IMMUNOREGULATORY ROLE OF MATERNAL IDIOTYPES

Ontogeny of Immune Networks*

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The phenomenon of idiotypy or individual antigenic specificity is one of the most fascinating in immunology (1–3). Clearly, this phenomenon appears to be intimately linked to the two major problems of the immune system: the origin of antibody diversity and the regulation of the immune system (4).

It has frequently been assumed that idiotypy could be explained by the somatic mutation theory. Different idiotypes were considered to be different somatic variants, appearing from very similar germ-line genes. However results from our laboratory (the Laboratory of Animal Physiology, Université Libre de Bruxelles, Rhode-Saint-Genèse, Belgium) and from the Pasteur Institute, Paris (4–11) indicate that, in fact, the total idiotypic repertoire is more or less the same in all rabbits and mice, with exceptions that have been discussed elsewhere (7). Briefly, these conclusions stem from experiments in which it was possible to elicit the synthesis of a specific idiotype in a randomly chosen animal. The rationale behind the experiment was suggested by network concepts (4, 12–14). If we suppose that rabbit X, which does not express idiotypes from rabbit 1, nevertheless contains silent lymphocyte clones precommitted to the synthesis of idiotypes from rabbit 1, it should be possible to relieve these silent clones from suppression by raising immunity against suppressor cells. In principle, specific suppressors should bear autoanti-idiotypic receptors. Therefore, conventional anti-idiotypic antibodies to anti-peptidoglycan antibody (Ab1)[1] (denoted Ab2) were raised, purified, and injected into other rabbits for the synthesis of anti-anti-idiotypic antibodies (Ab3). Rabbits who were making Ab3 were then injected with the original antigen. Using several antigenic systems, the data show that: (a) After injection of antigen, nearly all the rabbits synthesized antibodies that were idiotypically cross-reactive with the starting Ab1 (denoted Ab1'). (b) Although the bulk of Ab3 did not recognize antigen, Ab3 and Ab1' shared some idiotypic specificities because anti-anti-anti-idiotypic antibodies (Ab4) also recognized Ab1 and Ab1' antibodies. (c) Ab4 behaved like Ab2 and diversity did not seem to increase along the chain of immunization.

These data suggest clearly that suppression is dominant in the immune response.

[1] *Abbreviations used in this paper:* Ab1, anti-peptidoglycan antibody(ies); Ab1', antibody(ies) that were cross-reactive with the starting Ab1; Ab2, anti-idiotypic antibody(ies) to Ab1; Ab3, anti-anti-idiotypic antibody(ies); Ab4, anti-anti-anti-idiotypic antibody(ies); Ab1-F_1, Ab1 produced by the offspring; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

However, it could be supposed that the appearance of the cross-reactive idiotypes (Ab1') was not a result of the relief of suppression, but was the result of direct priming of Ab1-like clones by Ab2. Ab3 antibodies would in fact be irrelevant to the relief of suppression, according to this supposition. This supposition does not fit with data that show that Ab3 shared idiotypic specificities with Ab1 and that removal of T lymphocytes on Petri dishes that were coated with MOPC 460 led to the appearance of the MOPC 460 idiotype (7, 15).

We have investigated the putative immunoregulatory role of Ab3 by the following experimental procedure: Female rabbits were injected with Ab2. When the synthesis of AB3 was initiated, female rabbits were crossed with naive male rabbits. 3 mo after birth, both mothers and their offspring were immunized with the original antigen. Our results clearly indicate that both the mothers and ~40% of the offspring synthesize idiotypes cross-reactive with the starting antibody, Ab1.

Materials and Methods

Rabbits. Rabbits were obtained from different local suppliers. They had no familial relationship. Sera were typed for allotypes a_1, a_2, a_3, b_4, b_5, b_6, c_{21}, e_{14}, d_{11}, and d_{12}. Rabbit families were obtained by mating allotype-matched males and females. Some anti-allotype sera were kindly furnished by Dr. P. A. Cazenave, Institute Pasteur.

Preparation of Micrococcal vaccines and micrococcal antigens immunization procedures. The preparation of *Micrococcus luteus* vaccines and antigens was performed as described previously (5, 16). The Ab2 and Ab3 immunization schedule was the same as described previously (5, 7).

Radioimmunoassay. Two different methods of radioimmunoassay were used: (*a*) Radioiodination was performed according to the method of Hunter and Greenwood (17). Binding experiments of radiolabeled antibody were then performed with 10 ng of antibody and increasing amounts of cross-linked antisera. Inhibition curves were done at 50% binding of radiolabeled antibody to cross-linked antiserum (7). (*b*) For radioimmunoassay with polyvinyl chloride plates, anti-idiotypic antisera were precipitated with 18% sodium sulfate. The immunoglobulin solution was dialyzed against phosphate-buffered saline (PBS). Before use, the wells of polyvinyl chloride plates (SMWC-96; Linbro Scientific Co., Hamden, Conn.) were treated with 75% acetic acid for 2 min. This has been shown to increase the capacity of adsorption of the antisera to the plastic wells (J. D. Franssen. Manuscript in preparation.). After washing, the wells were incubated for 16 h with a solution that contained 2 mg/ml of immunoglobulin. The wells were washed with 0.2% bovine serum albumin (BSA)-PBS and incubated for an additional 6 h with 0.5% BSA-PBS to reduce nonspecific adsorption. The wells were then incubated with radiolabeled idiotype for one night. For inhibition experiments, cold putative inhibitor in variable amounts and a fixed amount of labeled antibody were mixed as described above. The plates are washed with 0.2% BSA-PBS, dried, and the individual wells were sliced and counted.

Results

The starting antibody, Ab1, was prepared as follows. Serum from rabbit 2977 hyperimmunized against *M. luteus* was adsorbed on an *M. luteus* immunoadsorbent column. Anti-carbohydrate of *M. luteus* antibodies were obtained by elution with 5% glucose in PBS. Antibodies still adsorbed on the column after this treatment were recovered by acid elution with 0.2 M acetic acid. This fraction contained mostly Ab1. Further purification was achieved with preparative liquid isoelectric focusing (18).

The experimental scheme is illustrated in Fig. 1. Ab1, corresponding to a major homogenous antibody fraction, was used as antigen to raise isoanti-idiotypic and auto-anti-idiotypic antisera (Ab2). The specificity of these idiotypic reactions has been
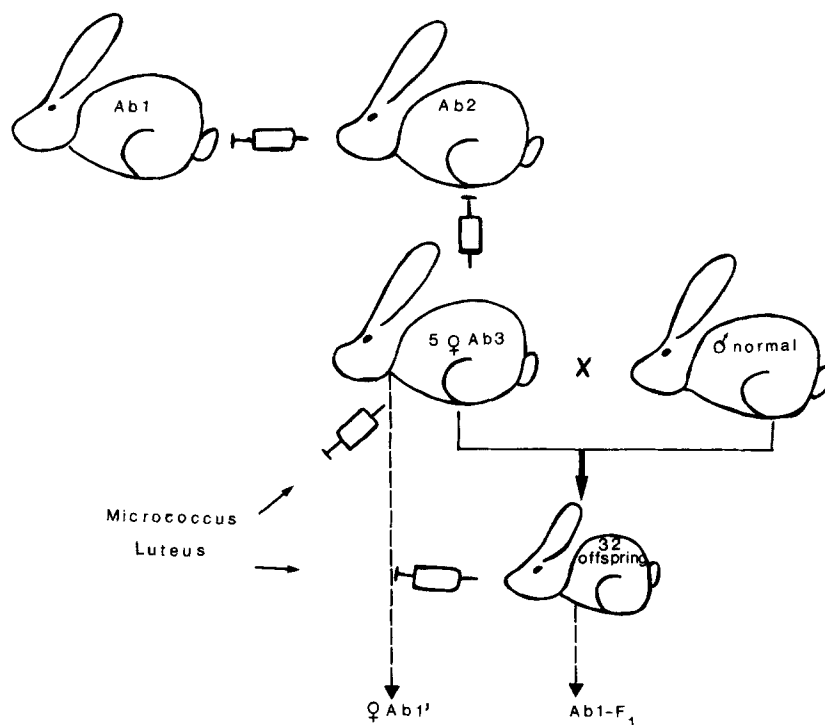
Fig. 1. Experimental scheme.

described previously (5, 7, 18). Ab2 that had originated from rabbit 2242 was isolated and injected into three female rabbits to elicit anti-anti-idiotypic antibodies (Ab3). Two other female rabbits received isolated auto-anti-idiotypic Ab2 that had been elicited in rabbit 2977 (Materials and Methods; and [18]). The appearance of Ab3 in the sera of five rabbits was followed by inhibition of the reaction between radiolabeled Ab1 and cross-linked Ab2 serum. All the females were mated when their Ab3 antiserum was able to inhibit at least 50% of the Ab1-Ab2 reaction. All together, the 5 litters yielded 32 surviving siblings. 2 mo after birth, both the mothers and their offspring were immunized against *M. luteus* according to a schedule previously described (16, 18). This resulted in the production of antibodies against peptidoglycan and carbohydrates. Ab1 synthesized by the mothers are denoted Ab1'; Ab1 produced by the offspring are denoted $Ab1-F_1$.

In an initial screening procedure, anti-idiotypic antisera (2242, 2244, and 2977) against Ab1 were tested in immunodiffusion with anti-micrococcal antibodies from the five mothers (Ab1') and from their progeny ($Ab1-F_1$). The same anti-idiotypic Ab2 sera were also tested with anti-micrococcal antibodies from 60 unrelated rabbits. This was done by the gel immunodiffusion technique, using the hyperimmune sera of each rabbit. Precipitin lines were observed with the antibodies from 2 out of 60 unrelated rabbits, whereas 4 out of 5 Ab1' antisera precipitated with Ab2. Moreover, 12 of the 32 $Ab1-F_1$ antisera showed a precipitin line with Ab2 antiserum. Thus, ~40% of $Ab1-F_1$-antisera cross-precipitated with Ab2 antisera.

Antibodies were then isolated from those antisera that were shown to contain antibodies with shared idiotypic determinants with Ab1 by immunodiffusion tests.

Immunologic relationships among these isolated antibodies were further examined by immunodiffusion analysis. The experiments illustrated in Fig. 2 demonstrate the sharing of idiotypic specificities among Ab1, Ab1', and Ab1-F$_1$ antibodies. The four examples show that only antibody directed to the peptidoglycan isolated from Ab1' and Ab1-F$_1$ antisera cross-reacted with Ab1 by immunodiffusion. Antibody specific to the *M. luteus* carbohydrate showed no significant precipitin line with Ab2. It should be recalled that the anti-idiotypic antibodies (Ab2) used to stimulate Ab3 in the mother were raised against an Ab1 antibody of the anti-peptidoglycan specificity. The results also demonstrate the absence of an idiotypic reaction in Ab1-F$_1$ antisera absorbed on *M. luteus* immunoadsorbent column. Antibody isolated from a pair of
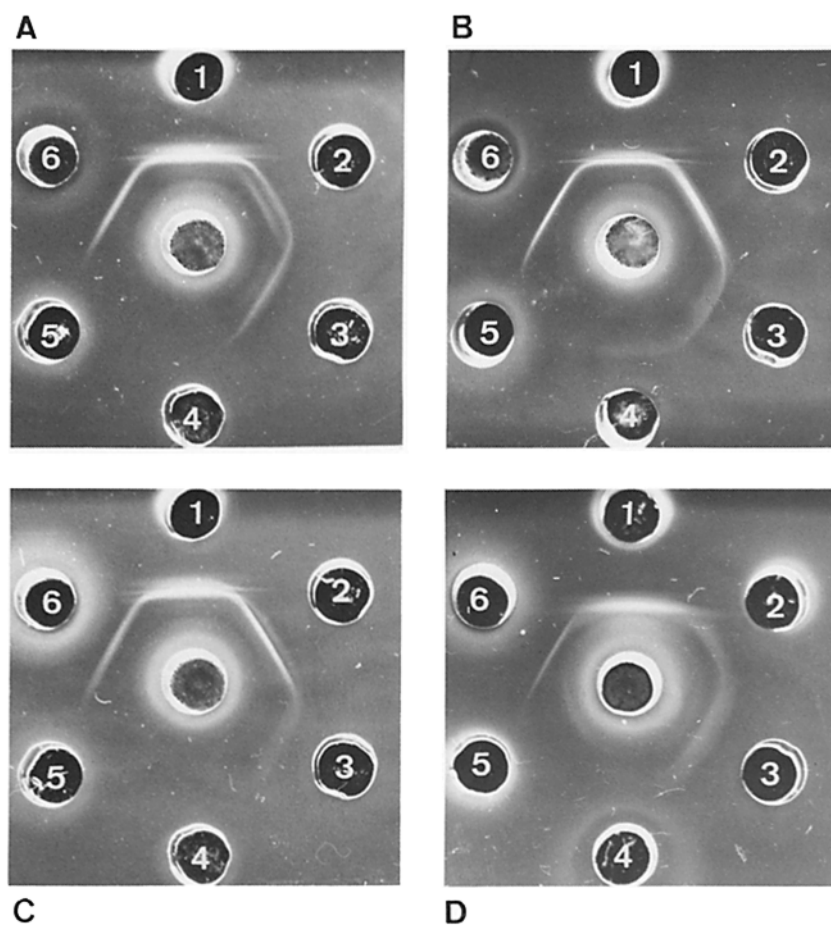

FIG. 2. Immunodiffusion analysis in agar gel that contained 2% polyethylene glycol. The center well was filled with the isologous anti-idiotypic antiserum (Ab2) raised against the idiotype (Ab1) produced in rabbit 2977. Well 1 contained purified Ab1. (A) Wells 2 and 3 contained Ab1-F$_1$ (2476) and Ab1-F$_1$ (2475). Well 4 was filled with anti-carbohydrate antibody isolated from the immune serum 2476. Well 6 contained the immune serum 2476. Immune serum 2476 that was adsorbed on an immunoadsorbent Sepharose column was in well 5. (B) Well 2 contained Ab1' (76), wells 3, 4, 5, and 6 contained Ab1-F$_1$, antibody to carbohydrate of *M. luteus*, adsorbed serum, and immune serum from rabbit 2483, respectively. (C and D) wells 3, 4, 5, and 6 were the same as for (B): immune serum 92 (Ab1') and fractions from F$_1$ serum 2464 and 2467 (D).
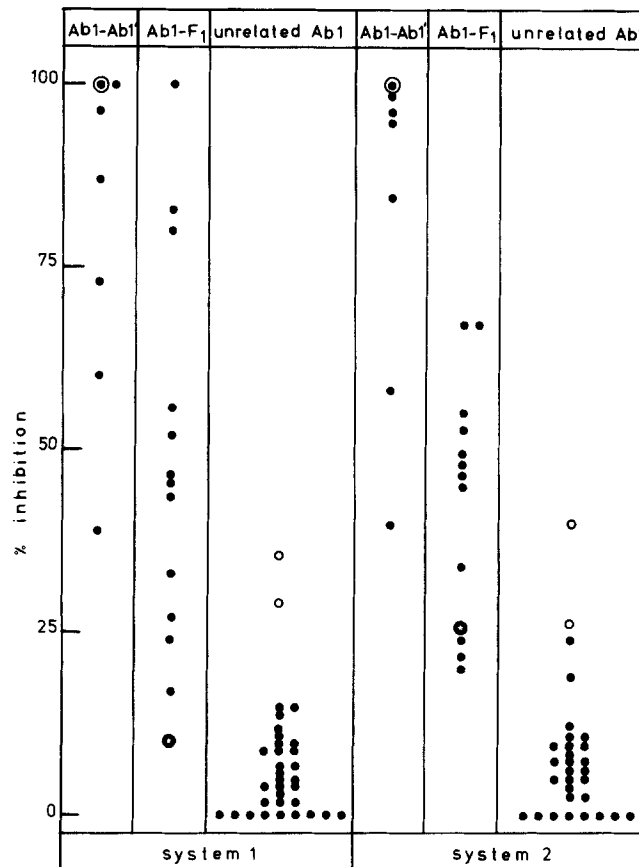
Fig. 3. Inhibition of idiotypic binding. Inhibitory capacity of Ab1, Ab1′, and Ab1-F₁ immune sera that were positive in immunodiffusion tests (●) and nonrelated sera from rabbits immunized with *M. luteus*. The inhibition of binding of radiolabeled Fab fragments of Ab1′ (76) to Ab2 (system 1) and Fab fragments of Ab1 to Ab2 (system 2) is shown. 20 μl serum was used as putative inhibitor of the idiotypic reaction performed on polyvinyl chloride plates. ●, Ab1 serum; O, unrelated Ab1 sera that reacted with Ab2 by immunodiffusion.◉, Calculated mean value for the inhibition by 20 Ab1-F₁ sera negative in immunodiffusion tests.

siblings that belonged to the F₁ generation (Fig. 2 A) form a line of identity. Partial identity is also revealed between Ab1, Ab1′, and Ab1-F₁. However, the results suggest clearly that some idiotypes displayed by Ab1 are absent in Ab1-F₁.

Isolated idiotypes Ab1, Ab1′, and Ab1-F₁ were radioiodinated and tested for their binding activity to Ab2. Although Ab1 binding was 70%, Ab1′ (rabbit 76) bound up to 80% to Ab2 serum. Two other Ab1′ (rabbits 78 and 92) exhibited a binding capacity of ~35% to Ab2. Only 25% of Ab1′ (rabbit 58) and Ab1′ (rabbit 62) were bound to Ab2. Nine radiolabeled Ab1-F₁ antibodies were assayed for their binding to Ab2. Two idiotypes bound ≤25% (sera 2461 and 2467), F₁ idiotypes from sera 2464, 2475, 2476, 2481, and 2483 exhibited a binding capacity of 36–39%. About 50% of Ab1-F₁ serum 2485 was bound to Ab2.

Immune sera that exhibited a precipitin line in immunodiffusion experiments were used as putative inhibitors of the reaction between Ab1 and Ab2 and between Ab1′
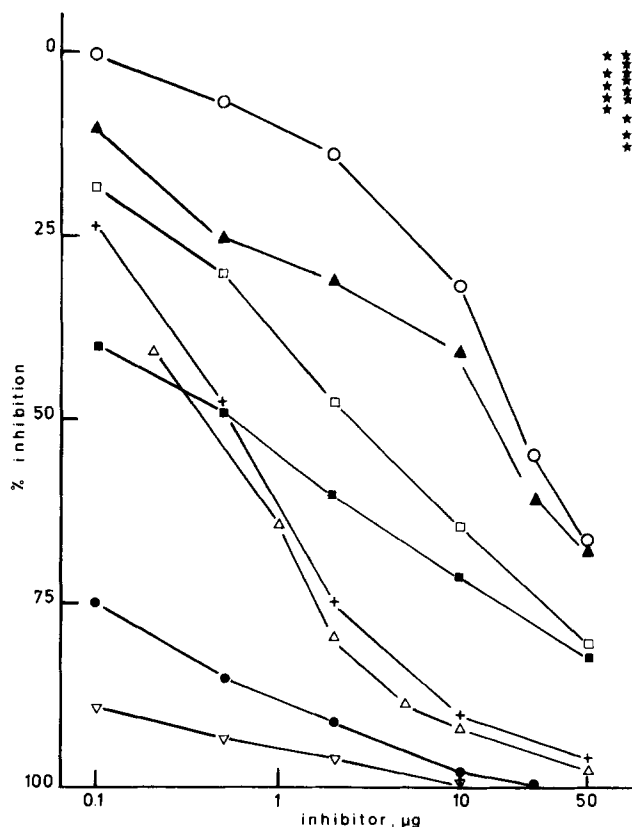
Fig. 4. Inhibition of binding of ^{125}I-labeled Ab1' from rabbit 76 to anti-idiotypic antiserum Ab2. Inhibitors are unlabeled Ab1 (●) and anti-peptidoglycan idiotypes isolated from five females producing Ab1': 58 (▲), 62 (○), 76 (▽), 78 (□), 92 (■), and Ab1' from rabbit 55 (+); specific inhibition by Ab2 (2242) is also shown (△). Controls at right top give inhibition values obtained with preimmune sera from Ab1 and Ab1' rabbits (★), and Ab1 isolated from nonrelated rabbits.

(rabbit 76) and Ab2. The percentage of inhibition obtained using 20 μl of antisera for the two idiotypic systems is shown in Fig. 3. (The Ab1-F_1 sera, negative in immunodiffusion tests, are now under investigation.) Thus of the 12 Ab1-F_1 antisera, 8 were able to achieve at least 50% inhibition of one or the other precipitin reactions. 33 antisera from unrelated rabbits were screened as inhibitors of the reaction between Ab1-Ab2 and Ab1'-Ab2. The results are also shown in Fig. 3. The calculated mean value for the inhibition by control sera of the system that employed Ab1 was 11.5%. The mean value for inhibition of the system that employed Ab1'-Ab2 was 8%. It is evident from these observations that the idiotypic cross-reactivities observed among the siblings and Ab1' are highly significant.

Further inhibition experiments were carried out to characterize idiotypic cross-reactions. Fig. 4 demonstrates that the original Ab1 and Ab1' (rabbit 76) are able to inhibit completely the binding between radiolabeled Ab1' and the anti-idiotypic serum Ab2. The five other Ab1' are capable of inhibiting, to various extents, this reaction. This is in agreement with previous results (7) where a different antibody specificity (anti-carbohydrate antibody) was used as the reference idiotype Ab1. Our
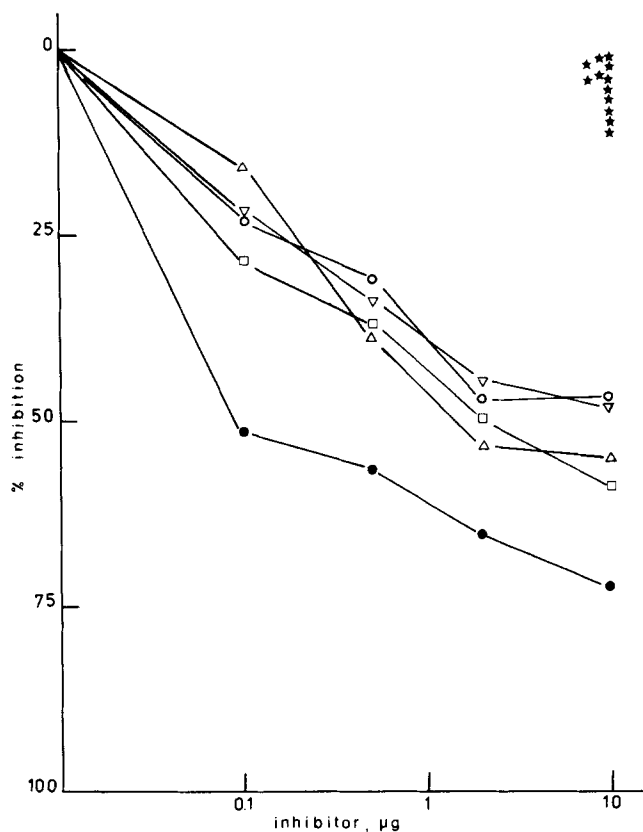
Fig. 5.   Inhibition of binding of radiolabeled Fab fragments of Ab1 (2977) to homologous anti-idiotypic antiserum Ab2 (2242). Inhibitors are unlabeled specifically purified anti-peptidoglycan antibodies Ab1′ from the female 76 (●), and anti-peptidoglycan antibodies from four siblings (Ab1-F_1) 2481 (□), 2483 (○), 2485 (△), and 2489 (▽). Controls at top right give inhibition values obtained with preimmune sera from Ab1-F_1 rabbits and anti-peptidoglycan antibodies from nonrelated rabbits (★).

study also confirmed the lack of antigenic recognition in Ab3 sera. No significant binding of radiolabeled peptidoglycan was obtained with any of the six Ab3 sera of the anti-peptidoglycan series. The idiotypic comparison of four Ab1 isolated from F_1 rabbits that all belonged to the same family is shown in Fig. 5. Inhibition curves were obtained using the reaction between radiolabeled Fab fragments of Ab1 and Ab2 serum.

The capacity of seven antibodies isolated from F_1 rabbits belonging to four different families to inhibit the reaction between Ab1′ and Ab2 is shown in Fig. 6. Two Ab1-F_1 are able to completely inhibit the reaction.

All these data suggest that Ab1, Ab1′, and some Ab1-F_1 represent a family of related, but nonidentical, idiotypes.

Discussion

The most salient features of this study are that both mothers and ~40% of the offspring synthesized antibodies that were strongly idiotypically cross-reactive with the starting antibody, Ab1. These results are documented using several methods.
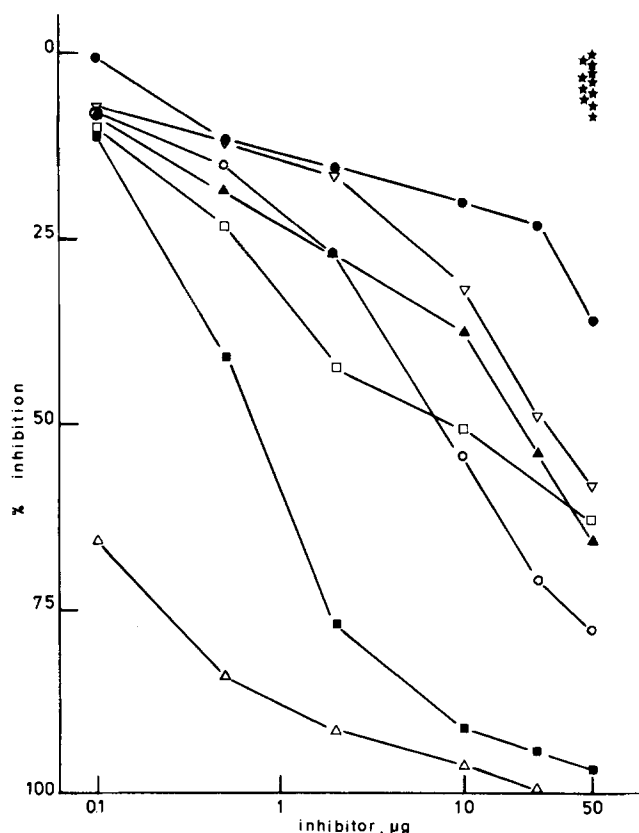
Fig. 6.   Inhibition of binding of ^{125}I-labeled Ab1′ from rabbit 76 to anti-idiotypic antiserum Ab2. Inhibitors are specifically purified Ab1-F$_1$ from two siblings: Ab1-F$_1$ 2461 (■) and 2492 (∇) from female 58; Ab1-F$_1$ 2476 (○) and 2477 (●) from female 62; Ab1-F$_1$ 2456, the (□) sibling of rabbit 78; and Ab$_1$-F$_1$ 2464 (△) and 2467 (▲) from female 92. Controls at top right give inhibition values obtained with preimmune sera from Ab1-F$_1$ rabbits and anti-peptidoglycan antibodies isolated from unrelated rabbits (★).

First, the results obtained with the mothers confirm and extend our previous results using the Ab1-Ab2-Ab3 scheme (5–7). Rabbits can learn to make idiotypes that are very different from those made when these rabbits are confronted only with antigen. It should be stressed that in this case, all five mothers' antibodies were able to inhibit the reaction between the starting idiotype (Ab1) and anti-idiotypic antibodies (Ab2). The new findings shown here indicate that an important proportion of the offspring, after immunization, with *M. luteus*, developed antibodies idiotypically cross-reactive with the starting idiotype (Ab1) and with the mothers' antibodies (Ab1′). It should be stressed that Ab1, Ab1′, and Ab1-F$_1$ are generally not identical but strongly idiotypically cross-reactive. They form a collection of interrelated immunoglobulins.

We think that this result can be expected because often even within one individual, the antibodies that appear during immunization are not just a random collection of immunoglobulins that happen to fit with antigen, but are made up of subpopulations that are idiotypically related. Even immunoglobulins that are devoid of antibody function but that display idiotypic specificities which are also found on antibodies are

present during some stages of immunization (4). It is also striking to note that major or recurrent idiotypes, which were long believed to be the products of a few germ-line genes are, in fact, a large family of idiotypically related, but nonidentical, molecules (when analyzed through the hybridoma technique (19, 20). Anti-idiotypic antibodies were injected into female rabbits, cross-linked with glutaraldehyde. It is therefore unlikely that such cross-linked Ab2 can cross the placental barrier. Most probably, maternal immunoglobulins of Ab3 type have crossed the placental barrier (or are absorbed by the offsprings via milk suckling) and have had a tremendous influence on the emergence of the idiotypic repertoire accessible to the antigen. These results argue strongly against direct priming of silent clones by Ab2 and suggest that the expression of clones of Ab1' is a result of relief of suppression.

It has been known for a long time that it is possible to strongly influence the expression of immunological repertoires by maternal immunoglobulins (21). In most experiments published until now, a suppressive effect of maternal immunoglobulins was clearly demonstrated. It has been shown by Herzenberg et al. (22) that it is possible to inhibit, in a long-term fashion, the synthesis of paternal allotypes in young mice that originate from mothers immunized against paternal allotypes. Similarly, allotype suppression has been documented in rabbits (23). Neonatal injection of anti-allotype and anti-idiotype antisera can lead to disappearance of the target allotypes or idiotypes (24, 25). This case of tolerance induction in the fetal or neonatal period is probably correlated with the special susceptibility of immature B lymphocytes to negative signals (26). Immature B lymphocytes are unable to express their endogenously synthesized receptors (27, 28) when incubated with anti-immunoglobulin antisera or antigen and their exposure to anti-immunoglobulin antisera or antigen seems to lead to clonal abortion.

In our experiments, exposure of immature immune systems to Ab3, which do not bind antigen but share idiotypic specificities with Ab1, seemed to promote an enhancing effect: the appearance of idiotypically cross-reactive antibodies. These effects could be more simply explained by stating that maternal Ab3 immunoglobulins inhibit the suppressors of the expression of cross-reactive idiotypes (Ab1 and Ab1'). Our results and the above conclusion fit well with the observation of Bona and Paul (15) who showed that removal of T lymphocytes that recognized the idiotypic specificities of the MOPC 460 protein lead to an enhanced expression of the idiotype after stimulation with trinitrophenylated Nocardia. Bona et al. also showed that the phenotype of the putative suppressors was of the Ly-2, Qua-1$^+$ type. Furthermore, anti-anti-idiotypic antibodies (Ab3) seem to be able to block the inhibitory activity of these cells (29).

Taken together, our results, those of P. A. Cazenave, (6) and the work of Bona and Paul (15) and Bona et al. (29) support the following newly emerging picture of the immune system: (a) Although different individuals synthesize different idiotypes when confronted with the same antigen, the total idiotypic repertoire in all individuals of the same species is approximately the same. Important limitations to this rule have been previously discussed. A large part of the total individual repertoire is silent during the lifetime of one individual. Silent clones are not insignificant minorities, but are kept under active suppression. This again raises the problem of selection in the immune system. (b) The immune system is a functional idiotypic network in the sense that idiotypes are involved in clonal interactions, even though positive or

negative signals are not delivered by idiotypic interactions. Idiotypic interactions could allow the meeting of complementary partners, and the nature of the signal could be given by the compartment to which interacting cells belong. (c) The idiotypic network is not open-ended, but rather it turns back on itself. The units of the network are made up of circular circuits whose size is unknown. (d) The functional adult network is strongly influenced by early signals that reach the initial network. These early signals could be maternal immunoglobulins, as shown here, or self antigens or external antigens that are accidentally present during a critical period. This last point deserves consideration for a number of reasons. An analogy has been put forward between the immune system and the nervous system. It is known in the nervous system that the initial network is genetically programmed and that the connectance is greater than in the adult nervous network. The final structure seems to depend on the selective stabilization of some pathways that function during an early critical period. An imprint of the environment is built in the initial nervous system (30). Similarly, in the immune system, even if different individuals start out with the same basic idiotypic repertoire, the presence of different self antigens in an outbred species, the occurrence of different maternal immunoglobulins, and the unpredictable arrival of external antigens will drive the initial network into different functional states in different individuals. Different pathways of response will be favored in different individuals, and this could well be one of the reasons why different individuals use a different idiotypic repertoire when confronted with the same antigen. Recently Cazenave and Voegtlé have obtained results similar to those described in this paper (P. A. Cazenave. Personal communication.).

Summary

    Specific idiotypes can be induced in randomly chosen rabbits by preimmunization with anti-idiotypic antibodies (Ab2). Rabbits that synthesize anti-anti-idiotypic antibodies (Ab3) when injected with antigen produce antibodies that display idiotypic specificities that are also found on the starting idiotype. When female rabbits actively producing Ab3 are crossed with naive males, a significant proportion of the offsprings (~40%) produce antibodies that were idiotypically cross-reactive with the starting idiotype, as compared to 3% of the controls. This conclusion was obtained using 5 female rabbits and their 32 surviving offspring. Maternal idiotypes have therefore strong immunoregulatory properties and influence the emergence of the available idiotypic repertoire.

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