10% are real and represent weak cross-reactions.

In several systems cross-reactions were also investigated by measuring the amount of binding of ^{125}I-labeled F(ab')$_2$ fragments from antibenzoate antibodies of various rabbits by heterologous anti-D sera (Table IV). In only one instance was a large extent of cross-reaction observed; i.e., with ^{125}I-F(ab')$_2$ fragments from rabbit AZ11 reacting with antiserum 7D. 12% of the fragments were bound by the heterologous antiserum. The same antiserum bound 35% of the homologous D fragments (from rabbit A5). However, the percentage of fragments of antibody AZ11 bound by the heterologous antiserum was as great as that bound by the anti-D serum homologous to AZ11, i.e., antiserum 2X. In the reciprocal test, binding was not observed; antiserum 2X did not bind F(ab')$_2$ fragments from rabbit A5. It therefore appears that the two antisera, 2X and 7D, recognize different subpopulations of F(ab')$_2$ fragments derived from rabbit AZ11.

The same cross-reaction is noted in the inhibition data of Table III. Unlabeled antibenzoate antibody from rabbit AZ11 caused 20% inhibition of binding of ^{125}I-F(ab')$_2$ fragments from rabbit A5 to its homologous antiserum, 7D.

Additional data concerning the specificity of anti-D sera are given in Table V. Whole immune sera from individual rabbits, all of which contained precipitating anti-*p*-azobenzoate antibodies, were tested as inhibitors of the reaction of ^{125}I-F(ab')$_2$ fragments of specifically purified antibenzoate antibodies (D) from two individual rabbits with their homologous anti-D sera. For each test 10 μl of the serum to be tested as an inhibitor, and 10 μg of F(ab')$_2$ fragments, containing 0.5 μg of ^{125}I-F(ab')$_2$ fragments of the donor antibody, were mixed. This was

followed by the addition of 10 μl of anti-D serum, incubation at 37°C, and precipitation with excess goat anti-rabbit Fc. In the control tests, 10 μl of antiovalbumin serum of high titer was used in place of the inhibitor.

Maximum inhibition in each test system occurred in the presence of the homologous serum. The small degree of inhibition by heterologous sera observed in a few instances is difficult to assess because the amount of IgG present in mixtures was variable, owing to differences in IgG content of sera used as inhibitors. The amounts of precipitate formed by the anti-Fc therefore also varied; this may have introduced small differences in amounts of radioactivity precipitated nonspecifically. And if, for example, 20% of the radioactivity is bound in the absence of inhibitor, an error of 1% in the amount of radioactivity bound corresponds to an error of 5% in the calculated relative extent of binding; therefore, small variations are magnified.

Reactions of Anit-D Sera from Different Recipients Immunized with The Same Donor Antibody Population.—Table VI presents data on the reactions of anti-D sera prepared in different recipients by immunization with the same preparation of specifically purified anti-*p*-azobenzoate antibody from an individual rabbit. Tests were carried out by the method of indirect precipitation, utilizing ^{125}I-F(ab')$_2$ fragments. In each case a second precipitation was performed by adding to a portion of the supernatant from the first precipitation more of the same anti-D serum; this was followed by the addition of excess goat anti-rabbit Fc. The cumulative results of the two precipitations are shown in the last column of Table VI; results are given as per cent of the total radioactivity initially present.

In some, but not all instances different recipient sera reacted with nearly the same percentages of molecules from a given donor population (e.g., antisera 7C and 7D reacting with antibodies of rabbit A5; and antisera 7B and 7M reacting with antibodies of rabbit A7).

To explore the question as to whether different anti-D sera reacted with the same D molecules, a portion of the supernatant, after precipitation with a given anti-D serum, was allowed to react with homologous anti-D serum from a second recipient and the percentage of radioactivity precipitable by the indirect method was determined. In the case of D antibodies from rabbit AZ1, 34% of the F(ab')$_2$ molecules were precipitable by anti-D from rabbit 1I and 42% by anti-D from rabbit RD8. Anti-D from rabbit 1I failed to react with the supernatant after an initial precipitation by RD8. However, anti-D from rabbit RD8 reacted with an additional 15% of the total population after the initial precipitation by antiserum 1I. These data indicate that antisera 1I and RD8 react with the same subpopulation of molecules, constituting 30–34% of the total, and that anti-D from rabbit RD8 reacts with an additional 11–15%.

A similar relation is evident for the two recipient sera (7O and 7E) of D antibodies from rabbit A6A. Anti-D from rabbit 7O reacted with essentially all of the D molecules bound by antibodies of serum 7E, plus an additional 13–17%.

Sera from the two recipients of the D antibodies of rabbit A7 appeared to

react with essentially the same subfraction of the D population, since neither recipient serum reacted with an appreciable fraction of the molecules after an initial precipitation by the other antiserum.

TABLE VI

*Successive Indirect Precipitations by Recipient (Anti-D) Sera from Different Rabbits Immunized with the Same D Preparation**

^{125}I-F(ab')$_2$ of D from rabbit	Rabbit anti-D serum	% ^{125}I-F(ab')$_2$ precipitated		Cumulative % of ^{125}I-F(ab')$_2$ precipitated
		1st ppt.	2nd ppt.	
AZ1	1I	30→	4	34
	(RD8)	└→	15	
	RD8	42→	0	42
	(1I)	└→	1	
A6A	7O	48→	10	58
	(7E)	└→	4	
	7E	38→	3	41
	(7O)	└→	16	
A7	7B	64→	5	69
	(7M)	└→	4	
	7M	66→	8	74
	(7B)	└→	4	
A5	7A	24→	5	29
	(7C)	├→	16	
	(7D)	└→	15	
	7C	37→	6	43
	(7A)	├→	0	
	(7D)	└→	6	
	7D	39→	4	43
	(7A)	├→	1	
	(7C)	└→	7	

* 0.5 μg ^{125}I-F(ab')$_2$ prepared from specifically purified anti-*p*-azobenzoate antibody (D), was precipitated by the indirect method with homologous anti-D serum. A portion of the supernatant was then similarly precipitated with the same antiserum or with an anti-D serum prepared against the same D antibody in a different recipient. The arrows show the order of precipitation. The numbers given for the second precipitation are based on the total radioactivity initially present. (See text).

Similarly, recipient antisera 7C and 7D reacted with approximately the same subpopulation of D antibodies from rabbit A5. Also, both antisera, 7C and 7D, reacted with essentially all of the molecules precipitable by the third recipient

antiserum, 7A (since 7A failed to react with the supernatant after precipitation with 7C or 7D); however, 7C and 7D each reacted with an additional 11%–16% of the $F(ab')_2$ molecules not bound by antiserum 7A.

DISCUSSION

In a previous investigation only a portion (4%–56%) of the molecules in specifically purified anti-*p*-azobenzoate antibody populations from individual rabbits reacted with homologous anti-idiotypic (anti-D) sera when tested by a method of exhaustive, direct precipitation (7). The data presented here indicate that considerably greater percentages of such D populations are in general precipitable by an indirect method, which utilizes goat antibody to rabbit fragment Fc to precipitate soluble complexes of anti-D antibody bound to ^{125}I-labeled $F(ab')_2$ fragments of specifically purified anti-*p*-azobenzoate (D) antibody. In the case of two of the D populations investigated, substantial fractions (28%–42%) of the molecules were precipitable by the indirect method from populations which failed to form precipitates with anti-D serum in agar or agarose gel when tested over a wide range of concentrations. For both D preparations, the same result was obtained with each of two recipient antisera. (4% of the radioactivity was, however, brought down in the precipitin test from one of these D preparations).

Thus, methods of direct precipitation, used in earlier investigations of idiotypic antibody specificity, usually fail to detect some or all of the reactive molecules in a donor population. It appears that the use of an indirect method of precipitation is essential for a complete description of certain idiotypic antibodies.

The increased precipitability by the indirect method may reflect a deficiency of antigenic determinants on D molecules. A univalent, and probably a bivalent antigen may fail to precipitate directly but would form soluble complexes precipitable by an antiglobulin reagent. A similar observation of increased precipitability by the indirect method was made in quantitative studies of certain rabbit allotypic specificities (11) and is frequently noted in investigations of antigens of low molecular weight (e.g., 12–14). Most human allotypes are also not detected by direct precipitation.

The reactive molecules in the sera of donors were shown to be antibodies directed to the *p*-azobenzoate hapten. Whole donor serum inhibited the reaction of ^{125}I-$F(ab')_2$, derived from specifically purified antibenzoate antibody (D), with each anti-D serum; this inhibitory capacity was largely removed in each case by prior absorption of the D serum with hemocyanin-*p*-azobenzoate but not by hemocyanin (Table II). These results indicate that the reactions are not allotypic, since immunoglobulins other than anti-*p*-azobenzoate antibodies did not interact effectively with anti-D sera. In three systems sera taken from the donor prior to immunization were also tested as inhibitors and found to have no significant effect.

The failure of hemocyanin-*p*-azobenzoate to remove all inhibitory activity may well be due to the presence of some soluble complexes, containing antibenzoate antibodies, in the supernatant after absorption. In our experience complete precipitation of antibenzoate antibodies is very difficult to accomplish. When tests were carried out by the method of direct precipitation (7), absorption with hemocyanin-*p*-azobenzoate removed all precipitating activity. The detection of residual activity in the present experiments is probably attributable to the much greater sensitivity of the indirect method, in which a large excess of inhibitor over labeled ligand is used.

Further evidence indicating that the observed reactions are idiotypic and not allotypic was the absence (with one exception) of any strong cross-reactions with a large number of antibenzoate antibody preparations or whole sera from individual rabbits, or with nonspecific IgG from the pooled sera of several rabbits. The antibodies tested included all the allotypic specificities of the donor antibodies for which tests were carried out. That the reactions of anti-D sera are not directed to hidden antigenic determinants is shown by the capacity of undigested D antibody to inhibit the reactions essentially completely.

Specificity of the anti-D sera was demonstrated by testing, as inhibitors of the reaction of ^{125}I-F(ab')$_2$ derived from D antibody with anti-D sera, unlabeled specifically purified antibenzoate antibody from the donor or heterologous rabbits, or nonspecific IgG from heterologous, pooled sera (Table III). Strong inhibition was observed only with the homologous, unlabeled purified antibody; inhibition by heterologous preparations did not exceed 21%. In 13 of 138 tests significant inhibition (greater then 10%) was observed with heterologous antibenzoate antibody preparations. It should be noted that a 60-fold excess of competitor over ^{125}I-F(ab')$_2$ was used, so that weak cross-reactions could be detected.

One definite cross-reaction was noted when ^{125}I-labeled antibenzoate antibodies from individual rabbits were tested for binding to various anti-D sera (Table IV). 12% of the ^{125}I-F(ab')$_2$ fragments from rabbit AZ11 reacted with antiserum 7D. Antibody of rabbit AZ11 also inhibited to a small extent the reaction of antiserum 7D with F(ab')$_2$ fragments of the homologous donor rabbit (A5, Table III).

In our previous investigations of idiotypic antibody, by methods of direct precipitation (7), no evidence for cross-reactivity was obtained by the Ouchterlony test or by coprecipitation of radioactive, heterologous antibodies with unlabeled D antibody and homologous anti-D sera. However, the method of inhibition of indirect precipitation should be more sensitive, since a large excess of inhibitor can be employed; also, a heterologous antigen with a single cross-reacting determinant would fail to form precipitates but could cause inhibition of binding.

Very weak cross-reactions of nonspecific IgG with antibodies directed to individually specific antigenic determinants in myeloma proteins have been

noted by Grey et al. (15) and by Hurez et al. (16). In the present investigation a 60-fold excess of nonspecific IgG did not inhibit strongly in any of the systems tested (Table III). It should be noted that each ^{125}I-F(ab')$_2$ donor preparation already contained a 20-fold excess of unlabeled nonspecific F(ab')$_2$, since nonspecific IgG was added as carrier prior to digestion with pepsin.

With two anti-D sera (Table V), specificity was also investigated by testing whole donor serum, or heterologous sera from individual rabbits hyperimmunized to the azobenzoate hapten, for capacity to inhibit the reaction of ^{125}I-F(ab')$_2$ fragments of D antibody with homologous anti-D serum. Again, a large degree of inhibition was observed only with homologous D serum, and 3 or 4 weak cross-reactions were noted.

Cohn et al. (17) have observed a much greater frequency of idiotypic cross-reactions among antibodies of the same specificity from different mice of an inbred strain. It should be of interest to ascertain whether such cross-reactions are also observed among mice of unrelated strains.

In several instances antisera were prepared in more than one recipient rabbit against anti-azobenzoate antibody from a single donor. Analysis of the supernatant with one anti-D serum after indirect precipitation with another serum showed that large, identical subpopulations of the D antibody were immunogenic in different recipients. In cases where one anti-D serum reacted with a larger fraction of the D population than an antiserum from another recipient, the larger subpopulation of reactive D molecules included most or all of the smaller population. In two systems two different recipient sera reacted with virtually the same subfraction of D molecules (from rabbits A7 and A5, Table VI).

These results confirm the conclusion, based on direct precipitations (7) that similar subfractions of the D population are immunogenic in different recipients. A possible explanation (7) is that those populations of antibody molecules with a particular structure which are present in appreciable concentration are immunogenic in different recipients. That portion of the donor population which is not immunogenic may be more heterogeneous so that the idiotypes present are not sufficient in concentration to elicit anti-antibodies.

Using the Ouchterlony method, Kelus and Gell (4) obtained lines of identity when different recipient antisera were placed in adjacent wells and allowed to react with a given D preparation. These results suggested that the same, indeterminate fraction of the D population elicited precipitating antibodies in both recipients.

It appears very likely from our results that the reactive portion of a donor population consists of more than one idiotype. In cases where one anti-D serum reacts with a substantially larger proportion of the D molecules than a second anti-D serum, the first antiserum must recognize at least one additional idiotypic population. Although similar data were obtained by methods of direct precipitation (7), it was difficult to draw the conclusion that more than one

idiotypic population was present. The proportion of D molecules precipitated by an antiserum may vary with the number of antigenic determinants recognized. Thus, different recipient sera might precipitate different percentages of D molecules even from a single homogeneous donor antigen. The recognition of a single antigenic determinant, however, should suffice for indirect precipitation. Further evidence for the presence of more than one idiotype in certain antisera is the multiple banding sometimes observed upon analysis by the Ouchterlony method (4, 7), and the isolation by Braun and Krause (6) of different idiotypes from specifically purified antibody of a single donor rabbit.

The much greater sensitivity of the indirect method, and its capacity to detect nonprecipitating idiotypic antibodies, suggest that it may be preferable to methods of direct precipitation for investigations of idiotypic populations. Another advantage is the very small amount of anti-D serum required for each test. The method has been used for the quantitative study of persistence of molecules of a given idiotype in a hyperimmunized animal. The results have been presented in preliminary form (8) and will be given in more detail in another report.

SUMMARY

Idiotypic antibodies were investigated quantitatively by a method of indirect precipitation, which utilizes labeled $F(ab')_2$ fragments of specifically purified antibenzoate antibody from the donor, anti-antibody, and an antiglobulin reagent. The contribution of allotypic and hidden determinants to these reactions was excluded.

Greater fractions of an idiotypic antibody population are precipitated by this method, as compared to direct precipitation, and in two instances large proportions of idiotypic antibodies were detected in populations which failed to form precipitates by double diffusion in agar gel. The greater sensitivity of the indirect method was attributed to its capacity to detect molecules bearing a small number of antigenic determinants.

Extensive studies of cross-reactions, carried out by an inhibition technique, failed to reveal any strong reactions of anti-idiotypic antibodies with heterologous antibenzoate antibody preparations, heterologous sera, or IgG, although a few weak cross-reactions were noted. One definite cross-reaction was observed by a direct binding measurement with heterologous antiserum.

Antisera prepared in more than one recipient against a single donor preparation reacted with identical or overlapping subpopulations of the donor molecules. Instances in which two recipient antisera reacted with different proportions of the molecules of a single donor provided evidence for the existence of more than one idiotypic antibody population in the antibenzoate antibody of an individual rabbit.

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