REGULATION OF NATURAL ANTIALLOTYPE ANTIBODY RESPONSES BY IDIOTYPE NETWORK-INDUCED AUTO-ANTIIDIOTYPIC ANTIBODIES*

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Immunoglobulin (Ig) idiotypes have been the focus of intense study since they were first recognized by Kunkel et al. (1) and by Oudin and Michel (2) in 1963. Idiotypic epitopes may be involved in the complex array of self-limiting regulatory mechanisms that the immune system imposes upon itself. Regulation at the level of idiotype recognition has been postulated by Niels Jerne (3) to be a fundamental specificity-associated immunoregulatory system capable of controlling immune responses at both the afferent and efferent limbs. Idiotype network interactions have been shown to involve both the B cell and T cell compartments (4). Cosenza et al. (5) showed that helper T cells can express idiotypes and Hetzelberger and Eichmann (6), Eichmann et al. (7), and Bona (8) have shown that helper T cells can express antiidiotypic specificity. Suppressor T cells can express idiotypes as shown by Lewis and Goodman (9) and are also capable of expressing antiidiotype specificity as shown by Bona and Paul (10). T cells mediating delayed-type hypersensitivity have also been shown to express idiotopes (11).

The allotypic markers on rabbit Ig have been used productively to obtain basic information on Ig structure, function, and regulation. Early work on the sharing of V-gene products by H-chains of several isotypes (12), on allelic exclusion (13), and on allotype suppression (14) has provided new insights and inspired continuing investigations. An additional area of study for which the allotypic markers of the rabbit appeared eminently suited was the question of whether exposure of the rabbit to a noninherited maternal Ig allotype *in utero* would induce, in the absence of experimental intervention, a state of specific tolerance. Gell and Kelus (15) found neither overt tolerance nor immunity in such offspring, while Adler and Noelle (16) and Hagen et al. (17), using more sensitive tests for antibody detection, reported long-lasting spontaneous antibody responses in a majority of the rabbits studied. In a more recent report, Adler and Adler (18) presented evidence that suggested co-existence of a state of active immunity and partial tolerance in such rabbits.

Data to be presented in this report provide evidence that auto-antiidiotype production contributed to the control of an antiallotype response that was initiated by natural *in utero* exposure to a noninherited maternal antigen under normal physiological conditions.

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Materials and Methods

Rabbits. The rabbits used in this study were obtained from a closed colony maintained at St. Jude Children's Research Hospital.

Radioimmunoassays. Assays to detect antiallotype antibodies and anti-(antiallotype) antibodies were done as described earlier (19). Briefly, antiserum and ^{125}I F(ab')$_2$ fragments were mixed and complexes were precipitated with either monospecific goat anti-Fc or with 18% PEGE 6000 dissolved in borate-saline buffer, pH 8.0. The precipitates were washed and the amount of precipitated radioactivity was determined.

Antibody Purification. Anti-a1 antibodies were purified from a column of a1b4 IgG-Sepharose prepared by the method of Cambiaso et al. (20). Anti-a1 antibodies were eluted with 0.2 M glycine-HCl buffer, pH 2.4.

Pepsin Digestion. Anti-a1 antibodies and other IgG preparations were digested in 2% pepsin at pH 4.1 for 8 h at 37 degrees C. F(ab')$_2$ fragments were separated from the pepsin and the Fc peptides by gel filtration.

Radiolabeling. F(ab')$_2$ fragments of antibodies or IgG were radiolabeled using the ICl method of McFarlane (21) at a ratio of 1.0–1.3 atoms I/molecule F(ab')$_2$.

Results

Onset and Persistence of Antiallotype Antibodies. Shown in Table I is a list of rabbits, all littermates, that were used in this study. They were the offspring of a female with the a1a2b4b5 allotype and a male of the a3b4 allotype. Thus the six young that had inherited the maternal a2 allotype might be expected to react against the noninherited maternal a1 allotype and the five young homozygous with respect to b4 allowed observations on the effects of exposure to maternal b5 Ig. Exposure to the maternal noninherited Ig was both *in utero* and through suckling.

Small serum samples were collected at intervals from each of the seven rabbits for over a year. All sera were initially examined for antibodies against one or more of the noninherited maternal allotypes by hemagglutination and by RIA. While hemagglutination tests revealed antibody specific for b5 in all 5 potential b5 producers, with peak titers of 8 to 256, the more sentitive RIA was required to demonstrate anti-a1 antibody formation in 3 of the 6 candidate animals. The natural antiallotype antibodies were detected as early as day 78 after birth in one rabbit and not until day 176 in another littermate. The responses in offspring 360 and 365 were found to persist for over 300 d.

TABLE I

Onset and Persistence of Natural Antiallotype Antibodies

Rabbit	Allotype	Specificity of antibody produced	Earliest day antibody detected	Length of time antibody was detectable
359	a2a3/b4	anti-b5	162	NT
360	a2a3/b4b5	anti-a1	78*	300+
361	a2a3/b4	anti-b5	162	NT
362	a2a3/b4	anti-b5	107	43
363	a1a3/b4	anti-b5	176	NT
364	a2a3/b4b5	anti-a1	107	71
365	a2a3/b4	anti-a1	78*	300+
		anti-b5	78*	300+

NT, Not tested.

* First available sample.

Qualitative Expression of Anti-b5. The sera from offspring expressing anti-b5 were assayed to measure the anti-b5 specificities expressed as a function of time. Each serum sample was assayed by mixing 20 μl of antiserum with 10 ng ^{125}I a3b5 F(ab')$_2$, precipitating the resulting complexes, and counting the radioactivity in the precipitates. Fig. 1 shows the result for the anti-b5 response of rabbit 365, which was typical of the anti-b5 responses of all five offspring that mounted anti-b5 responses. From day 160 to 380 antiserum from offspring 365 bound 70–75% of the labeled b5 molecules and the slight variation observed was ascribed to experimental error. The percentage of molecules bound was identical to the percentage bound by a standard anti-b5 antiserum for all bleeding dates tested (data not shown). Thus, at all times during the experimental period, all b5 molecules were bound by the naturally induced anti-b5 antibodies.

Qualitative Expression of Anti-a1. The sera from offspring expressing anti-a1 were assayed to measure anti-a1 specificities expressed as a function of time. Each serum sample was assayed by mixing 20 μl of antiserum with 10 ng ^{125}I a1b4 F(ab')$_2$, precipitating the resulting complexes, and counting the radioactivity in the precipitates. The results were dramatically different from those found for the anti-b5 responses. Fig. 2 shows the result of the RIA to measure anti-a1 from offspring 360, 364, and 365 as a function of time. Two of the rabbits showed a "cycling" effect. Rabbit 360 showed a first cycle of anti-a1 peaking on day 133 and a second cycle response that peaked on day 253. Rabbit 365 showed three cycles of anti-a1 resonses peaking on days 107, 239, and 364. The response of rabbit 364 dropped steadily after initial detection on day 107 and it was not studied further.

Quantitative Measurement of Natural Anti-1 Responses. Serum samples from the peaks of each anti-a1 response cycle from rabbit 360 and 365 were titrated against a constant quantity of labled a1b4 F(ab')$_2$ in an effort to determine whether the "cycles" of anti-a1 responses seen in Fig. 2 were due to variations in the concentration of anti-a1 in the sera or resulted from changes in the specificities of the anti-a1 populations that were expressed at different times. The titration results for rabbits 360 and 365 are shown in Fig. 3. Fig. 3*A* shows that the 133-d first-cycle serum of rabbit 360 bound only 60% of a1 molecules in the titration assay and that was the same as the result obtained in the qualitative assay. More importantly, the 253-d second-cycle serum, which bound only 40% of a1 molecules in the qualitative assay, bound a maximum

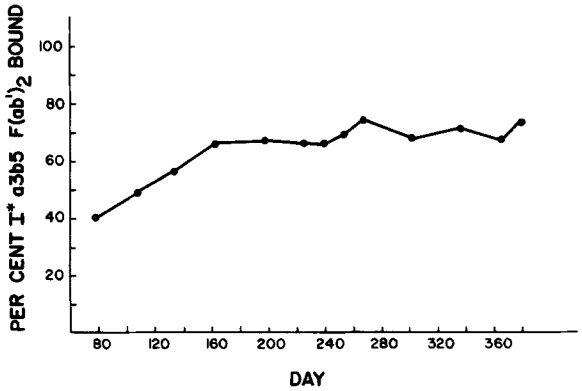

FIG. 1. RIA in which serum samples collected over a 380-d period were assayed for binding to ^{125}I a3b5 F(ab')$_2$ fragments.

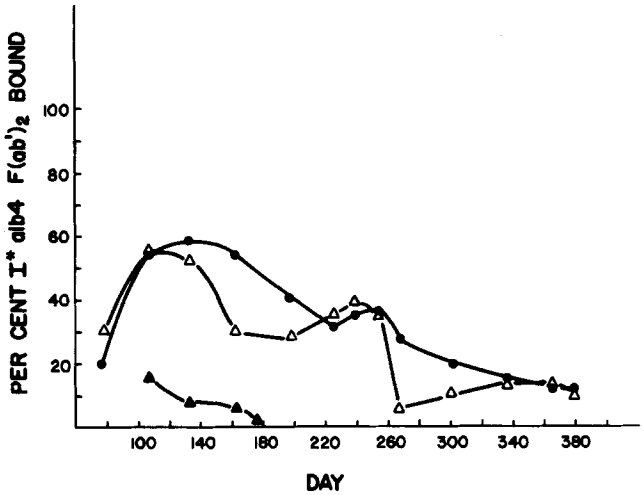

FIG. 2. RIA in which serum samples from offspring 360 (●), 364 (▲), and 365 (△) collected over a 380-d period were assayed for binding to ^{125}I a1b4 F(ab')$_2$ fragments.

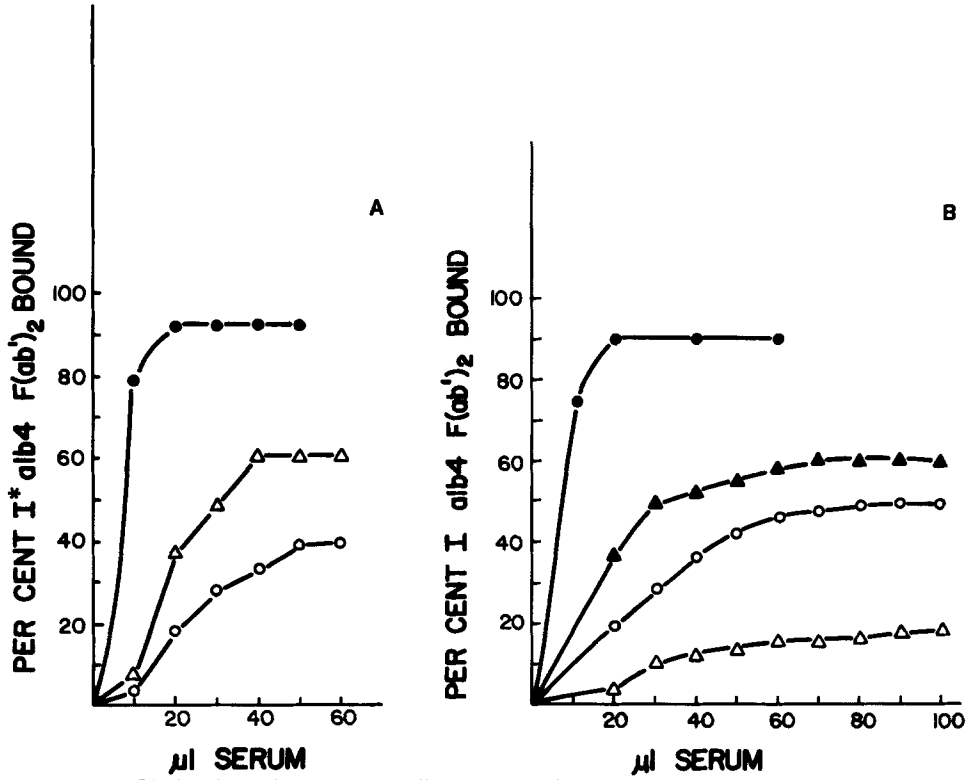

FIG. 3. RIA titrations of sera during different cycles of natural anti-a1 production from rabbits 360 (*A*) and 365 (*B*) using ^{125}I a1b4 F(ab')$_2$. (*A*) ●, anti-a1; △, 360 133 da; ○, 360 253 da. (*B*) ●, anti-a1, ▲, 365 107 da; ○, 365 239 da; △, 365 364 da.

of only 40% of a1 molecules in the titration assay. A standard anti-a1 antiserum bound >90% of the labeled a1 molecules used in the assays.

The results of the titration experiment for the three cycles of anti-a1 response from

rabbit 365 are shown in Fig. 3 *B*. The first cycle (day 107) serum was specific for only 60% of a1 molecules as compared with 90% of a1 molecules bound by the standard anti-a1. The second cycle (day 239) and third cycle (day 364) sera bound only 50% and 20% of a1 molecules, respectively. Thus, in offspring 365, each succeeding cycle of antibody response was specific for a smaller subset of a1 molecules than was the previous cycle. Further, the cycles of response were not a function of variations in the quantity of antibodies specific for a1 epitopes, as the titration curves for all cycles of both rabbits had similar volume maxima. These data showed that in both rabbits there was a clear-cut change in the specificities of the anti-a1 antibodies occuring as a function of time after the initial natural triggering of anti-a1 synthesis.

It is conceivable that the results shown in Fig. 3 could be explained by the presence of differing concentrations of antiallotype antibodies in the serum samples harvested at different times after birth. Additionally, since the sum of a1b4 molecules bound by cycle 1 plus cycle 2 was only slightly higher than the quantity bound by the standard anti-a1, it was possible that each cycle could be specific for a different subset of a1 epitopes. To clarify this point, an experiment was done in which a mixture of equal parts of cycle 1 and cycle 2 serum was made and was titrated with constant quantities of ^{125}I a1b4 F(ab')$_2$. The results showed that a maximum of 60% of a1b4 F(ab')$_2$ was bound by cycle 1 and also by the mixture of cycle 1 plus cycle 2 serum.

Detection of Natural Auto-Antiidiotypic Antibodies. We reasoned that a change in specificity of an antibody response might occur as a function of idiotype network triggering of auto-antiidiotypic antibodies. To test this hypothesis, the anti-a1 antibodies from the first cycle responses of rabbit 360 and 365 were purified on an immunoadsorbent column. Anti-a1 antibodies produced by rabbit 360 between days 120 and 162 and anti-a1 antibodies produced by rabbit 365 between days 107 and 133 were purified, digested with pepsin, and were radiolabeled. These labeled anti-a1 F(ab')$_2$ fragments were used in an RIA to detect auto-antiidiotype. Each labeled anti-a1 sample was assayed with 20-μl volumes of all subsequent sera from the same individual. Fig. 4 *A* shows that auto-antiidiotypic antibodies were detectable following each of the two cycles of anti-a1 responses in rabbits 360 and Fig. 4 *B* shows that auto-antiidiotypic antibodies were detected in sera from rabbit 365 following each of the three cycles of anti-a1 responses. In all cases, peaks of auto-antiidiotype responses occurred just before valleys of anti-a1 responses.

It was of interest to determine whether the idiotopes on the anti-a1 antibodies detected with the natural auto-antiidiotypic antibodies were cross-reactive between rabbits 360 and 365. An RIA was done in which the labeled 360 anti-a1 F(ab')$_2$ was mixed with auto-antiidiotypic antibody-containing serum from rabbit 360 and with serum from rabbit 365. The reverse assay was done in which labeled 365 anti-a1 F(ab')$_2$ was assayed using both 360 and 365 auto-antiidiotypic antisera. In both cases, each antiserum bound 25–30% of the labeled anti-a1 F(ab')$_2$ from each rabbit. These reactions were not inhibitable by adding unlabeled a1 F(ab')$_2$ fragments to the reactions, which suggests that the idiotopes were not binding-site associated. The results of this assay suggested that the idiotopes recognized by natural auto-antiidiotypic antiserum may be the public idiotopes associated with antiallotype antibodies.

Stimulation of Anti-a1 Synthesis After Natural Suppression. Rabbits 360 and 365 were given injections of 4 mg of a1b4 IgG in complete Freund's adjuvant on days 528 and 548 in an attempt to induce the re-expression of the anti-a1 specificities that had

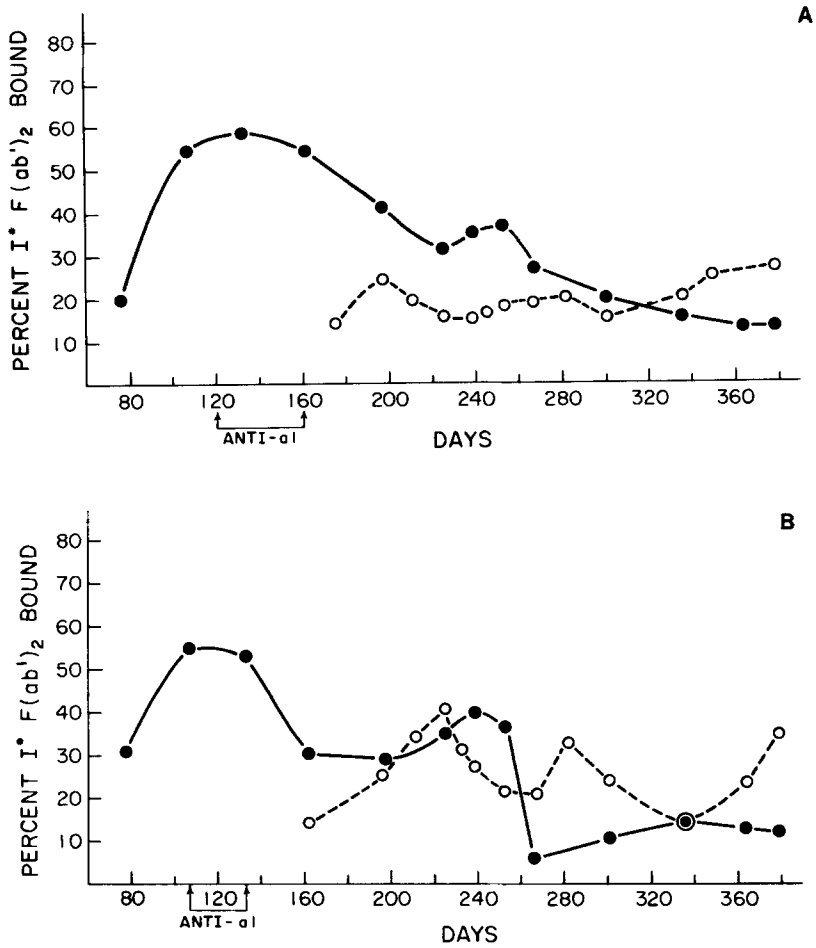

FIG. 4. Composite of natural anti-a1 response RIA (●) and of the auto-antiidiotype RIA (○) for rabbits 360 (A) and 365 (B). Sera pooled and used to purify anti-a1 are indicated by arrows.

apparently been suppressed by auto-antiidiotype. Serum samples were assayed for the presence of anti-a1 in an RIA titration assay using labeled a1b4 $F(ab')_2$. The results, shown in Fig. 5, reveal that these injections elicited antibodies of all the specificities present in the standard anti-a1 antiserum. In a further experiment, a pool of the day 556–570 anti-a1 from rabbit 360 inhibited completely a reaction between the labeled 360 anti-a1 $F(ab')_2$ and the day 384 auto-antiidiotype antiserum (data not shown). This showed that both the anti-a1 specificities and the idiotopes associated with first-cycle anti-a1 were re-expressed after deliberate immunization.

Discussion

Numerous examples of auto-antiidiotypic antibody formation in the course of immunization have been documented (4). Since extremely intense immunization protocols leading to hyperimmune levels of antibody production were used in most of these studies it was of interest to select a model in which auto-antiidiotype formation and function could be studied under strictly normal physiological conditions. We

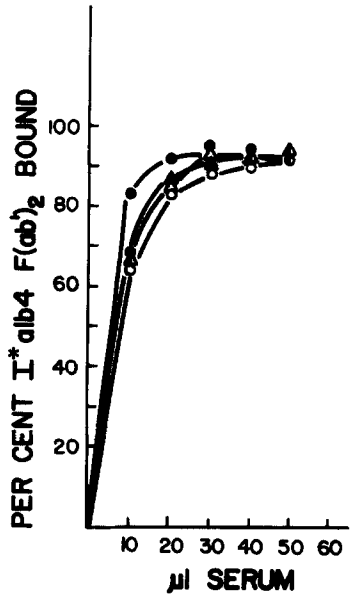

FIG. 5. RIA titration of sera from rabbit 360 taken after deliberate immunization to elicit anti-a1. ●, anti-a1; △, 360 556 da; ○, 360 563 da; ▲, 570 da.

turned to the immune response against noninherited maternal Ig allotypes, which is a normal and frequent event in rabbits (16–18), swine (22), mice (23), and humans (24). The rabbit, with its well-characterized allotype markers encoded in the *a* locus (V_H) and *b* locus (Cκ) appeared particularly suitable, especially so in view of evidence that "spontaneous" antibody formation is frequent (16, 17) and long lasting, and that a state of partial or complete specific unresponsiveness, the former concurrent with low level antibody formation (18) can also be observed in this species.

Onset of spontaneous antiallotype formation in the young rabbit can be detected in some animals within 2 wk of the disappearance of detectable maternal Ig, but may occur much later as has been shown previously (16) and may be noted in the littermates used in this study (Table I). Since both *in utero* and *per os* (nursing) exposure to noninherited allotype can stimulate spontaneous antiallotype synthesis (18), the wide variation in time of onset of detectable antiallotype may be attributable to longer persistence of maternal Ig in some offspring that are weaned later than others. It is also possible that the transient appearance of latent allotypes could trigger antiallotype synthesis in those offspring where responses were detected only after an extended time. To demonstrate the anti-a1 antibody in the six rabbits expected to produce such antibody, a highly sensitive RIA procedure had to be used which revealed the antibody in three of the six animals. The antibody specific for b5 was readily demonstrable in the five animals genotypically capable of producing such antibody by hemagglutination, a procedure estimated by us to be approximately 10-fold less sensitive than RIA in this allotype system. It is well known from deliberate immunization studies that epitopes specified by the *b* locus are stronger immunogens than *a* locus epitopes, and this appears to be also the case in the natural immunization system studied here.

Another important and relevant difference between determinants specified by *a*

and *b* loci is the marked heterogeneity of the former, which is recognized through the existence of distinct subsets of molecules (25–27). If, for example, antibody for one subset of a1 molecules is deleted from an anti-a1 serum, then the remaining antibody, even in excess of antigen, will bind only a portion of the a1-bearing Ig molecules. This attribute of the immune response to *a* locus determinants provides a rare opportunity for an immunochemical approach to auto-anti-idiotypic regulation.

The natural responses observed specific for the b5 allotype gave no suggestion of specificity variation as compared to the anti-a1 responses. However, the *b* locus appears to code for kappa-type light chain constant regions (28, 29) and so each b5 molecule should have all allotype epitopes that are on all b5 molecules. Here antibodies specific for one of the b5 allotype epitopes could be deleted and the remaining antibodies would still react with 100% of b5 molecules. It is possible that antiidiotype responses specific for some anti-b5 molecules may have been triggered here. Since our assay method did not detect changes in the specificities of the anti-b5 population, we chose to focus these studies on the anti-a1 responses and did not attempt to detect auto-antiidiotypic responses for the anti-b5 molecules.

The evidence that we were measuring anti-a1 antibodies in our assays and not antibodies against other allotypic determinants is compelling. The use of $F(ab')_2$ fragments eliminates any interference by anti-*de* locus antibodies. The labeled a1b4 $F(ab')_2$ preparation was prepared from a pool of serum from 9 a1b4 rabbits and had been shown earlier to have 3% of c7 or c21 molecules in it. Further, the antibodies purified from the first wave of anti-a1 response from rabbits 360 and 365 had no reactivity for labeled a2b4 or a3b4 $F(ab')_2$ fragments. It might be argued that, based on the percentages of molecules bound in the first and second cycles of natural anti-a1 responses from each offspring (Figs. 3*A* and *B*), the second cycle response could have been specific for a completely different subpopulation of a1 molecules than the antibodies from the first cycle response or that differences in quantity of anti-a1 in the samples collected at different times could explain the results. An experiment was done in which serum from first cycle and second cycle anti-a1 responses were mixed and the mixture was titrated against constant quantities of labeled a1b4 $F(ab')_2$ fragments. Only the percentage of molecules bound by first cycle serum alone was bound by the mixture. Thus, the results could not be explained on the basis of quantitative differences in first and second cycle sera and that second cycle anti-a1 antibodies recognized some, but not all of the a1 epitopes recognized by first cycle anti-a1 antibodies. Other results showed that the mother possessed all a1 specificities and that the restricted responses mounted by offspring were not a function of initial stimulation by only a subset of a1 molecules.

The data in this paper suggest that natural auto-antiidiotype responses may be preferentially directed to the public idiotopes on a1 molecules. Public as well as private idiotopes have been detected in rabbit antibodies specific for *a*, *b*, and *e* locus markers (30–33). The data showed that the natural auto-antiidiotypic antibodies directed against first wave anti-a1 antibodies from rabbit 360 reacted equally well with first wave anti-a1 antibodies from rabbits 360 and 365 and the same was true for the reaction of the anti-a1 antibodies of rabbit 365. These present data concerning cross-reactivity of the idiotopes recognized with natural auto-antiidiotypic antibodies in the anti-a1 responses confirm and extend the similar observation on the cross-reactive idiotypes detected in antimicrococcal responses made earlier in one of our

laboratories (35). Thus, data are now available for two unrelated antigen systems in which natural auto-antiidiotypic antisera are strongly cross-reactive with idiotopes on antibodies of the same specificity from outbred individuals. In both cases, however, the rabbits expressing the cross-reactive idiotypes were littermates or close relatives. It is not known whether this cross-reactivity extends beyond close family relationships. This is now being studied.

Studies of network interactions using allotypic epitopes as antigens must carefully exclude the possibility that an unexpected burst of synthesis of latent allotype is not misinterpreted as antiidiotype. For example, an a2a3 rabbit making anti-a1 could conceivably produce "latent" a1 molecules which would bind anti-a1 in a manner superficially similar to the binding of anti-a1 by antiidiotypic antibodies. Fortunately the rabbit allotypes can be used to distinguish between these possibilities. If the suspected auto-antiidiotypic antibodies are latent allotypes they would not bear the allotypic marker(s) present in the preinjection serum. For example, an a2a3 rabbit would synthesize a1 molecules as latent allotypes but should have a2 or a3 epitopes on true auto-antiidiotype antibodies. We used a radioimmunodiffusion assay with late (day 300) serum by reacting it with either anti-a2 or anti-a3, washing, and then reacting it with radiolabeled 360 or 365 anti-a1 $F(ab')_2$ isolated from the first wave of anti-a1 response. There was a strong reaction with anti-a2 and a weak reaction with anti-a3. This showed that the observed reactions were due to true auto-antiidiotypic antibodies and were not due to the emergence of latent allotype bearing a1 epitopes.

The inverse cycles of idiotype and auto-antiidiotype found in this natural immune response (Figs. 4*A* and *B*) are quite similar to responses found by Kelsoe and Cerny in studying artificially induced responses (34). The T15 responses they studied cycled three times in a 14-d period. Our studies measured anti-a1 responses over a period of one year and the results were remarkably similar in both studies, although the time frame was quite different.

Our early attempts to detect natural auto-antiidiotypic antibodies in these anti-a1 responses were unsuccessful. We were only successful when we used a method for labeling the anti-a1 $F(ab')_2$ fragments that did not employ reducing agents in the protocol. In a previous paper the extreme sensitivity of antimicrococcal idiotopes to reducing agents was documented when natural auto-antiidiotypic antiserum was used (35). The reduction properties of the idiotopes is described fully in another paper (36). We found that the idiotopes in the anti-a1 antibodies that elicit the natural auto-antiidiotypes responses documented in this paper for the a1 system are also susceptible to destruction by low levels of reducing agent. Further unpublished experiments have shown no degree of cross-reactivity between the antimicrococcal and anti-a1 idiotypes that are sensitive to reducing agents. Thus, the idiotope recognized by the natural auto-antiidiotypic antibodies is a true idiotope and not an unrecognized isotypic or allotypic structure.

Data presented in this paper confirm and extend the concept of reversibility of antiidiotype-mediated suppression of immune responses in the adult. Strayer et al. (37, 38) have shown in the T15 system that antiidiotype suppression is long-lasting in the neonate but that it only exerts transient suppression in adults. Other studies from this lab (19) have verified the reversibility of natural auto-antiidiotype regulation in rabbits and the data from the experiment described in Fig. 5 suggest that the natural auto-antiidiotypic regulation that followed the natural antiallotype response was

reversible. It is, however, possible that the use of complete Freund's adjuvant may have prejudiced the results in favor of breaking suppression of anti-a1 synthesis.

It is tempting to speculate that the cross-reactive idiotopes found in this paper and in (19) and (35) that seem to be involved in idiotype regulation in normal outbred animals may be similar to, or identical with, the special class of regulatory idiotopes postulated by Paul and Bona (39). These idiotopes in (35) were found only in sera of rabbits which mounted auto-antiidiotypic responses. Both the antimicrococcal idiotopes (35) and the anti-a1 idiotopes described here were highly sensitive to low concentrations of reducing agent which suggests that these idiotopes may be a special subset in rabbits that is highly reduction-sensitive.

We conclude that the data support the hypothesis that auto-antiidiotype antibodies can be induced in natural immunological reactions. This conclusion supports the concept introduced first by Niels Jerne (3) of a receptor-driven idiotype network constituting a central immunoregulatory system.

Summary

This study was designed to determine whether natural immune responses could elicit immunoregulatory auto-antiidiotypic antibodies. Female rabbits heterozygous at the *a* and *b* Ig loci were bred to homozygous males. Offspring of one such breeding were studied for natural production of antibodies specific for the noninherited allotypes and for the production of immunoregulatory auto-antiidiotypic antibodies. All offspring mounted natural antiallotype responses. The anti-a1 responses cycled as a function of time whereas the anti-b5 responses were invariant. Anti-a1 responses from two offspring were shown to change specificity for different a1 subsets as they cycled. Anti-a1 was purified from the first cycle and was used to assay for auto-antiidiotypic responses. Auto-antiidiotypic antibodies were detected and were found to cycle in an inverse way with the anti-a1 cycles. The idiotopes detected using the natural auto-antiidiotypic antisera were strongly cross-reactive. Subsequent deliberate immunization showed that antibodies specific for all a1 subsets could be elicited after auto-antiidiotypic regulation had functioned. The data support the interpretation that idiotype network interactions indeed function in naturally occurring immunologic situations and are not merely laboratory curiosities or artifacts.

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