

STUDIES OF IDIOTYPIC ANTIBODIES

PRODUCTION AND CHARACTERIZATION OF AUTOANTIIDIOTYPIC ANTISERA*

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Individual antigenic determinants on antibodies were originally described by Kunkel et al. (1). Subsequently, the determinants, termed "idiotypes" by Oudin (2), have been described in antibody populations made in response to bacterial antigens (3) and to haptens (4). The antiidiotypic antisera are usually made in homologous or even heterologous species. Absorption of the antiidiotypic antisera with preinoculation serum or IgG from the individual whose idio- type is being studied might not remove antibodies specific for constant areas of variable (V)¹ regions of a particular V-region subclass represented in the antibody (idiotype) population. Certainly, allotype matching of donor and recipient restricts one to matching for only the presently known allotypes. Idiotypic probes of V-region genetics might be more useful if a technique could be used that would elicit only antibodies in the antiidiotypic antiserum specific for hypervariable regions in the idiotypic population.

Evidence is presented here that determinants characteristic of idiotypes can induce synthesis of "autoantiidiotypic antibodies" within the same individual. This system might serve as a tool for studying hypervariable-region genetics and provide a different approach to questions of autoimmune processes.

Materials and Methods

Animals.—Outbred New Zealand White rabbits were used.

Haptens.—The hapten *p*-aminophenyl-*N*-trimethylammonium chloride (R4N) was obtained from Dr. V. Peter Kreiter, Glenwood, N.Y. Further purification was done by recrystallizing R4N from methanol. *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (R3N) was obtained from the Eastman Kodak Co., Rochester, N.Y. and was purified further by recrystallization from ethanol. *p*-nitrobenzoic acid (PNBA) was obtained from Matheson Coleman and Bell, East Rutherford, N.J. and was recrystallized from ethanol.

Antigen.—The hapten R4N was coupled to keyhole limpet hemocyanin (KLH) by diazotization at pH 9–9.5 using a weight ratio of 40 mg/g KLH. The KLH-R4N conjugate was

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¹ *Abbreviations used in this paper:* BSA, bovine serum albumin; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant; KLH, keyhole limpet hemocyanin; PNBA, *p*-nitrobenzoic acid; R3N, *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride; R4N, *p*-aminophenyl-*N*-trimethylammonium chloride; V, variable region.

dialyzed exhaustively against cold neutral buffer. The KLH-R4N (4–8 mg) was emulsified in either complete (CFA) or incomplete (IFA) Freund's adjuvant (Difco Laboratories, Detroit, Mich.) and injected in 4–6 sites subcutaneously over the dorsum.

Anti-R4N Purification.—Blood was collected from the marginal ear vein and allowed to clot, then the serum was separated by centrifugation and stored without preservative at -20°C . Before purification all sera were clarified by centrifugation to remove lipid and red cell debris. R4N was diazotized to bovine serum albumin (BSA) as described for KLH. Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway N. J.) was suspended in an equal volume of 0.1 M bicarbonate buffer, pH 9.0. Sepharose was activated by adding cyanogen bromide to a final concentration of 0.42 M for a total time of 9 min. The cyanogen bromide was removed by filtration, then BSA-R4N was added (2 mg/ml Sepharose) and allowed to react for 24 h at 4°C . After incubation the Sepharose BSA-R4N was packed in a column and washed with 0.01 M NH_4OH (pH 9.0) and then washed again with neutral buffer until the optical density of the effluent dropped below 0.010 measured at 280 nm. Antiserum was applied to the Sepharose BSA-R4N column at a ratio of 2 ml/ml bed volume. The serum was washed through the column with neutral buffer until the OD at 280 nm dropped below 0.010. 1 vol of physiological saline was washed through the column to minimize buffering capacity. This step was followed by adding 1 M propionic acid to the column to elute antibody. The eluted protein was dialyzed against distilled water to remove propionic acid before exhaustive dialysis against neutral buffer. The eluted protein was concentrated by pervaporation.

F(ab')₂ Preparation.—F(ab')₂ fragments of purified anti-R4N antibodies were made using pepsin digestion at pH 4.3 followed by chromatography on Sephadex G-150 (Pharmacia Fine Chemicals, Inc.) (5).

F(ab')₂ Polymerization.—Samples of F(ab')₂ fragments (2 mg/ml in 0.1 M phosphate buffer, pH 7.0) were treated with a 250-fold M excess of glutaraldehyde (Fisher Scientific Company, St. Louis, Mo.) and the reaction was stopped by adding lysine to a final 0.1 M concentration.

F(ab')₂ Iodination.—F(ab')₂ was iodinated using carrier ICl (redistilled) and ^{125}I (New England Nuclear, Boston, Mass.) according to McFarlane (6). Incorporation of ^{125}I was 1.2–1.6 atoms I/molecule F(ab')₂. After dialysis, the samples were assayed for TCA-precipitable radioactivity. All samples showed precipitable activity exceeding 98%.

Indirect Radioimmunoassay.—The technique is essentially as detailed previously (7). Briefly, 0.25- μg aliquots of ^{125}I F(ab')₂ were mixed and incubated with 10–20 μl aliquots of anti-idiotypic antiserum and then 0.3–0.6 ml monospecific goat antirabbit Fc antiserum was added. Supernate and precipitates were assayed for radioactivity in a Nuclear-Chicago automatic gamma-well scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.) equipped with a 3-inch NaI(Th) crystal.

Competitive Inhibition Reactions.—Aliquots of BSA-R4N were added to the autoanti-idiotypic antiserum before the addition of [^{125}I]F(ab')₂. In the studies with free hapten, hapten was dissolved in neutral buffer and the pH was adjusted to 8.0. Hapten was added to [^{125}I]F(ab')₂ anti-R4N antibodies and incubated overnight in the cold. The following day, autoanti-idiotypic antiserum was added and the tubes were incubated at 37°C for 4 h. Goat anti-Fc was added and the tubes were incubated at 37°C for 2 h and overnight in the cold room. In experiments using whole serum for inhibition, [^{125}I]F(ab')₂ anti-R4N and whole serum inhibitors were mixed before adding the autoanti-idiotypic antiserum. The quantities of goat anti-Fc added to the tubes were adjusted to accommodate the increase in Fc present in the reactions.

Injection Schedule.—The overall bleeding and injection schedule is shown in Table I. The rabbits were immunized and serum was collected over a period of 189 days. Following this schedule, a 432-day rest period was allowed. During this time, anti-R4N antibodies from individual rabbits were purified from a pool of serum collected during the first 189-day period. The purified antibodies were assayed by immunoelectrophoresis at a protein concentration of

30 mg/ml using a broad-spectrum goat antiserum. Only the IgG2 line was developed. Neither IgG1 (8) nor IgM protein was detected. These antibody preparations were digested with pepsin to prepare F(ab')₂ fragments. F(ab')₂ fragments were tested for the absence of Fc determinants by immunodiffusion and immunodiffusion inhibition using a monospecific goat anti-Fc antiserum. Part of the F(ab')₂ preparation was polymerized with glutaraldehyde for reinjection.

Polymerized F(ab')₂ in CFA was injected back into the rabbit that produced the F(ab')₂ on day 621. The schedule in Table I was followed through day 660.

RESULTS

Sera collected between days 641 and 660 were tested by radioimmunoassay against [¹²⁵I]F(ab')₂ fragments of anti-R4N antibodies produced earlier. Table II shows the results of homologous and heterologous assays. Each rabbit responded well against anti-R4N antibodies produced in itself and its antibody was specific only for its own anti-R4N antibodies, not the anti-R4N antibodies made in other individuals. Pooled normal rabbit serum and preinoculation serum from the five test animals showed no reaction with the labeled anti-R4N F(ab')₂ fragments. In addition, direct immunodiffusion assays using either pooled anti-R4N antiserum or the purified F(ab')₂ fragments and the 660-day autoantiidiotypic antiserum were negative for all individuals.

Control experiments were performed to confirm both the absence of small bits of immunizing antigen from the anti-R4N F(ab')₂ fragments and the absence of BSA-R4N that might have coeluted from the affinity chromatography columns. Assays also were performed to further substantiate the im-

TABLE I
Bleeding and Injection Schedule for Autoantiidiotypic Antibody Production

Day	Treatment
0	Bleed, inject KLH-R4N (CFA)
21	Bleed
44	Bleed, inject KLH-R4N (CFA)
63	Bleed
84	Bleed
150	Bleed, inject KLH-R4N (IFA)
170	Bleed
189	Bleed
Pool antisera, specific purification of anti-R4N, pepsin digestion, glutaraldehyde polymerization	
621	Inject Glu-F(ab') ₂ (anti-R4N)
634	Inject Glu-F(ab') ₂ (anti-R4N)
641	Bleed, inject Glu-F(ab') ₂ (anti-R4N)
647	Bleed
653	Bleed
660	Bleed

munoglobulin nature of the iodinated proteins. Table III shows the results of these assays.

Indirect assays suggested that BSA-R4N was not bound to the labeled $F(ab')_2$ fragments because fragments from all individuals showed no reaction with a high-titer anti-BSA antiserum. The possibility that fragments of KLH or KLH-R4N complexes were carried through the purification process and residual anti-KLH-R4N antibodies in the 641-660-day sera might be reacting with this complex bound to $[^{125}I]F(ab')_2$ anti-R4N was tested using a heterologous high-titer anti-KLH-R4N antiserum. The lack of such reaction suggested that that possibility had not occurred. The immunoglobulin nature of the $F(ab')_2$ preparations was substantiated by reaction with antiallotype antisera (Table III). The allotype distribution of the whole serum as tested by standard immunodiffusion assays is given in Table III.

TABLE II
Reactions of Autoantiidiotypic Antisera with $[^{125}I]F(ab')_2$ fragments of Anti-R4N Antibodies

Antiserum	$[^{125}I]F(ab')_2$				
	B-18	B-19	B-21	B-22	B-26
Preinoc*	0.8	1.3	1.5	2.3	2.3
PNRS†	1.1	1.5	2.0	2.3	1.2
B-18 anti-B-18	17.0	2.3	0.6	2.3	2.7
B-19 anti-B-19	0.9	39.3	1.6	2.0	2.4
B-21 anti-B-21	0.6	3.4	40.7	1.3	2.3
B-22 anti-B-22	0.1	0.7	0.3	23.3	0.7
B-26 anti-B-26	0.2	1.6	0.1	0.3	23.3

* Preinoculation (day 0) serum from each individual.

† Pooled normal rabbit serum.

TABLE III
Assays of Antigenic Nature of ^{125}I -Labeled Proteins

Antiserum	$[^{125}I]F(ab')_2$				
	B-18	B-19	B-21	B-22	B-26
Anti-BSA	1.0	1.0	1.1	0.8	1.3
Anti-KLH-R4N	0.1	0.1	0.1	0.8	1.2
Anti-Aa1	91.3	91.5	86.4	77.5	91.9
Anti-Aa2	0.7	0.7	0.9	2.6	0.4
Anti-Aa3	1.8	2.6	2.8	37.1	1.3
Anti-Ab4	93.9	95.5	90.9	93.5	94.0
Anti-Ab5	0.8	1.3	4.5	1.1	0.5
ID allotype*	alb4	alb4	alb4	ala3b4	alb4

* Phenotype as determined by standard immunodiffusion (ID) assay.

The close association of the idiotypic determinant on the $F(ab')_2$ fragments with the antigen-binding site was examined. A standard reaction of labeled anti-R4N $F(ab')_2$ fragments with corresponding antiserum was inhibited with various amounts of BSA-R4N for the two individuals (B-19, B-21) with highest percentage homologous reactions. These results (Fig. 1) show that the reaction was inhibitable by BSA-R4N. BSA alone did not inhibit the reaction.

Free hapten inhibition studies were done to verify and extend the results obtained with BSA-R4N inhibition. $[^{125}\text{I}]F(ab')_2$ fragments of anti-R4N anti-

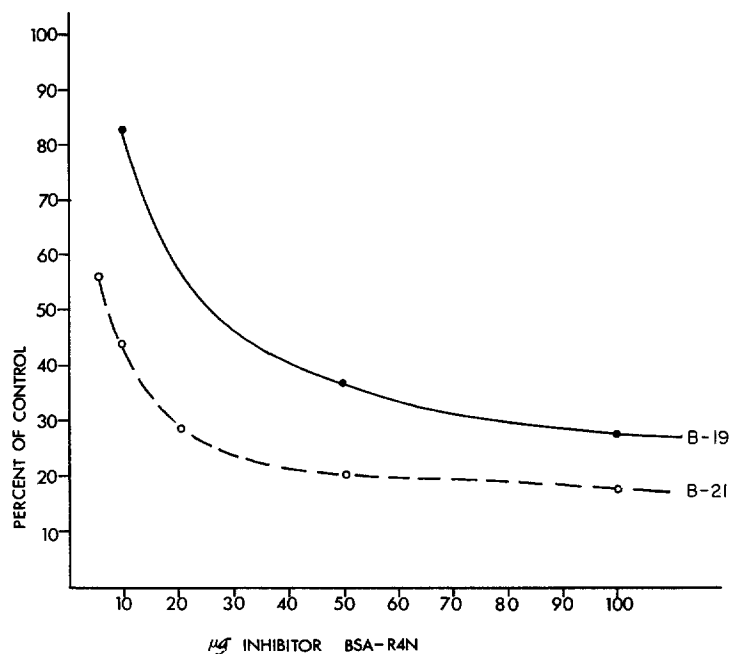


FIG. 1. Inhibition of homologous reactions of B-19 and B-21 $[^{125}\text{I}]F(ab')_2$ anti-R4N fragments with B-19 and B-21 autoantiidiotypic antisera with unlabeled BSA-R4N.

body from rabbits B-19 and B-21 were incubated with various concentrations of free haptens and the reactions obtained were calculated as percentage of uninhibited controls. These experiments are summarized in Table IV. Homologous free hapten (R4N) inhibited the reaction in each case to a much greater degree than did either a structurally similar hapten (R3N) or an unrelated hapten (PNBA). These results confirm the earlier results using BSA-R4N and show the specificity of the reaction for the R4N hapten.

Additional inhibition studies were done to determine whether or not the pepsin treatment had altered the native anti-R4N antibodies and perhaps had made a "buried" idiotypically specific determinant available for reaction. The $[^{125}\text{I}]F(ab')_2$ anti-R4N fragments were mixed with aliquots of untreated whole

serum followed by addition of the autoantiidiotypic antiserum. The results of these experiments are shown in Table V. Aliquots of the whole serum used to isolate the anti-R4N antibodies inhibited the reaction markedly, while the same serum showed no inhibition after passing it through the Sepharose BSA-R4N column. Normal rabbit serum showed no inhibitory capacity.

DISCUSSION

Idiotypes or "individual antigenic specificities" have served as immunochemical markers for studying antibody V regions in various systems since

TABLE IV
Effect of Free Haptens for Inhibition of the Reaction of [¹²⁵I]F(ab')₂ Anti-R4N Antibodies from Rabbits B-19 or B-21 and Homologous Autoantiidiotypic Antisera

	Final molar concentration of free hapten*				
	2.5×10^{-2}	2.5×10^{-3}	7.7×10^{-4}	2.5×10^{-4}	7.7×10^{-5}
Free R4N					
B-19 system ‡	—	36.1	40.7	47.0	57.2
B-21 system ‡	—	40.3	41.7	—	57.7
Free R3N					
B-19 system ‡	53.9§	66.7	—	77.6	—
B-21 system ‡	53.5	68.7	—	85.1	—
Free PNBA					
B-19 system ‡	96.7	102.4	—	102.9	—
B-21 system ‡	93.4	96.6	—	98.8	—

* Concentration of free hapten before adding goat anti-Fc.

‡ Refers to [¹²⁵I]F(ab')₂ reaction with homologous autoantiidiotypic antisera.

§ Percentage of the reaction obtained in the absence of inhibitor.

TABLE V
Effect of Undigested Whole Antibody on the Idiotype-Autoantiidiotypic Reaction of Rabbits B-19 and B-21

	Inhibitors			
	10 μ l untreated whole serum*	20 μ l untreated whole serum*	20 μ l absorbed whole serum ‡	20 μ l NRS§
B-19 system ¶	19.4	10.0	100.0	99.3
B-21 system ¶	17.1	14.7	97.1	100.0

* Whole serum from pool of 21–189 day antisera.

‡ Whole serum from pool of 21–189 day antisera after passage through Sepharose-BSA-R4N immunoadsorbent column.

§ Pooled normal rabbit serum.

¶ Refers to [¹²⁵I]F(ab')₂ reaction with homologous autoantiidiotypic antiserum.

|| Percentage of the reaction obtained in the absence of inhibitor.

their original description (1). Idiotypic antibodies found in artificially acquired active immune states in outbred animals usually are only a subpopulation of the total antibody with that particular specificity (9) and are unique to a single individual. Cross reactions have been noted among strains of inbred mice (10). The data in Table II suggest that the autoantiidiotypic reactions studied here are specific for individuals, and the percentage of anti-R4N F(ab')₂ fragments involved in the reactions suggest that the reactions involve only part of the total population of anti-R4N antibodies studied.

The immunoglobulin nature of the labeled radioimmunoassay test antigen was verified by analysis for allotypic determinants coded by the *a* and *b* locus. The results of the quantitative allotype assay of the labeled F(ab')₂ fragments paralleled the results of the standard immunodiffusion allotype assay performed on preinoculation serum.

The intimate involvement of V-region structures in determining idiotypic specificity was shown in hapten inhibition studies of idiotypes done by Briant and Nisonoff (11). Whether the inhibition was due to direct "masking" of the binding site (idiotypic site) or to perturbation of an area adjacent to the binding site is still controversial. Inhibition data do, however, support the contention that the idiotypic site (or sites) are contained within the V domain. The reaction reported here was specifically inhibitable with hapten coupled to BSA and with free hapten. It parallels other idiotypic systems in that respect. In addition, the reaction appeared to be mediated by structures present on the surface of native untreated anti-R4N antibodies since whole untreated serum from the pool used to make [¹²⁵I]F(ab')₂ fragments inhibited the reaction of the labeled fragment with the autoantiidiotypic antiserum. Whether some degradation might have occurred in vivo subsequent to secretion of the antibody from the plasma cell would be a possibility for all idiotypic systems.

Synthesis of antiidiotypic antibody by the same animal that produced the idiotypic antibody originally appears to pose a problem relating to the axiom of nonresponsiveness to "self"-antigens after immunological maturation. Autoresponsiveness to thyroglobulin (12) and components of the eye lens and spermatozoa is well recognized. This usually is explained (13) by assuming that (*a*) the components in question are normally inaccessible to blood and lymph and/or (*b*) they were not present as such in the body when tolerance to self-components was established during embryonic life. Clearly, explanation (*a*) does not fit the present circumstance because the antigen (idiotype) in question presumably was a B-cell receptor site and was part of the lymphatic system. The possibility that the determinant was not present during the "self-recognizing" period of development suggests that the B cells responsible for synthesizing these antibodies matured subsequent to immunological maturation.

Further studies using autoantiidiotypic antibodies are being directed toward genetic analysis of V regions. The ability of an individual to synthesize anti-

bodies specific for its own antibody V-region structures immediately increases theoretically the amount of genetic material needed for germ-line theories of antibody diversity (14). Other studies could be directed to determining whether autoantidiotype antibodies are elicited by an immune response without artificially reintroducing the antibodies. Such a study might suggest a mechanism for switching off synthesis of antibody by one clone of cells and establishing new clones.

SUMMARY

Rabbits were immunized with a hapten-protein conjugate and sera were collected for 189 days. The antihapten antibodies were purified by affinity chromatography, then the same animal that synthesized the antibody was reinjected with polymerized $F(ab')_2$ fragments of antihapten antibodies. Sera were collected after autoimmunization and tested by an indirect radioimmunoassay technique for reaction with $[^{125}I]F(ab')_2$ fragments of the original antihapten antibody. Results showed that each individual responded to its own $F(ab')_2$ and the antisera were specific for antihapten antibodies of that individual. Quantitative allotype assays established the immunoglobulin nature of the labeled test antigen. Inhibition assays showed that the reaction was specifically inhibitable with hapten. The relationship of this system with other idiotypic systems and the possible autoimmune implications of autoantidiotypic antibodies are discussed.

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