Published November 1, 1970

QUANTITATIVE INVESTIGATIONS OF IDIOTYPIC ANTIBODIES

IV. Inhibition by Specific Haptens of the Reaction of Anti-hapten Antibody with its Anti-Idiotypic Antibody*

By BRUCE W. BRIENT,‡ M.D., and ALFRED NISONOFF, Ph.D.

(*From the Departments of Surgery and Biological Chemistry, University of Illinois at the Medical Center, Chicago, Illinois 60680*)

(Received for publication 6 July 1970)

Myeloma proteins (1) and antibodies from individual animals (2, 3) have been shown to possess individually specific or "idiotypic" (4) antigenic determinants. For example, anti-salmonella antibodies from an individual rabbit have determinants that are not detectable in other immunoglobulins of that rabbit nor, ordinarily, in antibodies of the same specificity from other rabbits (3). Quantitative studies have shown that various fractions (up to 80%) of purified anti-benzoate antibodies, designated D (5), from a given donor rabbit are reactive with anti-idiotypic (anti-D) antisera (6, 7). Precipitin lines in the Ouchterlony test were obtained when as little as 2% of the donor (D) population was precipitable.

Our present investigations indicate that the reactions of anti-*p*-azobenzoate antibodies (D) with their anti-idiotypic antisera are strongly inhibited by benzoate derivatives, i.e. by specific haptens, whereas the reactions of D with anti-Fab antibodies are affected to a much smaller extent. The possible relationship of idiotypic determinants to the antigen-combining site of a donor antibody is discussed.

Materials and Methods

The following methods and materials have been described previously (6, 7): preparation and specific purification of anti-*p*-azobenzoate antibodies of the IgG class; polymerization of these antibodies with glutaraldehyde for the purpose of immunization; preparation and labeling with ^{125}ICl of $F(ab')_2$ fragments of purified anti-benzoate antibodies (each such preparation containing a 20-fold excess of nonspecific $F[ab']_2$ fragments); preparation of goat antiserum specific for rabbit fragment Fc; and determination of allotypes of rabbit sera. Anti-allotype antisera were the generous gift of Dr. Sheldon Dray.

* This work was supported by grants from the National Science Foundation (GB-5424) and the National Institutes of Health (A1-06281). It was presented in part at the annual meeting of the Federated Societies for Experimental Biology and Medicine, April, 1970 (*Fed. Proc.* **29**:773).

‡ Supported by a National Institute of Medical Sciences Surgery Academic Training Grant, U. S. Public Health Service grant No. GM 1930-01A1.

Percentages of ^{125}I-F(ab′)$_2$ fragments of D antibodies precipitable were determined by an indirect method utilizing excess goat antibody directed to the Fc fragment of rabbit IgG to precipitate complexes of ^{125}I-F(ab′)$_2$ fragments with anti-D antibodies. The method used corresponds to that reported previously (7) with two exceptions. First, ovalbumin (50 μg per test) was used in place of bovine serum albumin to minimize adherence of the labeled protein to glass. Serum albumin was not used because it is known to bind various small molecules which were tested as inhibitors. Second, controls were run by utilizing anti-dinitrophenyl (anti-DNP) antiserum in place of anti-D. (Formerly anti-ovalbumin was used as a control). Percentages of radioactivity precipitated in control experiments did not exceed 2.8%. The control value was subtracted in each series from percentage values obtained with anti-D antiserum.

When small molecules such as haptens were tested as competitors of the reaction of F(ab′)$_2$ fragments of D with anti-D, the competitor was first incubated with the labeled F(ab′)$_2$ fragments and 50 μg of ovalbumin for 2 hr at 37°C, pH 8. Anti-D antiserum was added, incubation was continued for 1 hr, and goat anti-rabbit–Fc was then added to the mixture. After standing for 3 days in the refrigerator, the percentage of radioactivity precipitated was determined with a gamma scintillation counter (7). A minimum of 3000 counts was recorded for each precipitate-supernatant pair. Experiments were carried out in triplicate. All haptens and other small molecules tested as competitors were crystallized from water or from an ethanol-water mixture, with the exception of *p*-nitrobenzene sulfonic acid, and used as the sodium salts. Solutions were adjusted to pH 8.

Preparation of Anti-Idiotypic (Anti-D) Antibodies.—Specifically purified anti-benzoate antibodies (D) from individual rabbits were injected into recipients of allotype matched to the donor with respect to the following specificities: a1, a2, a3, b4, b5, b6, b9, c7, c21.

Rabbit 9Q was immunized with purified D antibody from rabbit AZ5, according to the second protocol given in reference 6; 3–5 mg of monomeric D were used for the initial inoculations with Freund's adjuvant, and polymerized D was used for subsequent intravenous inoculations. A pool of antiserum from rabbit 9Q was made from several bleedings which showed strong antibody activity by the method of indirect precipitation.

Purified D antibodies from rabbits A4 and I-14 were injected exclusively in the polymerized form into recipients 14E and 10X, respectively, and D antibody from rabbit A5 was injected into 3 recipients, 7A, 7C, and 7D. Two subcutaneous inoculations of 3 mg each in complete Freund's adjuvant were given 3 wk apart. These were followed by intravenous inoculations at intervals of 2–4 wk. Bleedings were taken 5–8 days after an injection. Antisera from individual recipients which gave strong reactions by the method of indirect precipitation were pooled. Evidence that antibodies produced were directed to idiotypic determinants, and not to allotypic or hidden determinants, has been described in detail for D antibodies of rabbits A5 and AZ5 (7, 8). This evidence includes the failure of sera taken from donor rabbits prior to immunization to inhibit reactions of anti-D with ^{125}I-F(ab′)$_2$ fragments of D, the loss of inhibitory capacity of whole D serum after precipitation of antibenzoate antibodies, and the specificity of the reactions when various anti-D sera and D antibodies were utilized.

Similar results were obtained in a large number of preliminary control experiments with the D antibodies of rabbits I-14 and A4.

The percentages of F(ab′)$_2$ fragments of specifically purified anti-*p*-azobenzoate antibodies that were precipitable by anti-D antisera, using the indirect method, were: rabbit AZ5, 57 ±3%; rabbit A5, 37 ±2%; rabbit I–14, 31 ±1%; rabbit A4, 23 ±1%.

RESULTS

The effects of specific haptens and other small molecules on the reactions of ^{125}I-F(ab′)$_2$ fragments of D antibodies with anti-D antisera are shown in Tables

Published November 1, 1970

TABLE I

*Effect of Haptens and Other Small Molecules on the Reaction of ^{125}I-F(ab')$_2$ Derived from D Antibodies of Rabbit AZ5 with Anti-D Serum**

Competitor	Final molar concentration of competitor‡			Rel. K(9)
	1.6×10^{-3}	5×10^{-4}	5×10^{-5}	
	^{125}I-F(ab')$_2$ *precipitated, % of control*§			
p-(*p'*-hydroxy)-phenylazobenzoate	57 (1)	61 (5)	74 (4)	22
benzoate	73 (4)	90 (4)	95 (1)	1.0
p-nitrobenzoate	69 (2)	70 (3)	82 (2)	11.5
m-nitrobenzoate	74 (3)	86 (3)	85 (4)	0.4
o-nitrobenzoate	90 (1)	98 (1)	89 (2)	<0.1
p-aminobenzoate	88 (1)	96 (1)	97 (2)	0.9
m-aminobenzoate	93 (3)	91 (1)	96 (3)	0.3
o-aminobenzoate	76 (1)	85 (1)	93 (2)	1.5
p-bromobenzoate	75 (1)	86 (1)	78 (2)	5.0
m-bromobenzoate	81 (2)	98 (1)	93 (4)	1.3
o-bromobenzoate	95 (1)	103 (1)	96 (1)	0.1
p-chlorobenzoate	64 (2)	77 (2)	91 (2)	3.7
m-chlorobenzoate	78 (7)	83 (3)	89 (2)	0.8
o-chlorobenzoate	81 (3)	97 (1)	87 (5)	0.2
p-iodobenzoate	64 (2)	75 (2)	90 (1)	
o-iodobenzoate	87 (1)	97 (1)		
p-methylbenzoate	71 (2)	81 (1)	91 (1)	2.6
m-methylbenzoate	87 (2)	97 (3)	102 (2)	0.7
o-methylbenzoate	92 (2)	100 (1)	102 (1)	0.1
Sodium acetate	94 (1)	99 (1)	99 (1)	
Potassium iodide	98 (1)	102 (2)	101 (3)	
Potassium bromide	97 (1)	102 (1)	95 (1)	
p-(*p'*-dimethylamino)-phenylazobenzene sulfonate	112 (1)	104 (1)	94 (2)	

* Anti-D serum was from rabbit 9Q. The indirect method of precipitation was used. Each test contained 0.5 μg ^{125}I-F(ab')$_2$ of the D antibody, 9.5 μg nonspecific F(ab')$_2$, and 50 μg ovalbumin in a final volume of 0.31 ml. 10 μl of anti-D serum and 0.4 ml goat anti-rabbit Fc were then added. In the absence of competitors 57 ± 3% of the radioactivity was precipitated with anti-D serum and 1.2% in the control utilizing 10 μl anti-DNP antiserum. Experiments were in triplicate with average deviations given in parentheses.

‡ Refers to concentration prior to the addition of goat anti-rabbit Fc antiserum.

§ Expressed as percentage of the quantity precipitated in the absence of competitor.

I–IV. Each table reports data obtained with a different donor–recipient pair of rabbits. The last column in each table presents relative binding affinities of haptens, obtained by Pressman et al. (9), who measured inhibition of specific precipitation by haptens.

It is evident that specific haptens inhibit the reactions of anti-D sera. By far the best inhibitor in each system was *p*-(*p'*-hydroxy)-phenylazobenzoate. This

compound also combines with greater affinity with anti-*p*-azobenzoate antibodies than any of the other compounds tested. The percentage inhibition observed in the four systems ranged from 43 to 69% when *p*-(*p'*-hydroxy)-phenylazobenzoate was present at a final concentration of 1.6×10^{-3} M.

In three of the four systems (Tables I–III), all para-substituted benzoate derivatives tested were quite effective as inhibitors, and were more effective than unsubstituted benzoate. This is consistent with the greater binding affinities of *p*-substituted benzoate derivatives. At a final concentration of $1.6 \times$ 10^{-3} M, *p*-nitrobenzoate decreased the percentage of ^{125}I-F(ab')$_2$ fragments bound to 68–90% of the control value.

TABLE II

*Effect of Haptens and Other Small Molecules on the Reaction of ^{125}I-F(ab')$_2$ Derived from D Antibodies of Rabbit A4 with Anti-D Serum**

Competitor	Final molar concentration of competitor‡			Rel. K(9)
	1.6×10^{-3}	5×10^{-4}	5×10^{-5}	
	^{125}I-F(ab') *precipitated, % of control*§			
p-(*p'*-hydroxy)-phenylazobenzoate	43 (1)	42 (1)	86 (1)	22
Benzoate	92 (<1)	95 (<1)	106 (1)	1
p-nitrobenzoate	71 (<1)	92 (<1)	90 (2)	11.5
m-nitrobenzoate	87 (1)	102 (1)	101 (2)	0.4
o-nitrobenzoate	101 (<1)	101 (1)	103 (1)	<0.1
p-aminobenzene arsonic acid	105 (1)	103 (<1)	100 (1)	
p-nitrobenzene sulfonic acid	100 (<1)	102 (1)	102 (2)	
Sodium acetate	91 (1)	95 (<1)	98 (1)	
Potassium iodide	96 (1)	100 (1)	94 (2)	
Potassium bromide	84 (<1)	97 (1)	95 (<1)	
p((*p'*-dimethylamino)-phenylazobenzene sulfonate	98 (1)	99 (<1)	77 (<1)	

* Anti-D serum was from rabbit 14E. The indirect method of precipitation was used (see first footnote of Table I).

‡ Refers to concentration prior to the addition of goat anti-rabbit Fc antiserum.

§ Expressed as percentage of the quantity precipitated in the absence of competitor.

For benzoate derivatives with the same substituent group at various positions on the benzene ring, the order of inhibitory capacity in nearly all instances is $p > m > o$, which is the same as the decreasing order of combining affinities (9). The only exceptions are the amino derivatives (Table I); however a similar anomaly obtains with respect to their combining affinities. This was attributed (9) to the capacity of an *o*-amino substituent to form a hydrogen bond with the carboxylate group of benzoate ion.

A few tests were carried out in two additional systems in which the donor antibody again was from rabbit A5 and the recipients were rabbits 7A and 7C.

In these experiments *p*-(*p'*-hydroxy)-phenylazobenzoate gave significant inhibition (47 and 50%, respectively, at a concentration of 1.6×10^{-3} M) but *p*-nitrobenzoate had no effect on the reaction.

In general, small molecules other than benzoate derivatives had no significant effect on the percentage of ^{125}I-F(ab')$_2$ fragments precipitated. The only exception was methyl orange, *p*-(*p'*-dimethylamino)-phenylazobenzene sulfonate,

TABLE III

*Effect of Haptens and Other Small Molecules on the Reaction of ^{125}I-F(ab')$_2$ Derived from D Antibodies of Rabbit A5 with Anti-D Serum**

Competitor	Final molar concentration of competitor‡			Rel. K(9)
	1.6×10^{-3}	5×10^{-4}	5×10^{-5}	
	^{125}I-F(ab') precipitated, % of control§			
p-(*p'*-hydroxy)-phenylazobenzoate	51 (<1)	67 (3)	90 (6)	22
Benzoate	95 (3)	98 (1)	109 (1)	1.0
p-nitrobenzoate	78 (<1)	83 (1)	95 (1)	11.5
m-nitrobenzoate	88 (2)	95 (1)	99 (1)	0.4
o-nitrobenzoate	95 (1)	96 (1)	98 (2)	<0.1
p-aminobenzoate	90 (1)	94 (4)	99 (2)	0.9
m-aminobenzoate	91 (2)	99 (<1)	99 (1)	0.3
o-aminobenzoate	94 (2)	99 (2)	101 (2)	1.5
p-iodobenzoate	74 (1)	89 (5)	97 (<1)	
m-iodobenzoate	87 (2)	95 (<1)	94 (1)	0.9
o-iodobenzoate	92 (3)	96 (2)	96 (1)	
Sodium acetate	102 (2)	107 (3)	105 (2)	
Potassium bromide	106 (2)	106 (1)	109 (3)	
p-aminobenzene arsonate	95 (4)	96 (2)	85 (1)	
p-(*p'*-dimethylamino)-phenylazobenzene sulfonate	95 (4)	96 (2)	85 (1)	

* Anti-D serum was from rabbit 7D. The indirect method of precipitation was used (see first footnote of Table I).

‡ Refers to concentration prior to the addition of goat anti-rabbit Fc antiserum.

§ Expressed as percentage of the quantity precipitated in the absence of competitor.

which gave weak but consistent inhibition in two of the four systems but, surprisingly, inhibited only at the lowest concentration tested (5×10^{-5} M). We have at present no explanation for this result.

Other substances related structurally to benzoate, *p*-aminobenzene arsonate and *p*-nitrobenzene sulfonate, were inactive. Iodide, bromide, and acetate salts were also ineffective as inhibitors.

Additional experiments were carried out to determine whether *p*-(*p'*-hydroxy)-phenylazobenzoate inhibited the reaction of F(ab')$_2$ derived from D antibodies with an equivalent amount of goat anti-rabbit Fab antiserum. These experiments were carried out in triplicate with two systems (D from rabbits A5

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.