QUANTITATIVE INVESTIGATIONS OF IDIOTYPIC ANTIBODIES

V. Factors Affecting the Persistence and Replacement of Clones of Antibody-Producing Cells*

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There is substantial evidence that molecules of similar or identical structure are present in the serum of an individual animal during the course of prolonged immunization. Large amounts of specific activity were recovered in hybrid recombinants of heavy and light chains of anti-benzoate antibodies isolated from hyperimmunized rabbits at 6-month intervals (1). Also, a given idiotypic specificity often persists for long periods of time (2–5). Recently, Jaton et al. (6) have demonstrated persistence of identical molecules through amino acid sequence analysis of the light chains of relatively homogeneous antipneumococcal polysaccharide antibodies.

Since the half-life of an antibody molecule or of most antibody-producing cells is relatively brief (7–10), the continued presence of a given idiotypic specificity must reflect persistence of clones of cells, mediated by memory cells, which synthesize molecules of a particular structure.

This report presents results of quantitative investigations designed to measure the effect of several factors on the persistence and changes of idiotypic specificities in hyperimmunized rabbits. The parameters studied include the dose and frequency of administration of antigen and the effect of prolonged rest periods. The results are interpreted in terms of the effect of antigenic challenge on the persistence of clones of antibody-forming cells and on the induction of new clones.

Methods and Materials

Conjugates of bovine γ-globulin (BGG),[1] rabbit IgG, bovine serum albumin (BSA), and keyhole limpet hemocyanin (KLH) with *p*-azobenzoate hapten groups were prepared as previously described (2, 11). After extensive dialysis the KLH conjugate was filtered through Sephadex G-200 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) at pH 6.5; the protein eluted in the void volume was used. Other conjugates were dialyzed but not gel-filtered.

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[1] *Abbreviations used in this paper*: BGG, bovine γ-globulin; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin.

To prepare anti-hapten antibody, rabbits were inoculated subcutaneously with 3 mg of BGG–*p*-azobenzoate incorporated in complete Freund's adjuvant (Difco Laboratories, Detroit Mich.); inoculation was repeated after 3 wk. All subsequent injections were given intravenously according to the schedule in Table I. Bleeding preceded inoculation when both were done on the same day.

TABLE I

*Course of Immunization**

Rabbit	Week of immunization
	Dose: 1 mg every 2 wk
14R	1F,* 3F, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47–49P,‡ 66, 67–69P, 79, 84B§
14S	1F, 3F, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47–49P, 66, 67–69P
	Dose: 3 mg every 2 wk
16A	1F, 3F, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 41–43P, 59, 60–62P
16B	1F, 3F, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 35, 37, 41–43P, 59, 60–62P, 73, 78B
	Dose: 3 mg every 4 wk
Z4	1F, 3F, 5, 9, 13, 17, 21, 25, 29, 35, 37, 41, 45, 49, 53, 57, 61, 65, 69, 73, 77, 81, 85, 89
Z6	1F, 3F, 5, 9, 13, 17, 21, 25, 29, 33, 37, 41, 45, 49, 53, 57, 61, 65, 69, 73, 77, 81, 85, 89
Z9	1F, 3F, 5, 9, 13, 17, 21, 25, 29, 33, 37, 41, 45, 49, 53, 57, 61, 65, 69, 73, 77, 81, 85, 89
	Dose: 10 mg every 2 wk
13X	1F, 3F, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47–49P
14U	1F, 3F, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47–49P

* Unless otherwise indicated, a rabbit was bled and then inoculated with BGG-*p*-azobenzoate during the specified week. Immunization was given intravenously, except where noted by the symbol F, which indicates injection of 3 mg antigen in complete Freund's adjuvant.

‡ P: six bleedings were taken during this time interval and pooled; no inoculation was given at the end of the series of bleedings.

§ B: rabbit was bled once but not inoculated.

Antibody concentrations in hyperimmune sera were determined by precipitation with varying amounts of KLH–*p*-azobenzoate; typical precipitin curves were obtained and the maximum amount of antibody precipitable was ascertained. Each washed precipitate was dissolved in 1 ml of 0.04 N NaOH and the absorbance was determined at 280 and 400 nm. The contribution of antigen to the reading at 280 nm was determined from the known ratio of its absorbances at the two wavelengths; this value was subtracted from the total reading at 280 nm to give the absorbance attributable to antibody. To estimate the antibody concentration the extinction coefficient, $E^{1\%}_{1cm} = 15$, was used.

KLH–*p*-azobenzoate was also used to purify anti-hapten antibodies specifically. A washed antigen-antibody precipitate, prepared at equivalence in the presence of 0.01 M ethylenediaminetetraacetate (EDTA) to minimize uptake of complement components, was dissolved in 0.3 M *p*-nitrobenzoate and passed through a column of (diethylamino)ethyl (DEAE)-cellulose equilibrated with 0.04 M phosphate buffer, pH 6.8. The antigen, which is colored, was retained at the top of the column. The eluate was dialyzed for 2 days against borate-buffered saline, for 2 days against 0.1 M sodium benzoate, pH 8.0, and finally for at least 1 wk against multiple changes of the borate buffer. Chromatography on DEAE-cellulose insured that the isolated antibodies would be of the IgG class. A check on the specificity of the purified antibodies was carried out by absorbing ^{125}I-labeled F(ab')$_2$ fragments of specifically purified anti–*p*-azobenzoate antibodies to an immunoabsorbent consisting of BSA–*p*-azobenzoate coupled to Sepharose 4B (12). Under the conditions used, 75–85% of the ^{125}I-labeled F(ab')$_2$ fragments in the various preparations was absorbed, as compared to 6–8% of labeled nonspecific F(ab')$_2$ fragments. The uptake of the labeled antibody fragments was completely inhibited in the presence of 0.3 M *p*-nitrobenzoate. The same concentration of NaCl or NaI had no significant effect on the absorption. Purified antibodies were labeled with ^{125}I by the method of McFarlane (13); less than one atom was incorporated per molecule of protein. After exhaustive dialysis more than 98% of each preparation was precipitated by 5% trichloroacetic acid. The labeled purified antibody was mixed with a 20-fold excess of nonspecific rabbit IgG. 10 mg of the mixture was treated with pepsin for 18 hr at 37°C, pH 4.3, and the resulting F(ab')$_2$ fragments were purified by gel filtration on Sephadex G-150. The maximum degree of contamination by fragment Fc was determined by precipitation tests in which rabbit anti-ovalbumin antiserum was mixed with the labeled fragments; this was followed by an excess of goat anti-rabbit fragment Fc. The degree of precipitation of radioactivity in such control experiments was less than 3%.

Anti-idiotypic antisera were prepared, by one of two methods, in recipient rabbits matched to the donor with respect to allotypic specificities a1, a2, a3, b4, b5, b6, b9, c7, and c21. In the first method the purified anti–*p*-azobenzoate antibodies were polymerized with glutaraldehyde as described previously (14). 2 mg of the polymerized antigen was incorporated in complete Freund's adjuvant and injected subcutaneously; this was repeated 3 wk later. Subsequent inoculations of 2-mg portions were administered either subcutaneously in incomplete Freund's adjuvant or intravenously. These injections were given at 2-wk intervals. Anti-idiotypic antibody appeared in each rabbit after the third inoculation.

In two cases anti-idiotypic antiserum was prepared by inoculation of a saline suspension of a specific immune precipitate. Anti-benzoate antibodies were precipitated with a conjugate of *p*-azobenzoate with IgG prepared from the preimmune serum of the rabbit to be used as the recipient. This conjugate was mixed, at equivalence, with the serum of the donor rabbit hyperimmunized to the *p*-azobenzoate group. The washed precipitate was homogenized in 0.15 M saline, incorporated into an equal volume of complete Freund's adjuvant, and injected according to the schedule given above, utilizing incomplete Freund's adjuvant and subcutaneous inoculation for the third and fourth injections.

Anti-idiotypic antisera were assayed by indirect precipitation of ^{125}I-labeled F(ab')$_2$ fragments prepared from purified anti-benzoate antibodies of the donor rabbit (15). A typical reaction mixture comprised 0.5 μg of F(ab')$_2$-^{125}I fragments of anti-benzoate antibody, 9.5 μg of unlabeled nonspecific F(ab')$_2$ fragments, 6–20 μliters of anti-idiotypic antiserum, and an excess (0.3–0.5 ml) of goat anti-rabbit fragment Fc. Before the addition of the goat antiserum, the reaction mixture was allowed to stand for 1 hr at 37°C. The percentage of labeled fragments precipitated was determined by measurement of radioactivity in the washed precipitate and in the combined supernatants. Percentages of labeled fragments precipitated by anti-idiotypic antisera, using the indirect method, are given in Table II.

Tests of idiotypic specificity were carried out by using various unlabeled subtances as inhib-

itors of the binding of ^{125}I-labeled F(ab')$_2$ fragments to anti-idiotypic antibodies. Unlabeled substances to be tested as inhibitors were mixed with the labeled fragments before the addition of anti-idiotypic antiserum. Inhibitors tested included nonspecific IgG, hyperimmune antisera from rabbits, including the donor, immunized with BGG–*p*-azobenzoate conjugates, specifically purified anti–*p*-azobenzoate antibodies from individual rabbits, and preimmune serum of the donor rabbit. A large excess of nonspecific IgG had no effect on precipitation in any system studied. Similarly, 20 μliters of heterologous anti–*p*-azobenzoate antisera from each of a panel of at least six rabbits caused less than 10% inhibition of precipitation. In every instance, 20 μliters of the autologous hyperimmune antiserum gave essentially complete inhibition of indirect precipitation. Serum taken from the same donor rabbit before immunization had no significant inhibitory effect in any of the systems investigated.

TABLE II

*Anti-idiotypic Antisera Directed to Purified Rabbit Anti-p-azobenzoate Antibodies**

Donor rabbit	Recipient rabbit	Donor serum pool (Wk of Immunization)	^{125}I-donor F(ab')$_2$ precipitated‡ (% of Total)
Z4	G34	21§	35
Z6	17O	21	50
Z9	B10	23	15
13X	C20	47–49‖	40
14R	C43	47–49	54
14S	C21	47–49	44
14U	C47	47–49	53
16A	C44	60–62	45
16B	C46	41–43	28

* Assays were carried out by indirect precipitation using as antigen 0.5 μg of ^{125}I-labeled F(ab')$_2$ fragments of purified anti-*p*-azobenzoate antibody from the donor rabbit.

‡ Maximum (plateau) value obtained with increasing amounts of anti-idiotypic antiserum. The percentage of ^{125}I-labeled donor F(ab')$_2$ fragments precipitated by an equal volume of rabbit anti-ovalbumin antiserum (less than 3%) was subtracted to give the amount of specific precipitation.

§ Anti-*p*-azobenzoate antiserum was taken after 21 wk of immunization of the donor rabbit.

‖ Anti-*p*-azobenzoate antiserum was taken over a 2 wk period, 47–49 wk after the start of immunization of the donor rabbit. In the text this pool is referred to as week 48 antiserum.

A major difference between these and our earlier investigations of persistence of idiotype (2, 3) was the use of whole hyperimmune anti-benzoate antisera in many experiments, rather than purified anti-benzoate antibodies, to test for inhibition of anti-idiotypic antibodies. Because of the time required and loss of material in specific purification, the use of whole antisera enabled us to increase the scope of the investigation.

Justification for this procedure includes data indicating that preimmune serum of each donor rabbit failed to inhibit the indirect precipitation of F(ab')$_2$-^{125}I fragments of anti-benzoate antibody of the donor by its homologous anti-idiotypic antiserum; this indicates that anti-benzoate antibodies are responsible for the inhibition by serum. Also, removal of anti-benzoate antibodies from each donor serum, by precipitation at equivalence with KLH–*p*-azobenzoate, removed at least 95% of its inhibitory capacity. For each donor rabbit these ab-

sorption tests were carried out with that serum which provided the anti-benzoate antibodies used for eliciting anti-idiotypic antibodies and also with bleedings of the same rabbit taken at one or two other times during the course of immunization. In addition to showing that anti-benzoate antibody is responsible for the inhibition observed, these experiments demonstrated that the anti-idiotypic antibodies were not directed to nonprecipitating antibodies, since precipitation with KLH-azobenzoate removed nearly all inhibitory capacity. This may be attrib-

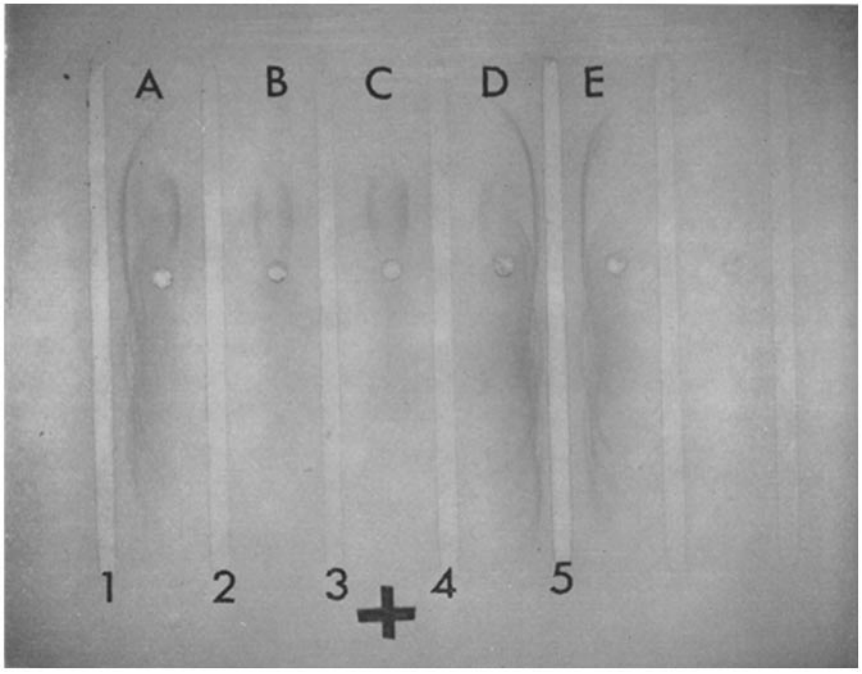

FIG. 1. Immunoelectrophoretic patterns of hyperimmune anti-benzoate antiserum from two rabbits. Well A, rabbit 13X, week 5 antiserum; well B, rabbit 13X, week 48 antiserum; well C, rabbit 14U, week 6 antiserum; well D, rabbit 14U, week 48 antiserum; well E, pooled normal rabbit serum. Troughs 1 and 5 were filled with sheep anti-rabbit antiserum; troughs 2, 3, and 4 were filled with KLH-*p*-azobenzoate.

utable to the fact that the immunogen (anti-benzoate antibody) was purified by a method which included specific precipitation as its first step.

The amount of anti-benzoate antibody present in each sample of antiserum used for inhibition studies was determined by the quantitative precipitin test as described above. The maximum amount of antibody precipitable was taken as its serum concentration.

Since IgM and IgG may share idiotypic specificities (4), anti-benzoate antibodies of the IgM class present in an antiserum may have contributed to its inhibitory capacity. While this possibility cannot be eliminated, we failed to detect appreciable amounts of IgM (or IgA) anti-benzoate antibodies by immunoelectrophoresis. Several examples are shown in Fig. 1. These tests included the first set of bleedings of each rabbit showing a significant titer of antibody.

Since IgM was not detected the change in idiotype later in the immunization period cannot be attributed to a switch from IgM to IgG. Although some contribution by IgM or IgA cannot be excluded, the error introduced by the assumption that idiotypic antibodies are IgG is probably small.

The inhibition data shown in Figs. 2–8 (rabbits 13X, 14R, 14S, 14U, 16A, and 16B) were all obtained by using whole hyperimmune antisera as inhibitors. Unlabeled specifically purified antibodies were utilized to secure the data in Figs. 9–11 (rabbits Z4, Z6, and Z9).

Immunoabsorbents were prepared by coupling the desired protein to Sepharose 4B by the method of Axen et al. (12), using approximately 2 mg protein per cm^3 of packed gel.

RESULTS

If, during the course of immunization, changes in idiotypic populations occur in part as a result of continued initiation of new clones of cells, one might expect that the dosage of antigen and frequency of challenge should influence the establishment of new idiotypic populations of antibody molecules. Experiments designed to investigate these two parameters are described below.

Effect of Dosage of Antigen.—In these experiments the lowest dosage of antigen used per intravenous inoculation was 1 mg, since previous experience had shown that this is about minimal for elicitation of the substantial quantities of anti-benzoate antibody (> 0.5 mg/ml), that were necessary for these studies. Rabbits were inoculated twice with antigen in Freund's adjuvant, with a 3-wk interval between inoculations, then intravenously at 2-wk intervals (Table I), with either, 1, 3, or 10 mg of antigen per injection. Anti-idiotypic antibodies were prepared against anti-*p*-azobenzoate antibodies isolated from bleedings taken either 41–43 or 47–49 wk after the start of immunization with BGG–*p*-azobenzoate. (The 41–43 wk bleedings and the 47–49 wk bleedings will be referred to as week 42 and week 48 antisera, respectively.) ^{125}I-labeled $F(ab')_2$ fragments were prepared from a portion of the batch of purified antibody used for immunization and were utilized for indirect precipitation tests. The amounts of antibody having the same idiotypic determinants as the immunogen were determined by inhibition of indirect precipitation, utilizing whole unlabeled antiserum from various bleedings as the inhibitor. The concentration of anti-*p*-azobenzoate antibody in each antiserum was quantified by the precipitin reaction (see Methods and Materials) and data are presented in the figures in terms of the amounts of anti-*p*-azobenzoate antibody present in the antiserum used as inhibitor.

Data obtained with antisera from rabbit 13X are shown in Fig. 2. This rabbit received 10 mg of BGG–*p*-azobenzoate per inoculation (high dose). Binding to anti-idiotypic antiserum of $F(ab')_2$-^{125}I fragments derived from the immunogen (week 48 antiserum) was almost completely inhibited by an excess of unlabeled antiserum from the same serum pool; antisera taken after 29 or 39 wk of immunization of rabbit 13X were nearly as effective on a quantitative basis. Antiserum from the week 17 bleeding was less potent as an inhibitor; nevertheless, the inhibition curve indicates that half of the idiotypic population in the

immunogen (week 48) was represented after 17 wk. In contrast, the week 9 antiserum gave maximum inhibition of only 20% and the week 5 antiserum was ineffective. The rapid change in idiotype which occurred after the initial period of immunization, and subsequent slower changes, are in accord with previous findings with rabbits immunized every 2 wk (2, 3).

The data are presented in another form in Fig. 3, which shows the degree of inhibition by a single concentration of unlabeled antibody (60-fold excess with respect to $F(ab')_2$-^{125}I) from a larger series of bleedings of rabbit 13X,

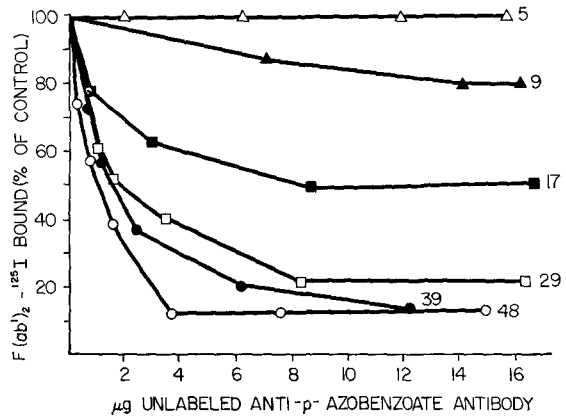

FIG. 2. Inhibition of binding of ^{125}I-labeled $F(ab')_2$ fragments (0.5 μg) of anti-*p*-azobenzoate antibodies from rabbit 13X to an approximately equivalent amount of anti-idiotypic antibodies. The immunogen was anti-*p*-azobenzoate antibody isolated from the serum of rabbit 13X 48 wk after the start of immunization. Competitors are unlabeled, whole antisera taken from bleedings of rabbit 13X at various times; the number of weeks after the start of immunization is indicated by the numeral on each curve. The following concentrations of precipitable anti-benzoate antibody were present: week 5, 2.4 mg/ml; week 9, 2.8 mg/ml; week 17, 3.4 mg/ml; week 29, 3.3 mg/ml; week 39, 2.5 mg/ml; week 48, 1.5 mg/ml.

including those represented in Fig. 2. The other curves in Fig. 3 represent data obtained with other rabbits (discussed below).

Fig. 4 presents the results obtained with another rabbit, 14U, which also received the high dose (10 mg) every 2 wk. Except for a somewhat more rapid change in idiotype between weeks 31 and 48, the patterns are very similar to those of rabbit 13X (Fig. 2), which had the same injection dose and schedule. (See also Fig. 3.)

Data for rabbit 14S, which was on the low dose schedule (1 mg per injection), are shown in Figs. 3 and 5. Quantitative changes in idiotype occurred somewhat more rapidly than in rabbit 13X (high dose) during the 10–15 wk period before week 48, but were very similar to those observed in the other rabbit, 14U, receiving the high dose. Thus, only 25–35% of the molecules present in the

immunogen (week 48) were represented in bleedings taken 15 or 21 wk after immunization. In rabbits 13X and 14U, on the high dose schedule, the corresponding values were approximately 50 and 20%, respectively.

Data obtained with antisera of rabbit 14R, which also received 1 mg per injection (low dose), are shown in Figs. 3 and 6. Again there is a somewhat more rapid change in expression of the idiotype of the immunogen (week 48) after

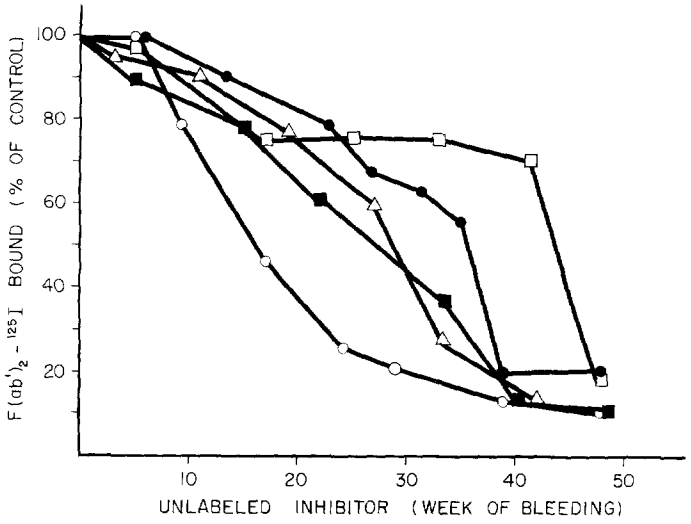

FIG. 3. Inhibition of binding of ^{125}I-labeled F(ab')$_2$ fragments (0.5 μg) of anti-*p*-azobenzoate antibodies to the homologous anti-idiotypic antiserum by 30 μg amounts of anti-*p*-azobenzoate antibodies present in the serum of the donor rabbit at varying times after the start of immunization. The number of weeks is indicated on the abscissa of the graph. Rabbits 13X (○) and 14U (●) received 10 mg of antigen every 2 wk; anti-idiotypic antisera were prepared against the antibodies present 48 wk after the start of immunization. Rabbits 14R (□) and 14S (■) received 1 mg of antigen every 2 wk; anti-idiotypic antisera were prepared against the antibodies present at week 48. Rabbit 16B (△) received 3 mg of antigen every 2 wk; anti-idiotypic antiserum was prepared against antibodies present at week 42.

week 25 than was observed with rabbit 13X (high dose), but the results were quite similar to those obtained with the second rabbit, 14U, on the high dose schedule.

These results are summarized in Fig. 3, which also includes data for one rabbit, 16B, on an intermediate dose schedule (3 mg per injection). In all rabbits very few molecules possessing the idiotype of the immunogen (week 48) were present in the earliest bleedings. Subsequently, in each rabbit there was a steady increase of the concentration of this idiotype in the immune serum. No clear correlation with dosage is evident; if anything, the idiotype appeared to change more rapidly after week 20 when a low dosage (1 mg) was administered

(cf. rabbits 14R and 13X). However, additional studies would be necessary to establish this point.

These results are somewhat unexpected since one might predict that a repeated high dose of antigen would continually initiate new clones of antibody-producing cells. This apparently is not the case.

Effect of Frequency of Inoculation on Changes in Idiotypic Specificities.—In this series of experiments groups of rabbits were inoculated with 3 mg quantities

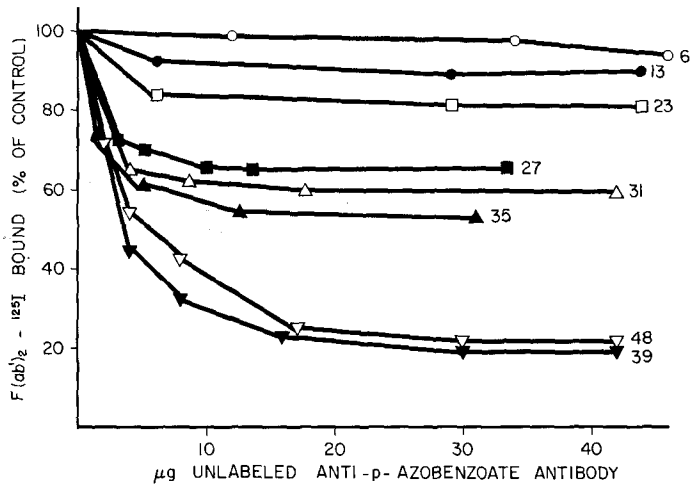

FIG. 4. Inhibition of binding of ^{125}I-labeled F(ab')$_2$ fragments (0.5 μg) of anti-*p*-azobenzoate antibodies from rabbit 14U to an approximately equivalent amount of anti-idiotypic antibodies. The immunogen was anti-*p*-azobenzoate antibody isolated from the serum of rabbit 14U 48 wk after the start of immunization. Competitors are unlabeled antisera taken from bleedings of rabbit 14U at various times; the number of weeks after the start of immunization is indicated by the numeral on each curve. The following concentrations of precipitable anti-benzoate antibody were present: week 6, 0.7 mg/ml; week 13, 0.8 mg/ml; week 23, 0.6 mg/ml; week 27, 1.3 mg/ml; week 31, 1.7 mg/ml; week 35, 1.3 mg/ml; week 39, 3.2 mg/ml; week 48, 1.7 mg/ml.

of BGG–*p*-azobenzoate at different time intervals. In a second series of experiments, the effect of a prolonged rest period between injections was investigated; 1 or 3 mg doses of antigen were used in this part of the experiment.

Fig. 7 shows quantitative inhibition data obtained with anti–*p*-azobenzoate antisera of rabbit 16B, which was inoculated with 3 mg of antigen every 2 wk. (Data obtained with the highest concentration of each inhibitory antiserum are shown in Fig. 3.) The anti-idiotypic antiserum was directed to specifically purified antibody from bleedings of rabbit 16B taken 42 wk after the start of immunization (Table I). The data indicate that approximately 40% of the

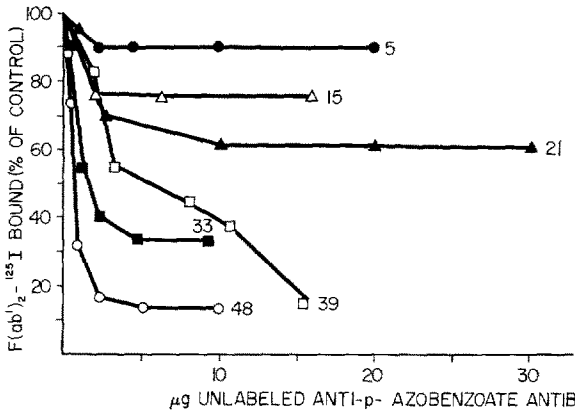

FIG. 5. Inhibition of binding of ^{125}I-labeled F(ab')$_2$ fragments (0.5 μg) of anti-*p*-azobenzoate antibodies from rabbit 14S to an approximately equivalent amount of anti-idiotypic antibodies. The immunogen was anti-*p*-azobenzoate antibody isolated from the serum of rabbit 14S 48 wk after the start of immunization. Competitors are unlabeled anti-benzoate antisera from bleedings of rabbit 14S taken at various times; the number of weeks after the start of immunization is indicated by the numeral on each curve. The following concentrations of precipitable anti-benzoate antibody were present: week 5, 0.5 mg/ml; week 15, 0.8 mg/ml; week 21, 1.0 mg/ml; week 33, 0.4 mg/ml; week 39, 0.8 mg/ml; week 48, 0.5 mg/ml.

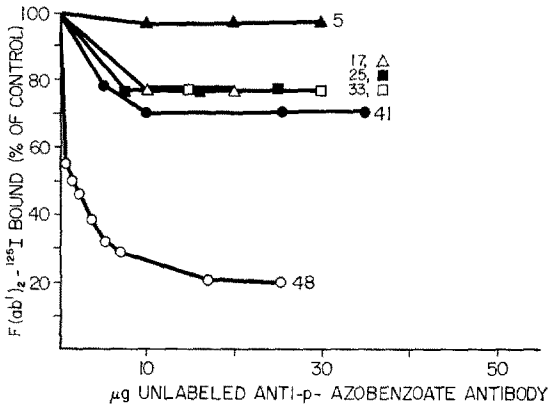

FIG. 6. Inhibition of binding of ^{125}I-labeled F(ab')$_2$ fragments (0.5 μg) of anti-*p*-azobenzoate antibodies from rabbit 14R to an approximately equivalent amount of anti-idiotypic antibodies. The immunogen was anti-*p*-azobenzoate antibody isolated from the serum of rabbit 14R 48 wk after the start of immunization. Competitors are unlabeled anti-benzoate antisera taken from bleedings of rabbit 14R at various times; the number of weeks after the start of immunization is indicated by the numeral on each curve. The following concentrations of precipitable anti-benzoate antibody were present: week 5, 1.0 mg/ml; week 17, 1.1 mg/ml; week 25, 0.8 mg/ml; week 33, 1.4 mg/ml; week 41, 1.0 mg/ml; week 48, 0.3 mg/ml.

idiotypic molecules in the immunogen (week 42) were represented at week 27; i.e., there was a fairly rapid change after week 27.

Fig. 8 shows similar data obtained with another rabbit 16A, also on a biweekly schedule of injections. The anti-idiotypic antiserum in this case was prepared against week 62 antibody. Again a continual and definite change in idiotypic specificities occurred between weeks 23 and 43. Thus, biweekly injections appear to result in gradual changes in idiotype after several months of immunization. Initially, however, there is a marked change in idiotype, evident

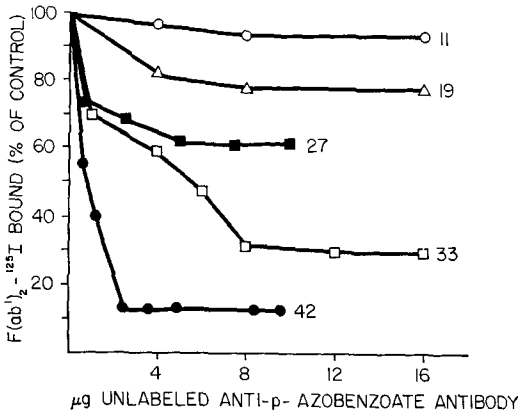

FIG. 7. Inhibition of binding of ^{125}I-labeled F(ab')$_2$ fragments (0.5 μg) of anti-*p*-azobenzoate antibodies from rabbit 16B to an approximately equivalent amount of anti-idiotypic antibodies. The immunogen was anti-*p*-azobenzoate antibody isolated from the serum of rabbit 16B, 42 wk after the start of immunization. Competitors are unlabeled antisera taken from bleedings of rabbit 16B at various times; the number of weeks after the start of immunization is indicated by the numeral on each curve. The following concentrations of precipitable anti-benzoate antibody were present: week 11, 1.6 mg/ml; week 19, 0.8 mg/ml; week 27, 0.5 mg/ml; week 33, 0.8 mg/ml; week 42, 0.5 mg/ml.

after 11–29 wk of immunization with this antigen (Figs. 7 and 8). Similar results have been reported previously for other rabbits on a biweekly schedule of immunization (2, 3).

Figs. 9–11 present data obtained with the sera of 3 rabbits that were inoculated with 3 mg portions of antigen every 4 wk, rather than biweekly. Anti-idiotypic antibodies were prepared against anti-benzoate antibodies of the week 21 or week 23 bleedings. Here the changes in idiotype are definitely slower than those observed with the biweekly schedule. It should be noted that the inhibitors used in these studies were specifically purified anti-*p*-azobenzoate antibodies rather than hyperimmune antisera. In the case of rabbit Z4 the inhibition curve obtained with week 21 antibodies (the immunogen) was very similar to that of the week 45 antibodies. In the 15 month period after week

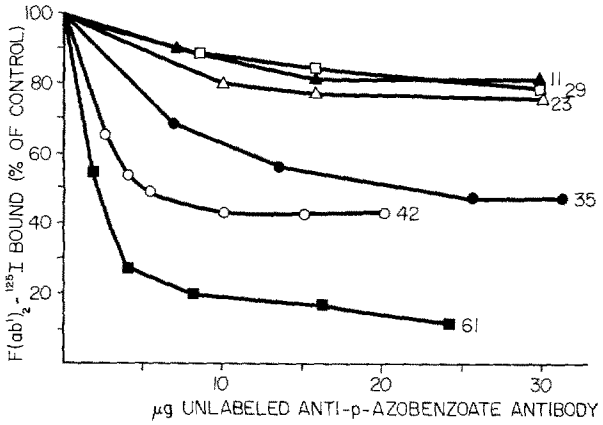

FIG. 8. Inhibition of binding of ^{125}I-labeled F(ab')$_2$ fragments (0.5 μg) of anti-*p*-azobenzoate antibodies from rabbit 16A to an approximately equivalent amount of anti-idiotypic antibodies. The immunogen was anti-*p*-azobenzoate antibody isolated from the serum of rabbit 16A, 61 wk after the start of immunization. Competitors are unlabeled antisera taken from bleedings of rabbit 16A at various times; the number of weeks after the start of immunization is indicated by the numeral on each curve. The following concentrations of precipitable antibody were present: week 11, 2.4 mg/ml; week 23, 1.5 mg/ml; week 29, 1.6 mg/ml; week 35, 1.3 mg/ml; week 42, 1.0 mg/ml; week 61, 1.6 mg/ml.

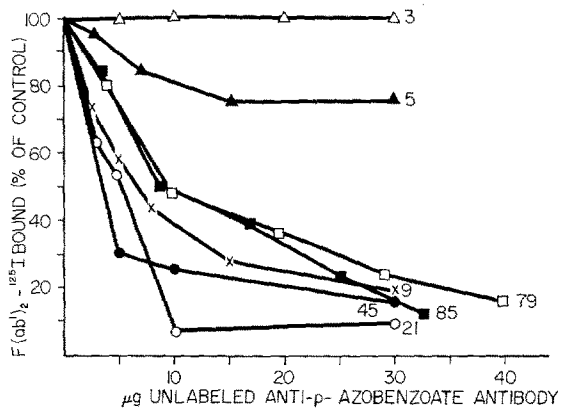

FIG. 9. Inhibition of binding of ^{125}I-labeled F(ab')$_2$ fragments (0.5 μg) of anti-*p*-azobenzoate antibodies from rabbit Z4 to an approximately equivalent amount of anti-idiotypic antibodies. The immunogen was anti-*p*-azobenzoate antibody isolated from the serum of rabbit Z4, 21 wk after the start of immunization. Competitor molecules are unlabeled specifically purified anti-*p*-azobenzoate antibodies isolated from bleedings of rabbit Z4 at various times; the number of weeks after the start of immunization is indicated by the numeral on each curve. The following concentrations of precipitable anti-benzoate antibody were present in the antisera: week 3, 0.5 mg/ml; week 5, 2.0 mg/ml; week 9, 2.6 mg/ml; week 21, 1.0 mg/ml; week 45, 0.5 mg/ml; week 79, 0.4 mg/ml; week 85, 0.3 mg/ml.

21, there was a quantitative change in the expression of idiotype, but essentially all of the idiotype identified at week 21 was still present at week 85; this is shown by the capacity of antibodies from week 85 antiserum to displace nearly all of the labeled $F(ab')_2$ fragments from homologous anti-idiotypic antiserum. The smaller slope of the inhibition curve for week 85 antibodies indicates that molecules having different idiotypic specificities were also present.

Very similar results were observed with the antibodies of rabbit Z6 (Fig. 10). Again there was little change in idiotype between weeks 21 and 49, and only a

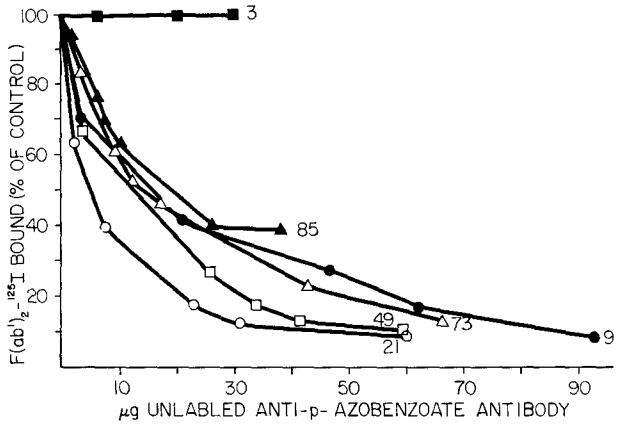

FIG. 10. Inhibition of binding of ^{125}I-labeled $F(ab')_2$ fragments (0.5 μg) of anti-*p*-azobenzoate antibodies from rabbit Z6 to an approximately equivalent amount of anti-idiotypic antibodies. The immunogen was anti-*p*-azobenzoate antibody isolated from the serum of rabbit Z6, 21 wk after the start of immunization. Competitor molecules are unlabeled specifically purified anti-*p*-azobenzoate antibodies isolated from bleedings of rabbit Z6 at various times; the number of weeks after the start of immunization is indicated by the numeral on each curve. The following concentrations of precipitable anti-benzoate antibody were present in the antisera: week 3, 0.7 mg/ml; week 9, 1.6 mg/ml; week 21, 0.8 mg/ml; week 49, 0.3 mg/ml; week 73, 0.3 mg/ml; week 85, 0.3 mg/ml.

gradual change to week 73; i.e., nearly all the idiotypic specificities in the antibody population at week 21 were still present a year later, although at somewhat lower concentration. There seemed to be a change in idiotype between weeks 73 and 85, but the low titer of the latter antiserum precluded tests at higher antibody concentrations with the use of amounts of inhibitory serum which would give very heavy precipitates with the goat anti-Fc.

The third rabbit, Z9, on the monthly injection schedule showed a somewhat different pattern (Fig. 11). About 70% of the idiotypic molecules identified at week 23 were still present at week 43, but the inhibition curve for the homologous bleeding was considerably steeper. Also, there was a continued change of the idiotype to week 85. Nevertheless, the latter antiserum still possessed 40%

of the molecules recognized in the immunogen (week 23) by its homologous anti-idiotypic antiserum.

Rabbits Z4 and Z6, which were inoculated every 4 wk, showed the most gradual change in idiotype of animals which we have investigated to date. (This observation refers to the rate of change after several weeks of immunization. In each rabbit the earliest antibodies produced differed markedly in idiotype from those synthesized later in the course of immunization.)

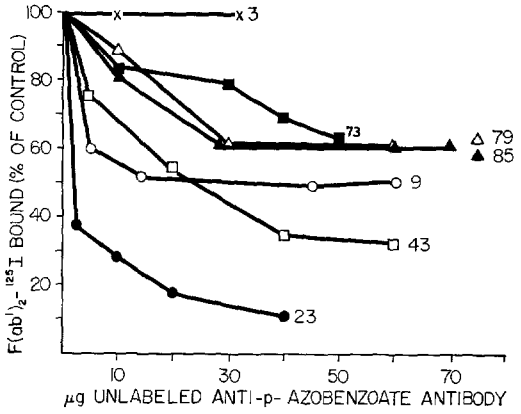

FIG. 11. Inhibition of binding of ^{125}I-labeled F(ab')$_2$ fragments (0.5 μg) of anti-*p*-azobenzoate antibodies from rabbit Z9 to an approximately equivalent amount of anti-idiotypic antibodies. The immunogen was anti-*p*-azobenzoate antibody isolated from the serum of rabbit Z9, 23 wk after the start of immunization. Competitors molecules are unlabeled specifically purified anti-*p*-azobenzoate antibodies isolated from bleedings of rabbit Z9 at various times; the number of weeks after the start of immunization is indicated by the numeral on each curve. The following concentrations of precipitable anti-benzoate antibody were present in each antiserum: week 3, 0.9 mg/ml; week 9, 2.2 mg/ml; week 23, 0.4 mg/ml; week 43, 0.8 mg/ml; week 73, 0.7 mg/ml; week 79, 0.7 mg/ml; week 85, 0.3 mg/ml.

Effect of an Extended Rest Period on the Quantitative Expression of Idiotypic Specificities.—After repeated inoculation over a period of 37–45 wk, four rabbits (14R, 14S, 16A, and 16B) were allowed to rest for 5 months, then were challenged again with BGG–*p*-azobenzoate. Data obtained before this rest period have already been presented (Figs. 3, 5–8). Two of the rabbits, 14R and 14S, had received 1 mg of antigen every 2 wk before the rest period and were given one injection of 1 mg immediately afterwards (Table I). Before this booster injection, no antibodies were detectable by the precipitin test. 1 wk afterwards, the amounts of precipitable anti-benzoate antibody in the sera of rabbits 14R and 14S were 1.8 and 2.6 mg/ml respectively. The other two rabbits, 16A and 16B, had received 3 mg of antigen on a biweekly schedule and 3 mg was administered after the rest period (Table I). Again there was no measurable titer

immediately after the rest; the concentrations of antibody in the two rabbits 1 wk after the booster inoculation were 1.6 and 0.45 mg/ml, respectively.

The anti-idiotypic antisera used for this series of investigations were prepared against antibody from bleedings taken at the start of the rest period (1 wk after injection), except for rabbit 16A; in this instance anti-idiotypic antiserum was prepared against antibody isolated 1 wk after the booster injection which terminated the rest period. Two of the rabbits, 16B and 14R, were given a second rest period of 14 wk, followed by another injection (Table I).

The results of these experiments are shown in Fig. 12. Each of the four graphs in this figure represents data obtained with a different donor-recipient pair of rabbits. The data in Figs. 12 A, B, and C were obtained with anti-idiotypic antisera prepared against purified anti-benzoate antibodies isolated at the start of the rest period. Results in Fig. 12 D were obtained with an anti-idiotypic antiserum directed to anti-benzoate antibody isolated after the injection which terminated the first rest period. Three curves, rather than two, are shown in Figs. 12 A and C, because these rabbits were allowed two rest periods (see figure legend). It is evident that the idiotypic antibodies present in rabbits 14R, 14S, and 16B before the rest period were still represented in the serum of the rabbit after the booster injection 5 months later. This is shown by the capacity of each anti-benzoate antiserum taken subsequent to the rest period to inhibit the binding of $F(ab')_2$-^{125}I fragments of anti-benzoate antibodies, isolated before resting, to their homologous anti-idiotypic antibodies. For rabbits 14R and 14S displacement by the antibodies from the later bleeding was essentially complete, although the homologous (prerest) antibodies were quantitatively more effective inhibitors on a weight basis. In the case of rabbit 16B (Fig. 12 C), a maximum of about 70% displacement was observed, although this value actually increased after the second rest period.

These results indicate that nearly all of the antibody-producing clones of cells that were active before the rest period were still present afterwards, but that one or more additional clones were stimulated, so that the inhibitory capacity of the anti-benzoate antibodies, per unit weight, was diminished. As indicated in the figure legend, the antibody titer increased for each rabbit after the first rest period. Thus, the decrease in the total amount of idiotype in the antiserum was less than would be suggested by the change in slope of each inhibition curve. When the titer of antibody is taken into consideration the concentration of idiotypic antibodies in the serum of rabbit 14R was approximately the same after resting; for rabbits 14S and 16B the concentration was somewhat decreased.

The results in Fig. 12 D were obtained with anti-idiotypic antiserum prepared against anti-benzoate antibodies isolated *after* the rest period (rabbit 16A). In this case the antibodies present before resting were not capable of completely displacing the homologous (postrest) antibodies. However, this observation may

not conflict with the data for the other three rabbits. Although all idiotypic antibodies identified in rabbit 16A at week 61 were not present at week 42, the

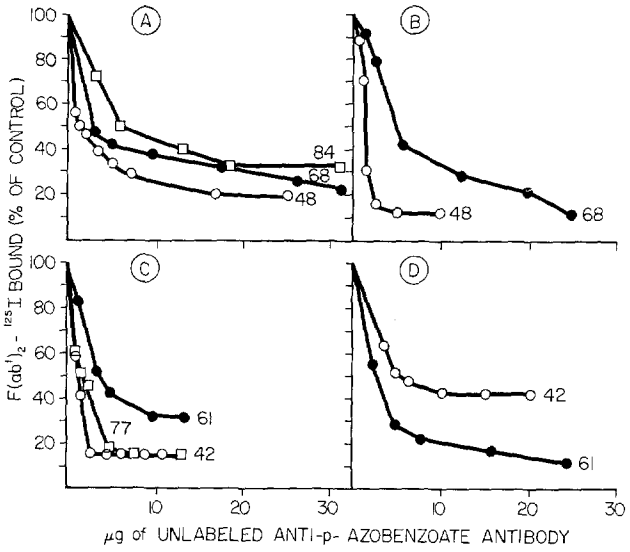

FIG. 12. Inhibition of binding of ^{125}I-labeled F(ab')$_2$ fragments (0.5 μg) of anti-*p*-azobenzoate antibodies to homologous anti-idiotypic antisera by anti-*p*-azobenzoate antibodies present before and after a 5 month rest period. Rabbits 14R (Fig. 12 A) and 14S (Fig. 12 B) were inoculated every 2 wk between weeks 1 and 45, with 1 mg of BGG–*p*-azobenzoate. They were allowed to rest between weeks 46 and 65; during week 66 they were bled and given a booster inoculation. Serum was again collected at weeks 67–69. Anti-idiotypic antisera were prepared against the antibodies present 48 wk after the start of immunization. Also, rabbit 14R was allowed a second rest period between weeks 67 and 79. The rabbit was given a booster inoculation during week 79 and antiserum was collected at week 84. Rabbit 16B (Fig. 12 C) and 16A (Fig. 12 D) were inoculated every 2 wk, between weeks 1 and 37, with 3 mg of BGG–*p*-azobenzoate. They were allowed to rest between weeks 38 and 58, then were bled and given a booster injection. Antiserum was again collected between weeks 60 and 62. Rabbit 16B was allowed a second rest period between weeks 60 and 72; it was inoculated again at week 73 and bled at week 77. Anti-idiotypic antisera were prepared against the antibodies present at week 41 in rabbit 16B and at week 61 in rabbit 16A. The following concentrations of precipitable anti-benozate were present in antisera: Rabbit 14S: week 48, 0.5 mg/ml; week 68, 2.6 mg/ml. Rabbit 14R: week 48, 0.3 mg/ml; week 68, 1.8 mg/ml; week 84, 0.6 mg/ml. Rabbit 16B: week 41, 0.5 mg/ml; week 61, 0.4 mg/ml; week 77, 0.2 mg/ml. Rabbit 16A: week 41, 1.0 mg/ml; week 61, 1.6 mg/ml.

converse is not necessarily true. That is, all molecular species present at week 42 may still have been represented, but at lower concentration, after the rest period, with the decrease in concentration attributable to new molecular species. The data thus indicate that new clones were active in rabbit 16A after

resting and a booster inoculation, and indeed, the same conclusion must apply to the other three rabbits on the basis of the decrease in concentration of idiotypic antibodies recognized by anti-idiotypic antiserum directed to the prerest antibodies.

DISCUSSION

The analysis of idiotypic specificities provides a convenient tool for following the continued presence or changes of molecular species during the course of prolonged immunization. It is generally thought that molecules of a given idiotype are the product of a single clone of cells (16). Since rabbit anti-benzoate antibodies are heterogeneous our conclusions are based on changes in multiple idiotypic populations. Studies reported heretofore have shown that:

(*a*) Molecules of a given idiotype may be present over long periods of time after hyperimmunization (2–6).

(*b*) After hyperimmunization, continued but gradual quantitative changes occur in populations of anti-benzoate antibodies in rabbits that are frequently challenged with antigen (3).

(*c*) Pronounced changes take place after the first 2–4 months of immunization with a protein-benzoate conjugate. The antibody population present at month 8, in rabbits repeatedly challenged with the antigen, was almost entirely distinct from anti-benzoate molecules present at month 2 (2, 3). Through qualitative measurements Oudin and Michel (4) similarly observed the loss of a set of idiotypic determinants, during the early stages of immunization of one rabbit with *Salmonella*; another set of idiotypic determinants persisted. Jaton et al. (6) obtained somewhat different results in a rabbit which had been immunized with a pneumococcal vaccine. Antibodies present after each of three 1-month courses of immunization, with a 1-month rest between courses, shared a major idiotypic component, although quantitative changes were noted. Similarly, Eichmann et al. (5) observed the presence of antibodies after primary immunization with streptococcal vaccine which shared idiotypic specificity with antibodies isolated as long as 17 months later. It seems possible that carbohydrate antigens may initially select out clones of cells that are not readily replaced during continued immunization.

(*d*) Idiotypic specificities persist not only during a prolonged rest period, but also in the face of repeated challenge by antigen over a period of as long as a year (2).

(*e*) In quantitative tests, the antibody population used as immunogen has invariably reacted more effectively with homologous anti-idiotypic antibodies than antibodies of the same specificity isolated from any other bleeding of the same rabbit (2, 3). This finding is confirmed in the present investigation.

The experiments reported here were designed to study the effects of dosage of antigen, of the frequency of its administration, and of a prolonged rest period

on the quantitative expression of idiotypic specificities. Although a limited number of rabbits was necessarily investigated certain patterns of response are evident.

In all rabbits there was a marked change in idiotypic specificities after the first 1–3 months of immunization. The earliest antibodies produced, in each of nine rabbits, carried almost none of the idiotypic specificities of the anti-benzoate population present after 20–60 wk of immunization (Figs. 2–11). In an earlier study of two rabbits, (2, 3) this change in idiotype was found to be associated with an increase in hapten-binding affinity in the "later" (8 month) antibodies. Such an increase in affinity was noted earlier by Eisen and Siskind (17), who worked with rabbit anti-dinitrophenyl antibodies.

In studying the effect of dosage of antigen (1, 3, or 10 mg administered every 2 wk) on the rate of change of idiotypic specificity, no marked trend was observed. Contrary to what might have been expected, the changes in idiotype seemed, if anything, to be somewhat more gradual when 10 mg of antigen was administered (Figs. 2–8). It thus appears that larger quantities of antigen do not necessarily favor initiation of new clones of antibody-producing cells, and that after hyperimmunization the antigen mainly serves to stimulate existing clones of cells, although new clones are slowly initiated. This could reflect a greater antigen-binding affinity of the receptors on memory cells as compared to precursor cells, or simply the presence of a larger number of memory cells. Alternatively, one might speculate that essentially all precursor cells are stimulated early in the course of immunization, and that some of these cells give rise to antibody-producing clones while the remainder are rendered tolerant. The gradual change in idiotype would reflect the appearance and stimulation of new precursor cells. This interpretation would be analogous to that often invoked to account for escape from tolerance.

The effect of frequency of administration of antigen was investigated by challenging rabbits with 3 mg of BGG–*p*-azobenzoate every 2 wk (two rabbits) or every 4 wk (three rabbits), or by allowing hyperimmunized rabbits to rest for long periods between injections (four rabbits). The pattern of response to the biweekly immunization schedule was very similar to that noted previously with two other rabbits on the same schedule (2, 3). A marked change in idiotypic specificities occurred after the first 11 wk and was followed by gradual changes during the next several months (Figs. 7 and 8).

The responses of two of the three rabbits challenged every 4 wk had different characteristics (Figs. 9 and 10). Again, the earliest antibodies formed differed in idiotype from those isolated later. However, in both rabbits the rate of change of idiotypic specificities after week 9 was very slow and essentially all of the idiotypic specificities identified at week 21 were still present, at only slightly lower concentration, a year later. The pattern of response for the third rabbit on the 4 wk schedule (Fig. 11), however, was quite similar to that of the rabbits that

were challenged every 2 wk. On the basis of these and earlier (2, 3) data, it would appear that idiotypic specificities tend to change more rapidly when antigen is administered frequently. The only rabbits that have shown extremely gradual changes so far are the two rabbits in the present study that were challenged with antigen once a month.

This conclusion is strengthened by the observations made in four rabbits that were allowed long rest periods after hyperimmunization (Fig. 12). In rabbits 14R, 14S, and 16B, nearly all of the idiotypic specificities identified at 42 or 48 wk after the start of immunization were still present after a rest period of about 5 months. However, the quantitative inhibition curves indicate that the concentration of molecules recognized by the anti-idiotypic antiserum was somewhat diminished in each instance. This indicates that some initiation of new clones occurred either during the rest period or as a consequence of the booster injection. It should be noted that precipitating antibody was not detectable at the end of the rest period, before inoculation.

The results for rabbit 16A (Fig. 12 D) appear superficially to be somewhat different, since the antibodies present at week 42 are deficient in idiotypic specificities recognized by the antiserum prepared against anti-benzoate antibodies isolated from the week 61 bleeding. However, in this instance the anti-idiotypic antiserum was prepared against anti-benzoate antibodies isolated after, rather than before, the rest period. If new molecular species appeared after resting, the "prerest" antibodies from rabbit 16A would not have been able to displace them from their homologous anti-idiotypic antibodies, even if all the idiotypic specificities present before the rest period were still present afterwards, but at reduced concentration. If this is the case, the pattern of response of rabbit 16A may not have differed from that of the other three rabbits.

Rabbits 14R and 16B each were allowed a second rest period of 16 wk. Again, only small quantitative changes in idiotypic specificities were observed (Figs. 12 A and C). In the case of rabbit 16B, some of the idiotypic specificities that were lost between weeks 42 and 61, evidently reemerged at week 77.

These quantitative studies confirm the report of Oudin and Michel (4), concerning persistence of idiotype in one rabbit after a long rest period and demonstrate that new populations also arise. Our results also indicate that frequent inoculations are associated with a greater tendency to stimulate new clones of cells. It is noteworthy, however, that even large quantities of antigen given at frequent intervals caused only gradual shifts in the idiotypic determinants present in antibody of a given specificity.

SUMMARY

The effect of challenge by antigen on persistence of clones of antibody-producing cells and on the induction of new clones was investigated through quantitative measurements of idiotypic specificities.

In each of nine rabbits idiotypic specificities present in the earliest bleedings were completely replaced after a few months; subsequent changes occurred much more slowly. On a quantitative basis the population of molecules used as immunogen always reacted most effectively with the homologous anti-idiotypic antiserum.

Little effect of increased antigen dose on the rate of change of idiotype was observed. Even large amounts of antigen administered every 2 wk caused only gradual changes in idiotypic specificities. This was attributed either to more effective capture of antigen by memory cells, as compared to precursor cells, or to the induction of tolerance in those clones that were not expressed. In two of three rabbits on a monthly injection schedule, the idiotypic specificities identified underwent very slow changes over a period as long as 17 months. Changes occurred more rapidly when antigen was administered every 2 wk.

In each of four rabbits investigated, all idiotypic specificities identified before a 5 month rest period were still present afterwards, indicating the survival of essentially all clones of antibody-producing cells during that interval. Quantitative inhibition data indicated that some new clones of cells were initiated.

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