IDIOTYPIC REGULATION OF THE IMMUNE SYSTEM

Common Idiotypic Specificities between Idiotypes and Antibodies Raised Against Anti-Idiotypic Antibodies in Rabbits*

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An animal uses only a small part of the immunological repertoire of the species in response to a given antigen (1-3). The most easy way to explain idiotypy is to assume that different somatic mutations have occurred in the germ-line V genes of different individuals. However recent data from this laboratory (4, 5) and from the Cazenave group (6) indicate that an individual normally expresses only a small fraction of its own immune repertoire and suggest that it is possible to make a given individual to synthesize an idiotype very similar to that of another individual.

Normally rabbits, say, n° 1 and n° X, synthesize different idiotypes when injected with the same antigen. If rabbit X possesses in its immune repertoire silent lymphocytes precommitted for the synthesis of idiotype 1, it is reasonable to assume that their nonexpression is a result of specific suppressors which bear autoanti-idiotypic receptors (Ab2).[1] If we suppress the suppressor of clone 1 in rabbit X, by inducing an immune response against the suppressor (Ab2), we would expect to favour the synthesis of idiotype 1 in rabbit X. Using this rationale, anti-idiotypic antibodies (Ab2) were raised in allotype-matched rabbits, against a first antibody (Ab1). These Ab2 were then injected into a third series of rabbits to obtain anti-anti-idiotypic antibodies (Ab3). These rabbits were then immunized with the original antigen. These experiments show clearly that the subsequent antibodies (Ab1') display idiotypic specificities similar to those displayed by Ab1.

These results raise interesting questions, one of which concerns the idiotypic specificities of anti-anti-idiotypic antibodies (Ab3). Do Ab1 and Ab3 display similar idiotypic specificities? This paper presents a detailed idiotypic analysis of Ab3 in the *Micrococcus lysodeikticus* and in the tobacco mosaic virus (TMV) systems. The most salient features of our results are that Ab4 (specific antibodies to Ab3) recognize specifically Ab1 and Ab1'. Ab4 behaves as Ab2 and diversity does not increase along the chain of immunization.

Materials and Methods

Rabbits. Were obtained from different local suppliers. They have no familial relationship. Sera were typed for allotypes a1, a2, a3, b4, b5, b6, c21, e14, d11, and d12.

Immunizations. Were performed as described previously. Ab3 antibodies were prepared by affinity chromatography on Sepharose (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) coupled to anti-idiotypic antibodies. The eluted Ab3 were cross-linked with glutaraldehyde and were injected into allotype-matched rabbits using immunization schedule described previously (4).

Radioimmunoassay. Two different methods were used. (*a*) Iodination was performed according to Hunter (7). The specific radioactivity of immunoglobulins ranged between 10^6 and 5×10^6 cpm/μg. Binding curves of radiolabeled antibody were performed with 25 ng of antibody and increasing amounts of cross-linked anti-idiotypic sera. Cross-linking of antisera was performed with ethyl chloroformate (8). All dilutions were done in phosphate buffer containing 1% ovalbumin. The tubes were incubated overnight at room temperature and centrifuged at 13,000 rpm for 5 min. After centrifugation, the top of the tube content was transferred to another tube and radioactivity in both tubes was assayed in a gamma scintillation counter (Beckman). Control tubes without solid antiserum were included in each experiment. Inhibition curves were performed as follows: a fixed amount of cross-linked antiserum, corresponding to 70% of the plateau (binding), the cold putative inhibitor in variable amounts, and a fixed amount of labeled antibody were mixed and the tubes were processed as described above (9). (*b*) Radioimmunoassay with polyvinylchloride plates. Suspension of TMV (0.1%) is incubated in the wells of a polyvinylchloride plate (Linbro Scientific Inc., S-MVC-96 Hamden, Conn.) during 30 min and dried. After washing and further incubation with gelatin-phosphate-buffered saline (PBS) 0.1% to reduce nonspecific adsorption, the wells are filled with the investigated sera. Sera are removed, the plates are washed thoroughly, and incubated with radiolabeled guinea pig anti-rabbit immunoglobulins one night at 4°C. The plates are washed, dried, and the individual wells are sliced and counted.

Detection of Anti-Carbohydrate Activity. Carbohydrate was purified according to the Perkins' method (10). Carbohydrate was coupled to tyramin as described by Cramer and Braun (11) and labeled with iodine ^{125}I (7). 20 ng of carbohydrate are incubated with varying amounts of investigated sera during one night at 4°C. After addition of *Staphylococcus aureus* and one night at 4°C, the tubes are centrifuged and the radioactivity of the pellet is measured in a gamma scintillator counter.

Results

Immunization Chain. The experimental scheme employed is illustrated in Fig. 1 for the *Micrococcus* system. Starting from a first anti-carbohydrate antibody (Ab1), anti-idiotypic antibodies were raised in a series of allotypically matched rabbits (rabbits II). The corresponding Ab2 will be denoted Ab2a, Ab2b, Ab2c.... Several Ab2 were isolated and injected into rabbits III to get anti-anti-idiotypic antibodies (Ab3). One Ab3 was purified on a Sepharose column coupled to Ab2, and Ab3 was injected into two rabbits to get Ab4 (anti-anti-anti-idiotypic antibodies). The original antigen was injected into rabbits III and the resultant antibodies were called Ab1'. Ab3 sera in the *Micrococcus* system react not only with their own specific immunogen but also with all the other ab2s directed against the same original Ab1. They do not react with Ab2s raised in rabbits against other anti-*Micrococcus* antibodies nor with Ab2s of the TMV series. Furthermore, Ab3 antibodies are able to inhibit the reaction between Ab1 and Ab2 (4). It should be noted that Ab3 of the TMV series do not inhibit the reaction Ab1-Ab2 of the *Micrococcus* system.

Lack of Antigenic Recognition in Ab3 Sera. Several systems were used to test for a possible recognition of antigen by Ab3. Ab3 antibodies of the *Micrococcus* system were passed repeatedly on a Sepharose column coupled to *Micrococcus* antigens. After
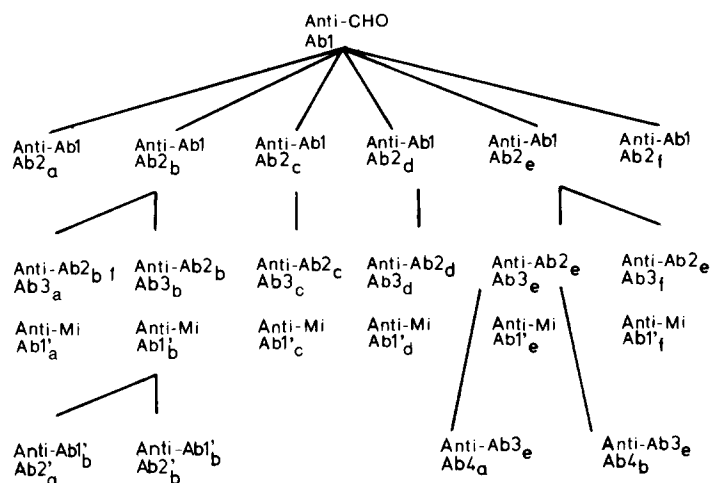
Fig. 1. Immunization scheme of rabbits.

exhaustive adsorptions, the Ab3 sera were assayed for inhibition of the reaction between radiolabeled Ab1 and Ab2. No significant loss of the inhibitory activity was observed. A modified Farr assay was used for the determination of the antigen-binding capacity of Ab3. No significant binding of radiolabeled carbohydrates was obtained even when using a 100-fold excess of Ab3 sera (as compared to Ab1 and Ab1' sera). Ab3 antibodies of the TMV system were also checked for their capacity to inhibit the reaction between TMV and anti-TMV antibodies; no significant inhibition was observed (data not shown).

Finally, Ab3 sera of the TMV series were assayed for their capacity to bind directly to TMV in a sensitive test which is able to detect 0.2 ng of anti-TMV antibodies (Fig. 2). TMV is first adsorbed to plastic trays. Ab3 sera are incubated in the wells. After washings, bound immunoglobulins are revealed using radiolabeled antibodies derived from a guinea pig anti-rabbit immunoglobulin serum. In these tests, no differences were observed between preimmune sera and Ab3 sera.

These results all show that the vast majority of Ab3 antibodies do not recognize the antigen and could be interpreted as follows. (a) There could be a sharing of idiotypic specificities between Ab1 and Ab3. (b) Ab1 and Ab3 could be idiotypically different but the different Ab2 could display the same idiotopes.

Induction of Ab4 Antibodies. Ab3 antibodies from the *Micrococcus* system were isolated on Ab2 columns and injected into two rabbits (rabbits IV). The precipitating antisera (Ab4) obtained were checked with preimmune sera, sera from unrelated rabbits hyperimmunized with *Micrococcus* or TMV and Ab3 sera of the TMV system. No precipitation lines were observed in immunodiffusion. Interestingly, besides recognizing their immunogen Ab3, Ab4 sera recognize Ab1 and Ab1' antibodies (Fig. 3). It appears therefore that Ab4 which was raised against Ab3 displays a behavior identical to Ab2.

Properties of Ab4 Antibodies. Because Ab4 antibodies recognize Ab1 and Ab1' antibodies in immunodiffusion tests, it was important to analyze the specificity of these reactions by quantitative methods. Both Ab1 and Ab1' are able to bind the cross-linked Ab4 serum to an extent of 60%. Fig. 4 demonstrates that the two Ab4 are
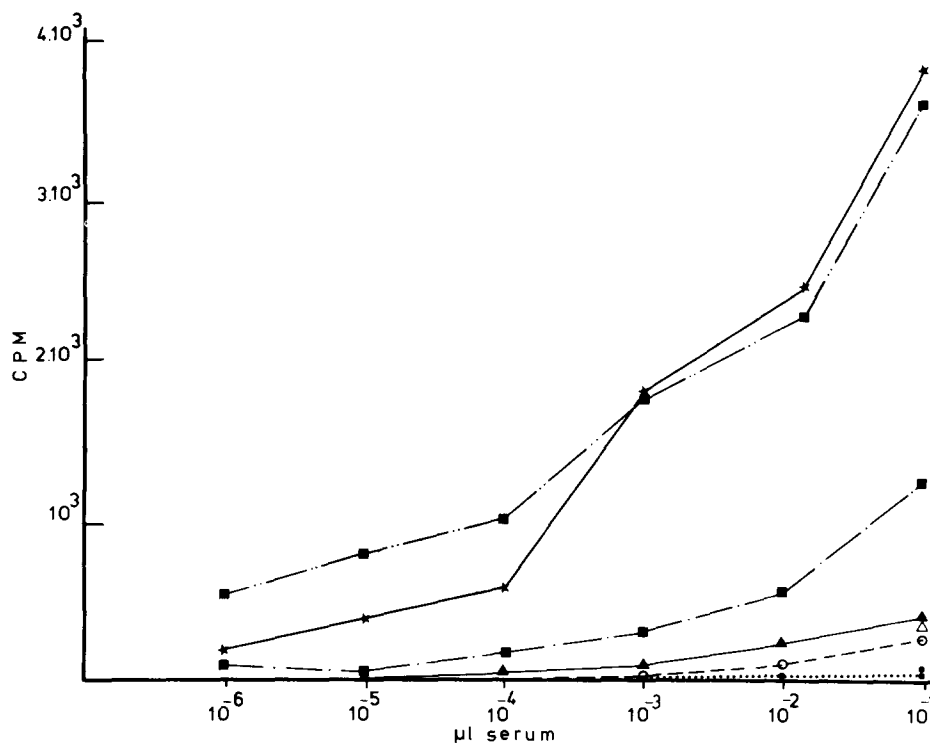
Fig. 2. Study of the anti-TMV specificity by solid phase radioimmunoassay. Detection of anti-TMV antibodies in Ab1 (★), Ab3 (▲), Ab1'1 (primary response, ■–·–■), Ab1'II (secondary response, ■–··–■) sera, preimmune serum (○), and anti-bungarotoxin serum (△). Ordinate, number of counts per minute of radiolabeled guinea anti-rabbit immunoglobulin in each well. Abscissa, dilution of investigated serum. The symbols (●) at right bottom give fixation values obtained with horse or goat anti-TMV antibodies.

able to inhibit completely the binding between radiolabeled Ab1 and the solid anti-idiotypic serum Ab2. A complete inhibition of this reaction is also obtained with Ab1 and Ab1' antibodies, as was shown previously (4). Appropriate controls are included in this figure (preimmune sera of rabbits I, II, III, and IV; heterologous anti-idiotypic sera, anti-bacteria antibodies raised in other rabbits). Tests for inhibition of the reaction between radiolabeled Ab4 and Ab1 were also performed. Ab1 and four Ab1' of the same series were found to inhibit specifically this reaction (Fig. 5). Only one Ab1' of the series is a bad inhibitor of this reaction. All these findings indicate that Ab1, Ab3, and Ab1' are strongly idiotypically cross-reactive.

Idiotypic Comparison of Ab1, Ab1', and Ab3 Antibodies in the TMV System Using Purified Ab2 Antibodies. It should be stressed that Ab1, Ab3, and Ab1' constitute a family of interrellated but nonidentical idiotypes. This is especially evident in the comparison between Ab1 and Ab3 because Ab3 does not recognize the original antigen. It is therefore likely that Ab3 lacks some idiotopes which are only found on Ab1. The following experiments illustrate this point.

One Ab2 serum of the TMV system was adsorbed on an Ab1 column (anti-TMV antibodies), Ab1' column, or on Ab3 column. The eluted Ab2 antibodies were then iodinated and compared for their capacity to bind to insoluble Ab1, Ab1', and Ab3
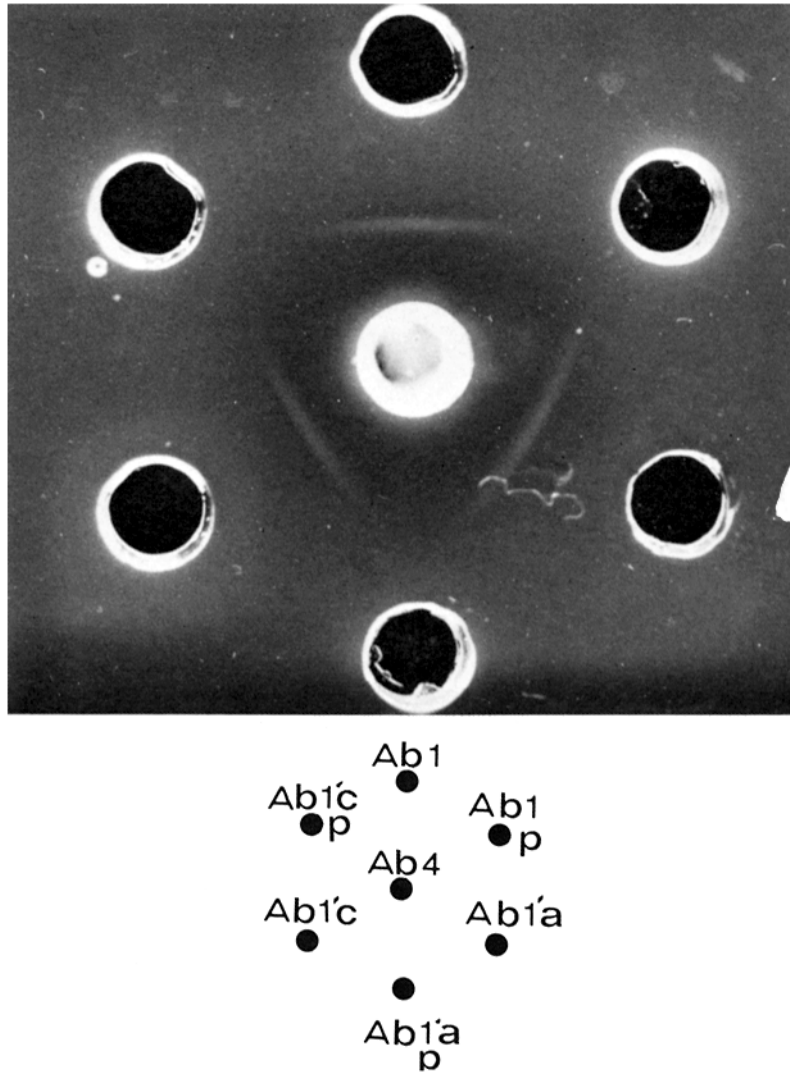
FIG. 3. Ouchterlony assay. The center well was filled with serum Ab4 (10). Wells marked Ab1 (2975), Ab1$_a$' (2281), and Ab1$_c$' (2731) contained anti-CHO purified by affinity chromatography. Wells marked Ab1$_p$, Ab1$_{ap}'$, and Ab1$_{cp}'$ were filled with preimmune sera of the same rabbits.

sera. Fig. 6 shows that only part of the Ab2 antibodies which recognize Ab1 do in fact recognize Ab3, whereas all Ab2 which recognize Ab3 also recognize Ab1 and Ab1' (Fig. 6, 6c). These results confirm those obtained in the *Micrococcus* system: sharing of idiotypic specificities between Ab1, Ab1', and Ab3 antibodies. Moreover, the results show that Ab3 is lacking some idiotopes of Ab1 or Ab1' which may be those intimately involved in the active site.

Discussion

The results here confirm and significantly extend our previous conclusions (4, 5). Our results show that randomly chosen rabbits, which have been immunized with
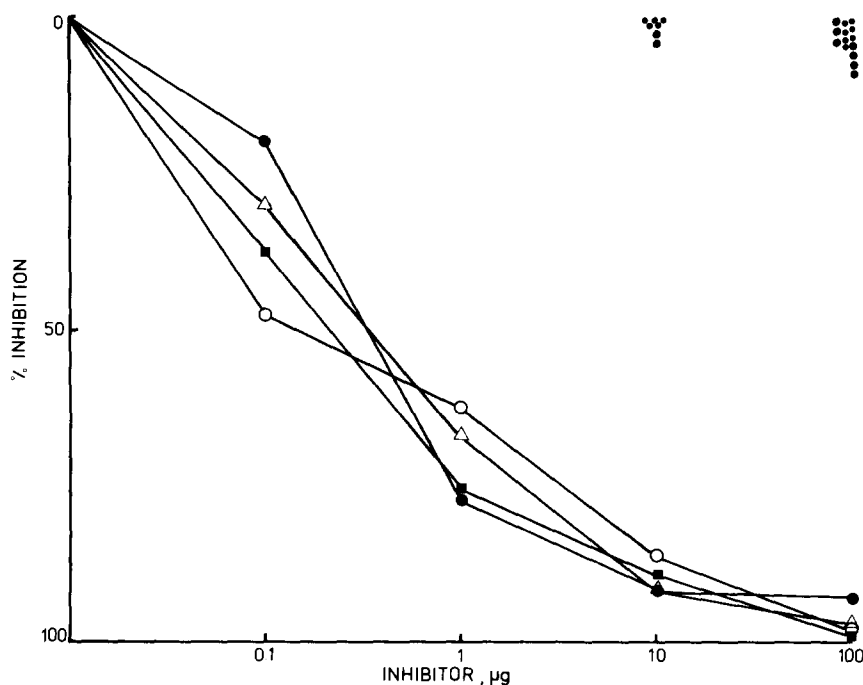
Fig. 4. Inhibition of the binding of purified radiolabeled Ab1 (2975) to cross-linked homologous anti-idiotypic antiserum Ab2 (2238). Inhibitors are unlabeled, specifically purified, anti-CHO antibodies from rabbit I (● Ab1 2975) and rabbit III (O Ab1' 2731). Symbols △ and ■ give inhibition values obtained, respectively, with sera Ab4 (10) and Ab4 (12). Controls are also shown at right top. (●) give inhibition values obtained with preimmune sera from rabbits 2975, 2731, 10, and 12 and heterologous anti-idiotypic antisera. (●) represents inhibition values obtained with specifically purified antibacterial antibodies from nine different rabbits.

anti-idiotypic antibodies when given the original antigen, synthesize antibodies bearing idiotypic specificities similar to those of the original idiotype. Furthermore, these results show that anti-anti-idiotypic antibodies most probably share idiotopes with Ab1 because Ab4 antibodies, raised against Ab3, do recognize specifically Ab1 and Ab1' antibodies. Despite this idiotypic similarity between Ab1 and Ab3, Ab3 does not recognize the original antigen to a significant extent.

The idiotypic similarity between Ab3 and Ab1' which are both synthesized in the same rabbits (rabbits III) bears a striking analogy with the simultaneous expression of nonspecific immunoglobulins and antibodies simultaneously induced by one antigen (12-15). The pioneering work of Oudin and Cazenave (14) showed that there was a sharing of idiotypic specificities between antibodies and immunoglobulins without known antibody function. These findings have subsequently been confirmed at the cellular level (15). This derepression of several sets of cross-reactive idiotypes is easy to understand in the light of network concepts (16-18).

There is a striking similarity between Ab2 and Ab4. Both sets of antibodies (anti-idiotypic antibodies and anti-anti-anti-idiotypic antibodies) recognize Ab1 and Ab1' antibodies. The Ab2, used to raise Ab3 antibodies, when checked with the sera from 60 rabbits immunized with *Micrococcus* only recognized 1 of these 60 sera.

This cross-reactive idiotype was, however, unable to inhibit the reaction. Two Ab4 sera were obtained and checked with the same rabbit sera. Only one antibody was
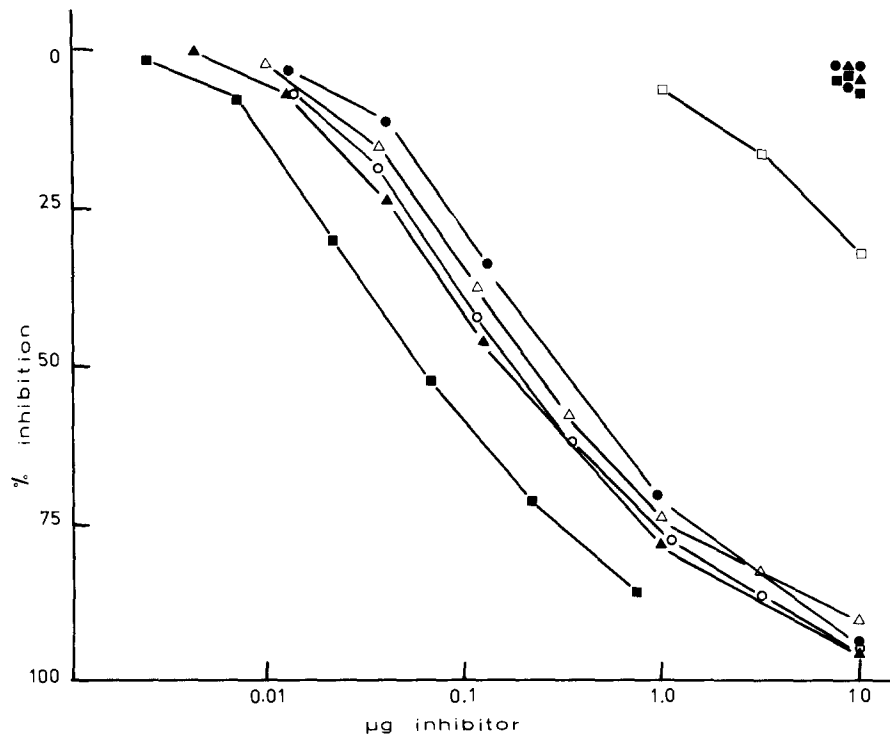
Fig. 5. Inhibition of the binding of purified radiolabeled Ab4 (10) to insolubilized serum Ab1 (2975). Inhibitors are unlabeled, specifically purified, anti-CHO antibodies from rabbit I (▲ Ab1 2975) and from rabbits III (■ Ab1′ 5083, △ Ab1′ 2731, O Ab1′ 2732, □ Ab1′ 2733, and ● Ab1′ 2281). Controls are also shown: ▲ represent average inhibition values obtained with sera from rabbits hyperimmunized with *Micrococcus* or with preimmune sera from rabbits III (● 2731, ▲ 2281) and IV (■ 10).

found to react and this was the one also recognized by Ab2. Thus, Ab4 and Ab2 have exactly the same pattern of reactivity.

The rules which are valid at the Ab1 level (each individual is making its own Ab1) do not appear to hold at the Ab3 level. In a way, Ab3 is an image of Ab1 and Ab4 is an image of Ab2. However, it should not be forgotten that there is also a lack of symmetry, because Ab3 is idiotypically similar to Ab1 but does not recognize antigen. Thus, although the immune repertoire available to an antigen is strongly different from one individual to another, the total repertoire is similar in each rabbit. These facts lead to the conclusions that suppression is dominant in the functioning of the immune system and that each individual is using not only a small part of the total repertoire species but also a small part of its own repertoire. They also show that the part of immune repertoire available to the antigen depends on the functional state of the idiotypic network. One important question is how generally applicable to other systems is the device used in our experiments to force the expression of a randomly chosen idiotype. It must be stressed that this kind of results has now been found for four antigens and two species (4, 6, 22). However some important limitations should be born in mind.

(a) the success of idiotypic manipulations is limited by the genetic polymorphism. The rabbit must possess the relevant genetic information. For example, a linkage of
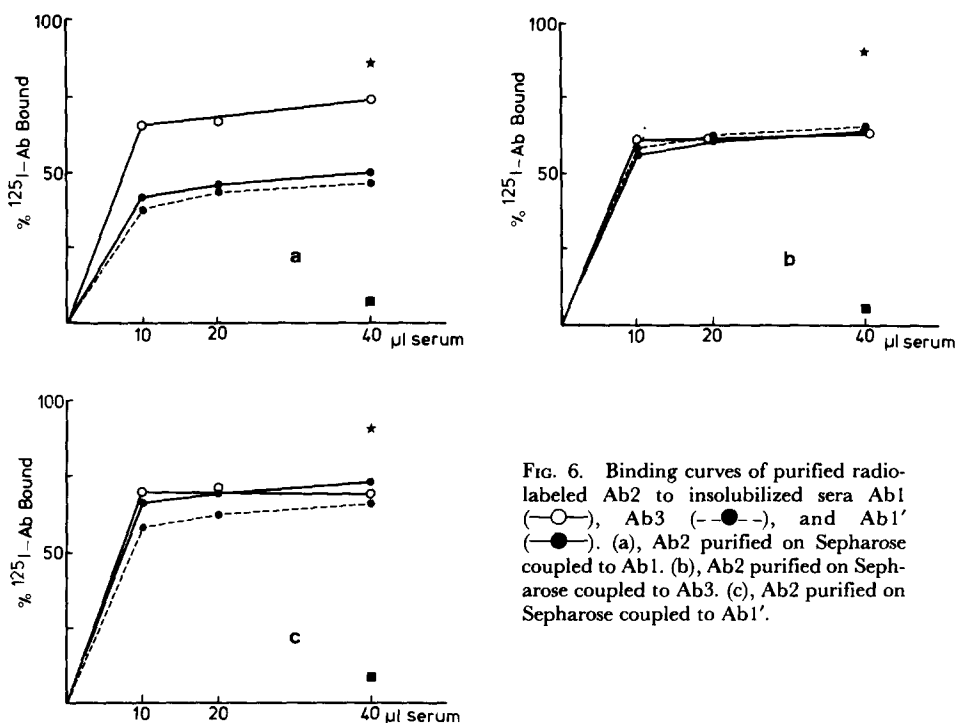
Fig. 6. Binding curves of purified radio-labeled Ab2 to insolubilized sera Ab1 (—O—), Ab3 (- -●- -), and Ab1' (—●—). (a), Ab2 purified on Sepharose coupled to Ab1. (b), Ab2 purified on Sepharose coupled to Ab3. (c), Ab2 purified on Sepharose coupled to Ab1'.
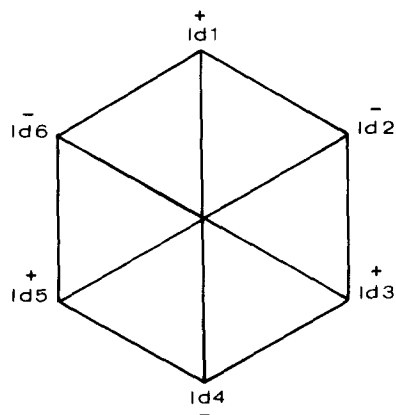

Fig. 7. A circular idiotypic network.

idiotype to the a allotype has been found both in the *Micrococcus* and in the RNase system (4, 6).

(*b*) the experimental design leads to the appearance in rabbits III of a family of cross-reactive idiotypes (Ab1'). In general, these idiotypes are similar to Ab1 but not identical.

In some cases, Ab1 bears idiotypic specificities which are absent from Ab3. Suppose for example that Ab1 bears idiotopes a and b and Ab3 display the idiotopes a and c. It could be that Ab1' is also expressing a and c (like Ab3). If so, the idiotypic similarity between Ab1 and Ab1' will be revealed more easily, when using Ab4 or Ab2' which

is the anti-idiotype against Ab1' or the fraction of Ab2 antibodies bound to a column of Sepharose coupled to Ab1' antibodies (to eliminate Ab2 antibodies directed against the private idiotope b of Ab1).

Another important limitation could be a result of the kind of anti-idiotypic serum used. It has been shown by Coutinho, Forni, and Blomberg (19) that some anti-idiotypic sera directed against some myeloma protein recognize a large subset of B cells and behave as polyclonal B-cell activators (induction of DNA synthesis and immunoglobulin secretion) (20). This is observed for anti-idiotypic sera directed against the myeloma protein J 558 which itself binds to dextran. A simple interpretation of this result is that the anti-idiotypic antibodies are in fact an internal image of dextran. This would be similar to the results obtained by Sege and Peterson in which they showed that anti-idiotypic antibodies directed against anti-insulin antibodies could partially mimic insulin (21). The study of idiotypic regulation requires the choice of antigen and anti-idiotypic antibodies which are not polyclonal B-cell activator (PBA). The Ab2 sera used in our studies have been assayed for PBA properties and do not display any of the properties ascribed to polyclonal B cells activators (no binding of fluorescent Ab2 to a large subset of B lymphocytes, no induction of DNA synthesis and immunoglobulin secretion) (G.Urbain-Vansanten, C. Bruyns, and M. Wikler. Unpublished results.)

The fact that Ab3 and Ab1 are bearing similar idiotypic specificities has implications for understanding the mechanism underlying our idiotypic manipulations. Rabbits III which are synthesizing Ab3 should possess T_H lymphocytes whose immunological receptors display idiotopes similar to those of Ab3. When antigen is injected into rabbits III, these T helper lymphocytes would counteract suppressor T cells displaying immunological receptors with Ab2 activity. This conclusion is strongly reinforced by the recent finding of Bona and Paul who showed that removal of normal suppressors which recognize a given idiotype allows the expression of clones secreting that particular idiotype (22).

Taken together our previous findings, the results shown in this paper, the work of Bona and Paul and others (23–29) strongly suggest that idiotypes are involved in clonal interactions. Our findings that Ab3 is idiotypically similar to Ab1 and that Ab4 displays the same behavior as Ab2 suggest strongly that the diversity of the repertoire does not increase as one moves along the chain of immunization. These facts could have far reaching implications for the structure and the stability of the functional idiotypic network (30).

The immune system is a physiological network, made up of two networks: the compartment network and the idiotypic network.

(a) Compartment network. The immune system is divided into compartments performing different functions, B, T_H, and T_S lymphocytes. As a rule, T cells only see an antigenic determinant when this antigenic determinant is located in the proper membrane environment. This is a result of the fact that T lymphocytes use a dual recognition system based on two receptors, a physiological receptor which recognizes self-makers (31) (H2K, H2D, I ... protein) and an immunological receptor, whose exact nature is still unclear. Signals are effectively delivered by T lymphocytes only if both kinds of receptors are occupied. This dual recognition system might only allow interactions between certain sets of lymphocytes.

(b) The idiotypic network allows the meeting of complementary partners (lymphocytes bearing idiotypes and lymphocytes bearing autoanti-idiotypes). Signals are

probably not given through these interactions, but depend upon the compartment to which these lymphocytes belong. For example, T_H lymphocytes can give positive signals of differentiation to B lymphocytes. They can also induce the activation of the inhibitory set (T_S). It is likely that the physiological receptor to T_H (call it protein a) is able to recognize a surface protein b from the T_H lymphocytes. Therefore, T_H cells will activate T_S cells bearing autoanti-idiotypic receptors.

The finding of idiotypic similarity between Ab1 and Ab3 on one hand, and Ab2, Ab4 on the other hand, lead us to the concept of circular idiotypic networks. A small idiotypic community is illustrated in Fig. 7 id1, id3, and id5 could represent different antibody subpopulations displaying similar idiotopes or even id3 or id5 could represent nonspecific Ig sharing idiotypes with Ab1. The set of cross-reactive idiotypes (id1, id3, and id5) is induced by antigen. When these idiotypes on the surface of B and/or T_H lymphocytes reach a threshold, they induce the activation of T_S lymphocytes bearing the autoanti-idiotypic set id2, id4, and id6, leading to a normal feedback mechanism of the immune response. The finding of a possible correlation between the decrease in binding affinity and the appearance of lymphocytes bearing autoanti-idiotypic receptors during an immune response (33) is in complete agreement with the ideas expressed in this paper. A detailed network model, taking into account the occurrence of T helper cells bearing autoanti-idiotypic receptors has been presented elsewhere (18).

Summary

Anti-idiotypic antibodies (Ab2) were raised in allotype-matched rabbits against anti-carbohydrate or anti-tobacco mosaic virus antibodies (Ab1). Several Ab2 were purified and injected into a third series of rabbits III which synthesized antianti-idiotypic antibodies (Ab3). Antigen was then given for the first time in those rabbits who had synthesized Ab3. The specific antibody synthesized in rabbits III was called Ab1'. Anti-idiotypic antibodies were raised against purified Ab3 antibodies (Ab4). In most cases, Ab1' antibodies are sharing idiotypic specificities with Ab1. Ab3 did not react with antigen but shared idiotopes with Ab1 and Ab1' because Ab4 antibodies, which are anti-idiotypes to Ab3 do recognize specifically Ab1 and Ab1' antibodies belonging to the same chain of immunization. It seems therefore that Ab3 looks idiotypically like Ab1 and Ab4 displays the same behaviour as Ab2. A general view of the functioning of the immune system is presented.

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