INDUCTION OF ANTI-TOBACCO MOSAIC VIRUS ANTIBODIES IN MICE BY RABBIT ANTIIDIOTYPIC ANTIBODIES

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Since their original discovery (1, 2), idiotypes have been studied for two main purposes. As markers of immunoglobulin variable regions, recurrent idiotypes have been used to unravel the genetics of antibody diversity. Furthermore, it is likely that the immune system uses its own idiotypic diversity for regulatory purposes: idiotypic-antiidiotypic interactions play a role in the logics of lymphocyte interactions (3). Idiotype network concepts have inspired many works and have allowed specific manipulations of the immune system (4, 5). For example, minute amounts of antiidiotypic specificities raised against recurrent idiotypes can profoundly modify subsequent immune responses (6). In the case of idiotypes as individual markers, it is possible to induce silent idiotypes by the idiotypic cascade. The first studies were done in rabbits (7, 8) and were extended to mice systems (9–12) with very similar results.

All these experiments show that it is possible to reprogram the immune response towards a predetermined goal. To further investigate these problems and to study the evolution of idiotype networks, we addressed the following question: can we bypass the species barrier and induce the expression of private rabbit idiotypes in mice? Our results show that this approach is indeed feasible using antiidiotypic antibodies that do not display at all the properties of internal images, using either the idiotypic cascade or rabbit antiidiotypic antibodies coupled to lipopolysaccharide (LPS)[1] (13, 14).

Materials and Methods

Mice. All experiments were carried out in BALB/c female mice obtained from CEN, Mol, Belgium.

Antigens and Immunizations. LPS was a phenol extract of *Escherichia coli* 055:B5, obtained from Difco Laboratories, Inc., Detroit, Mich.

Rabbit Antiidiotypic Antibodies (Ab2). Ab2 were raised against rabbit anti-Micrococcus or anti-tobacco mosaic virus (TMV) antibodies as described previously (15). They were

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[1] *Abbreviations used in this paper:* Ab1, antibody raised against antigen; Ab2, antiidiotypic antibody; Ab3, anti-antiidiotypic antibody; Ab4, anti-anti-antiidiotypic antibody; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide from *E. coli*; Mi, *Micrococcus lysodeikticus*; PBS, phosphate-buffered saline; RIA, radioimmunoassay; TMV, tobacco mosaic virus.

purified by affinity chromatography on Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) coupled to idiotypic antibodies.

LPS-Ab2 Conjugates. These were prepared by a procedure adapted from Scibienski (16): 20 mg of LPS were dissolved in 5 ml H_2O and adjusted to pH 11 with 0.1 M NaOH. 10 mg of cyanuric chloride (J. T. Baker Chemical Co., Phillipsburg, NJ) were added to this solution with constant stirring for 6 min during which pH was maintained at 11 with 0.1 M NaOH. This solution was mixed with 20 mg purified antiidiotypic antibodies dissolved in 5 ml of 0.5 M carbonate buffer at pH 8.5. The reaction was allowed to proceed at 4°C overnight. The conjugates were then dialyzed against saline. LPS-Ab2 complexes were separated from uncoupled antibodies by chromatography on Sephadex G200. LPS-Ab2 conjugates were also prepared by a carbodiimide coupling procedure adapted from the method described by Scibienski (16). For this procedure, the LPS and purified rabbit Ab2 antibodies were mixed at a 1:1 ratio in normal saline and an amount of *N*-cyclohexyl-*N'*-(2-morpholinoethyl)carbodiimide methyl-*p*-toluenesulfonate (Fluka A. G., Basel, Switzerland) equivalent to one-half the amount of protein was added. Coupling was allowed to proceed for 4 h at room temperature and then overnight at 4°C, after which the complexes were dialyzed against saline and then isolated on Sephadex G200. This carbodiimide procedure appeared much less efficient than the cyanuric chloride one, because of incomplete coupling of the rabbit Ab2 antibodies. BALB/c mice were immunized intravenously with 10 μg of LPS-Ab2 complexes, once a week during 4 wk. The same immunization schedule was followed with the control mice, which received intravenously 10 μg of LPS or Ab2 alone.

Radioimmunoassays. These were performed as described previously (15).

Mitogenic Stimulation of Spleen Cells. 2×10^5 mouse spleen cells were cultured in microtiter plates (Linbro Chemical Co., Hamden, CT) in 0.2 ml of RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. 25 of 50 μg/ml of LPS, Ab2, or LPS-Ab2 complexes were added at the start of the cultures. The cultures were set up in triplicate and incubated at 37°C with 5% CO_2. After 48 h, each culture was pulsed with 1 μCi of [^{3}H]thymidine and harvested 17 h later on glass fiber filters using an automated cell harvester (Titertek; Flow Laboratories, Inc., McLean, VA).

Cell Fusion, Cloning, and Antibody Screening. Hybridomas were produced according to the method of Köhler and Milstein (17) with the modifications described by Franssen et al. (18). Soft agar cloning and in situ detection of hybridomas were performed according to the procedure of Hérion et al. (19). Cloning in agarose semi-solid medium was performed using a slight modification of the technique described by Coffino and Charff (20).

Culture supernatants were screened by solid phase immunoenzyme assay. The enzyme-linked immunosorbent assay (ELISA) test was carried out as follows: Polyvinyl chloride Linbro microtiter plates were coated overnight at 4°C with 25 μl of purified rabbit $AB2_{TMV}$ (0.2 μg), normal rabbit IgG (0.3 μg), or TMV (50 μg). The remaining adsorption sites were blocked by incubation with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) for 2 h at room temperature. Plates were washed several times and incubated with 25 μl of culture supernatants for 4 h at room temperature. Peroxidase-labeled Ig from a goat anti-mouse serum was used to reveal the mouse antibodies (25 μl of a solution containing 0.5 μg/ml of labeled Ig in PBS 0.1% Tween 80, 1% BSA, and 10% horse serum, per well). After 4 h incubation at room temperature, the wells were thoroughly washed. A 0.04% orthophenylenediamine, 0.02% urea peroxyde, 0.1 M phosphate, pH 5 solution was added to reveal the bound enzymatic activity. The optimal density was scored by a Microelisa Automatic Reader (AM 120; Dynatech Laboratories, Inc., Alexandria, VA).

Results

Immunization Chain. Antiidiotypic antibodies raised against rabbit Ab1 (anti-TMV or anti-Micrococcus) antibodies were isolated and injected into allotypically

matched rabbits, to get anti-antiidiotypic antibodies (Ab3). These rabbits were then immunized with the original antigen and the induced antibodies were called Ab1′. The production and detailed characterization of these antibodies were described elsewhere (15).

In the TMV system, antiidiotypic antibodies against $Ab1_{TMV}$ were prepared in two rabbits (rabbits IIa and IIb). These Ab2 were shown to have the same specificity. The antiidiotypic antibodies of the TMV system ($Ab2_{TMV}$) or of the Micrococcus system ($Ab2_{Mi}$) are usual discriminatory antibodies that do not behave at all like internal images. Furthermore, they do not recognize anti-TMV or anti-Micrococcus antibodies from mice, as shown in Fig. 1. Complexes of LPS-$Ab2_{Mi}$ and LPS-$Ab2^a_{TMV}$ were prepared and injected into BALB/c mice.

Mitogenic Properties of LPS-Ab2 Conjugates. To insure that the covalent binding of Ab2 molecules to LPS did not modify the mitogenic activity of LPS, we compared the capacity of LPS and LPS-Ab2 conjugates to stimulate the incorporation of [^{3}H]thymidine by murine spleen cells. BALB/c female spleen cells were cultured with 25 or 50 μg/ml of LPS, LPS-Ab2 complexes, or Ab2 alone. Activation of DNA synthesis was measured after 2 d in culture. The results of this stimulation showed no significant difference among LPS and LPS-Ab2 preparations, although LPS-Ab2 coupled by the cyanuric chloride method may have lost some mitogenicity in comparison with the LPS (data not shown).

Immunogenicity of LPS-Ab2 Conjugates. Each purified Ab2 ($Ab2^a_{TMV}$ or $Ab2_{Mi}$) was coupled to LPS and the conjugates were then isolated by chromatography on Sephadex G200. In each system, three groups of 10 mice were injected intravenously with 10 μg of LPS, Ab2, or LPS-Ab2. After four successive immunizations, the sera of these mice were tested for the presence of specific antibodies to rabbit Ab2 by radioimmunoassay. Each assay was carried out in the presence of normal rabbit serum. As shown in Fig. 1, all BALB/c mice injected with LPS-$Ab2^a_{TMV}$ conjugates synthesized Ab3 antibodies that are recognized by the immunogen $Ab2^a_{TMV}$ but not by $Ab2_{Mi}$. Similarly, sera from LPS-$Ab2_{Mi}$-immunized BALB/c mice contained significant amounts of antibodies specifically

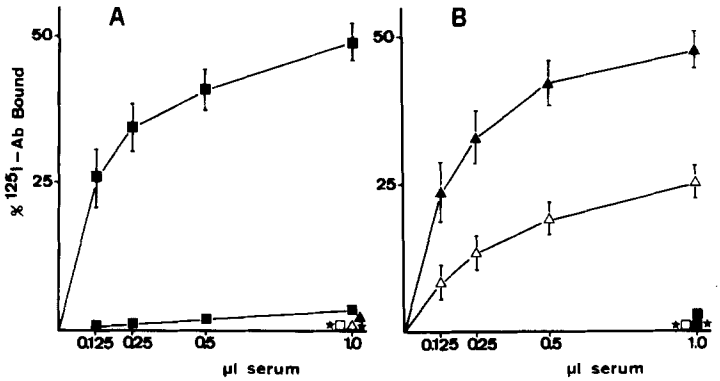

FIGURE 1. Binding curves of ^{125}I-labeled rabbit $Ab2_{Mi}$ (*A*) and rabbit $Ab2^a_{TMV}$ (*B*) to sera from mice immunized with LPS-$Ab2_{Mi}$ (■), $Ab2_{Mi}$ (▣), LPS-$Ab2^a_{TMV}$ (▲), $Ab2^a_{TMV}$ (△), and LPS (□). Each point represents the mean (±95% confidence level) of 10 mice. (★) Average binding values of tested sera from 15 anti-TMV or anti-Micrococcus BALB/c mice.

bound by the $Ab2_{Mi}$ molecules. Mice that received LPS or $Ab2_{Mi}$ alone did not produce Ab3 antibodies. An antibody response to $Ab2^a_{TMV}$ was also observed in mice injected with $Ab2^a_{TMV}$ alone, but the response of these mice was consistently lower compared with the response after conjugate injection. The Ab3 antisera from mice immunized either with LPS-$Ab2^a_{TMV}$ or $Ab2^a_{TMV}$ alone reacted not only with their own specific immunogen ($Ab2^a_{TMV}$), but also with the other Ab2 directed against the same original $Ab1_{TMV}$ (data not shown).

LPS-$Ab2_{TMV}$ Conjugates Induce the Synthesis of Anti-TMV Antibodies in Ab3 Mice. All Ab3 sera were tested for a specific anti-TMV activity. The bleedings were assayed for their capacity to bind directly to TMV adsorbed to plastic trays. Fig. 2 shows that 7 out of 10 sera from mice immunized with LPS-$Ab2_{TMV}$ displayed a specific binding activity to TMV, as compared with controls (normal BALB/c sera, sera from mice injected with LPS, LPS-$Ab2_{Mi}$, or $Ab2_{Mi}$). The levels of anti-TMV antibodies in Ab3 sera in positive mice injected with LPS-$Ab2_{TMV}$ were as high or higher than those observed in BALB/c mice sera during a primary response against TMV (Table I). In the other groups (LPS, $Ab2_{Mi}$,

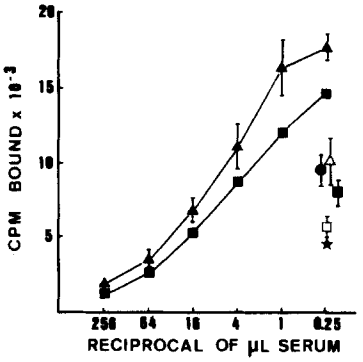

FIGURE 2. Antigen-binding capacity of BALB/c sera. Sera from individual mice were tested for anti-TMV antibodies with a solid phase RIA. Each point represents the mean (±95% confidence level) for 10 mice. Detection of anti-TMV antibodies in sera from mice immunized with LPS-$Ab2^a_{TMV}$ (▲) (7 mice out of 10); $Ab2^a_{TMV}$(△), LPS-$Ab2_{Mi}$ (■), $Ab2_{Mi}$ (□), and LPS (●). ■ Binding values of a pool of BALB/c anti-TMV primary response antisera. Fixation values obtained with a pool of normal BALB/c sera is also shown (★).

TABLE I

Anti-TMV Titer of Ab3 Mice and Control Mice

Treatment of the mice*	Anti-TMV titer				
			µg/ml		
None			0.1‡		
(5) Normal rabbit Ig ($Ab2_{Mi}$)	0.9	1.2	1.4	1	1.3
(5) Rabbit $Ab2_{TMV}$	6.2	5.3	5.1	6.8	4.8
(10) LPS-$Ab2_{TMV}$	10.5	12.2	13.3	15.3	16
	29.7	39.9	8.5	7.6	9.5
(5) LPS-$Ab2_{Mi}$	3.5	2.9	1.3	3.9	2.3
(5) LPS	2.4	4.1	3.5	4.5	3.9
(10) TMV (primary response)			10.5‡		

* The number of mice is in parentheses.

‡ Mean values obtained with the pooled sera of 10 BALB/c mice.

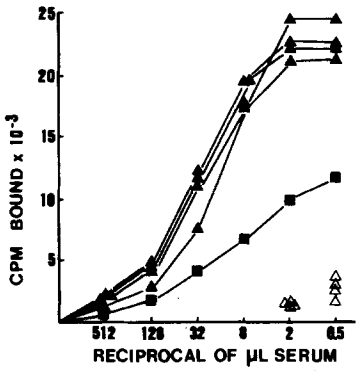

FIGURE 3. Detection of anti-TMV activity in antibodies from LPS-$Ab2^{a}_{TMV}$-immunized mice, isolated either on $Ab2_{TMV}$-Sepharose beads (▲), or on normal rabbit Ig–Sepharose columns (△). Individual sera from four immunized animals were submitted to affinity chromatography on these columns. The binding curve of pooled primary anti-TMV antisera from BALB/c mice is also shown (■).

etc.), no significant anti-TMV activity was observed with the exception of mice that had received $Ab2_{TMV}$ alone. To determine whether these anti-TMV antibodies were related to the rabbit $Ab2^{a}_{TMV}$ molecules, the following experiment was performed. The sera from Ab3 mice immunized with LPS-$Ab2_{TMV}$ conjugates were submitted to affinity chromatography on Sepharose columns coupled either to $Ab2^{a}_{TMV}$ antibodies or to normal rabbit Ig. As can be seen in Fig. 3, the antibodies that bound to $Ab2^{a}_{TMV}$ were found to be enriched in anti-TMV activity, whereas the antibodies isolated on the normal rabbit Ig column did not react with TMV. The fractionation of sera from mice immunized with $Ab2^{a}_{TMV}$ alone showed similar results, except that the amount of anti-TMV antibodies was twofold lower. These results indicate that the administration of LPS-Ab2 complexes or $Ab2_{TMV}$ in BALB/c mice led to the appearance of anti-TMV antibodies, specific to the rabbit $Ab2_{TMV}$, in the sera of these mice.

Idiotypic Characterization of Ab3 Antibodies in LPS-$Ab2_{TMV}$-Immunized Mice. To study the idiotypic specificities of Ab3 antibodies in mice, a competitive radioimmunoassay (RIA) was performed. The binding of ^{125}I-labeled rabbit antiidiotype to purified Ab3 antibodies was inhibited by serial dilutions of rabbit Ab1, Ab2, Ab3, or Ab1′ antisera from the TMV system. Purified Ab3 antibodies from LPS-$Ab2_{TMV}$-immunized mice were isolated by affinity chromatography, using Sepharose column coupled to rabbit $Ab2^{a}_{TMV}$. Antibodies against rabbit Ig were removed by passage on columns coupled with normal rabbit Ig. No loss of anti-TMV activity was observed after removal of anti–rabbit Ig antibodies. Various rabbit antisera were tested for their putative capacity to inhibit the binding of ^{125}I-labeled $Ab2^{a}_{TMV}$ to the purified Ab3 antibodies. As can be seen in Table II, the two $Ab2_{TMV}$ raised against the original Ab1 were able to inhibit completely this reaction. Preimmune rabbit sera or heterologous antiidiotypic antisera failed to cause significant inhibition. Antiidiotypic antisera from the Micrococcus system were also devoid of inhibitory capacity. Rabbit Ab1, Ab3, and Ab1′ antisera were tested as inhibitors in the same assay (Fig. 4*A*). The Ab1, Ab3, and Ab1′

TABLE II

Inhibitor	μl Inhibitor	Percent inhibition
Rabbit $Ab2^{a}_{TMV}$ antisera	0.5	99.9
Rabbit $Ab2^{b}_{TMV}$ antisera	0.5	99.8
Heterologous $Ab2_{TMV}$ antisera	5	2
Preimmune sera from rabbit II_a	5	0.5
Rabbit $Ab2_{Mi}$ antisera	5	1

Inhibition of the binding of ^{125}I-labeled rabbit $Ab2^{a}_{TMV}$ antibodies to purified Ab3 antibodies. Ab3 antibodies from LPS-$Ab2^{a}_{TMV}$-immunized mice were purified by isolation on a rabbit $Ab2_{TMV}$-Sepharose column, followed by passage through a Sepharose–normal rabbit Ig column.

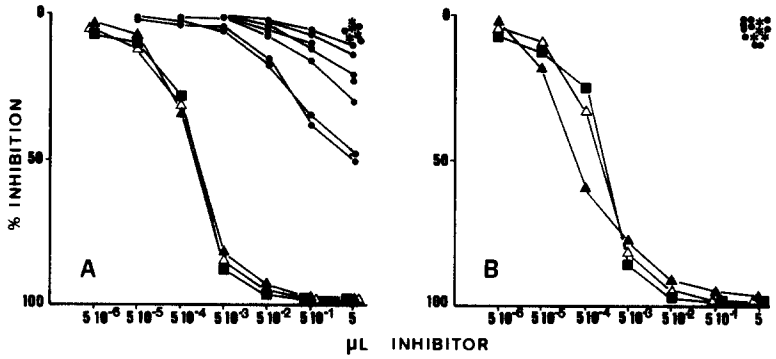

FIGURE 4. (*A*) Inhibition of the binding of ^{125}I-labeled rabbit $Ab2^{a}_{TMV}$ to mice purified Ab3 antibodies by antisera from rabbit I (Ab1) (▲) and rabbit IIIa [$Ab3^{a}$ (■) and $Ab1'^{a}$ (△)] of the immunization chain in the TMV system. Inhibition values obtained with anti-TMV antisera from 10 different rabbits are represented (●). (*) Inhibition values obtained with sera from three rabbits hyperimmunized with Micrococcus or with a pool of normal rabbit sera. (*B*) The same inhibition assays were performed in presence of pooled rabbit anti-TMV antisera.

antibodies of the immunization chain in the TMV system were strong inhibitors of the reaction between radiolabeled $Ab2^{a}_{TMV}$ to mice Ab3 antibodies. This is in agreement with previous results that indicated that these rabbit antibodies are idiotypically cross-reactive. Neither normal rabbit Ig nor sera from rabbits immunized with Micrococcus produced significant inhibition. 10 anti-TMV antisera from unrelated rabbits were screened as inhibitors of the same reaction. A weak cross-reaction was found for some of them (Fig. 4*A*). It is interesting to note that the most cross-reactive rabbit anti-TMV antibodies in this assay inhibit also the reaction between rabbit Ab1 and its corresponding rabbit antiidiotypic serum.

To compare further the idiotypic specificities of rabbit Ab1 and mouse anti-TMV antibodies, the same assay was performed in the presence of pooled rabbit anti-TMV antisera. The results, illustrated in Fig. 4*B*, indicated that only Ab1 and Ab1′ antibodies of the immunization chain completely inhibit the binding between rabbit Ab2 and mice Ab3 antibodies, whereas all other rabbit anti-TMV antisera failed to produce inhibition. These results clearly show the sharing of

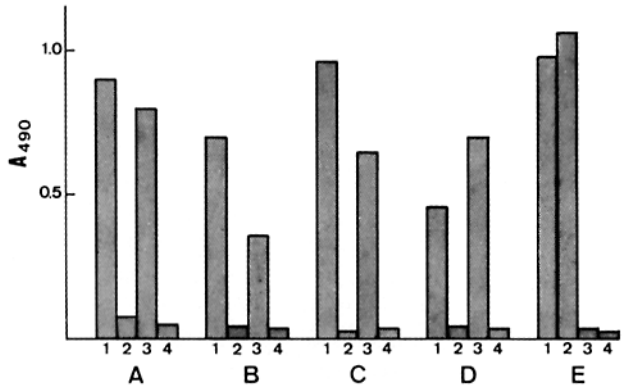

FIGURE 5. ELISA of culture supernatants of Ab1′ hydridomas. Culture supernatants of four cloned Ab1′ hybridomas (*A*, *B*, *C*, and *D*) were incubated on microtiter plates coated with rabbit $Ab2^{a}_{TMV}$ (*1*), normal rabbit IgG (*2*), TMV (*3*), or BSA (*4*). Culture supernatants of an anti-Ig rabbit hybridoma (*E*) was tested as control. The bound antibodies were detected by the addition of peroxidase-labeled goat anti-mouse Ig.

strikingly similar idiotypic specificities between the mice anti-TMV antibodies induced by LPS-$Ab2_{TMV}$ and the rabbit Ab1 (or Ab1′) anti-TMV antibodies.

Confirmation of these conclusions was sought, using monoclonal antibodies prepared from a mouse injected with LPS-$Ab2_{TMV}$ conjugates. A fusion experiment was performed and four monoclonal antibodies recognizing both TMV and rabbit $Ab2_{TMV}$ were obtained (Fig. 5). These monoclonal antibodies were affinity purified on rabbit Ab2-Sepharose column antibodies and injected into rabbits or mice to induce what we call, in operational terms, Ab4 antibodies. These Ab4 sera, checked by RIA, react specifically with the starting anti-TMV rabbit Ab1 (Francotte, M. and J. Urbain, manuscript in preparation).

Discussion

During the last years, our group and others have shown that one can guide the immunological repertoire towards a predetermined goal. Particularly idiotypes, specific for one antigen and expressed only in a few immunized individuals, can be induced by appropriate idiotypic manipulations. As recalled in the introduction starting with the idiotype (Ab1), one can obtain in a second animal antiidiotypic antibodies (Ab2) which, in turn, are injected into a third set of recipients. As a result, anti-antiidiotypic antibodies (Ab3) are raised. It is important to recall that Ab1, Ab2, and Ab3 are merely operational terms that indicate in what sense immunizations were alone.

At first sight, one should expect that immunization with Ab2 specific for a private idiotype would induce mainly a set of heterogeneous antibodies just recognizing idiotopes of Ab2. These Ab3 antibodies should be serologically and genetically unrelated to Ab1. However, studies performed both in rabbits and mice (9, 15) indicated that Ab4 antisera, raised against Ab3, recognize specifically Ab1, the starting idiotype. From these studies and others (21), it is clear that the major outcome following Ab2 immunization is the induction of Ab1-like Ig ($Id^{+}Ag^{-}$ and $Id^{+}Ag^{+}$ subsets). Mostly Ab3 is similar to Ab1 and Ab4 to Ab2 (4, 9, 22).

In this study, we have induced Ab3 antibodies in BALB/c mice by injecting Ab2 rabbit antibodies coupled to LPS or Ab2 alone. The rabbit Ab2 antibodies were raised against a rabbit anti-TMV antibody and are specific for nonrecurrent idiotypes. This idiotype is not expressed (or at very low levels) in BALB/c mice immunized with TMV alone. The most striking results of this study is the appearance of mouse anti-TMV antibodies, which are strongly idiotypically cross-reactive with Ab1, despite the fact that these mice have never seen TMV. Therefore, silent idiotypes can be induced, not only within the same species, but as well across species. The manipulation is extremely specific, since the injection of other antiidiotypic antibodies similarly coupled to LPS, but raised against anti-Micrococcus antibodies did not elicit anti-TMV antibodies in mice.

The results clearly show, by the use of affinity chromatography, that the same molecules that bind to Ab2 also bind to TMV. Furthermore, we obtained from mice injected with Ab2-LPS, monoclonal antibodies that bind TMV and share idiotypic specificities with the rabbit idiotype. Antiidiotypic antibodies (or Ab4 in operational terms) raised against mice anti-TMV antibodies bound specifically to the starting rabbit idiotype.

It is important to stress that the Ab2 used in our studies are specific for nonrecurrent idiotypes. Therefore, we can exclude the fact that the Ab2 used carry an internal image of the antigen (23, 24). Therefore, the present results should be distinguished from previous ones, in which we used a selected antiidiotypic antibody recognizing anti-TMV antibodies from all rabbits, chickens, mice, etc (24).

If we exclude the internal image as the explanation for our findings, how can we interpret our results? Several hypotheses have been proposed to explain the results of the idiotypic cascade. Bona et al. (9) and Paul and Bona (25) have developed the hypothesis of regulatory idiotopes (9, 25). Among all idiotopes (which are first defined in operational terms), they define a special class of idiotopes, the regulatory idiotopes which are autoimmunogenic and are the targets of regulatory lymphocytes. In this sense, our findings show the existence of interspecies cross-reactive regulatory idiotopes, recognized by a conventional rabbit antiidiotypic antiserum that can activate silent clones in both species.

We have proposed that the results of the idiotypic cascade are compatible with the existence of "germline circles" (4): the germline repertoire embodies an idiotypic network or, more simply, the germline repertoire encodes for families of idiotypically interacting Ig. In this latter hypothesis, no distinction is made between Ab1 and Ab2. The immune system does not know whether an Ig is an idiotype or an antiidiotype. As in the Paul and Bona (25) hypothesis, not all idiotopes are equal. Some are germline encoded and are the primordial targets for regulatory mechanisms. We can fuse the two and suggest that, in fact, regulatory idiotopes (those really involved in the idiotypic network) are germline encoded and conserved between species. As pointed out elsewhere, these ideas do not imply at all a simplistic germline theory (26).

On a more practical level, the results described here should provide interesting tools to manipulate in a fine and specific way, an immune response. Indeed, the induction of idiotypically cross-reactive anti-TMV antibodies is also found upon injection of Ab2 alone. It seems therefore possible to use conventional antiidi-

otypic sera that recognize private idiotypes as substitutes for vaccination. This extends further recent works (27–30) showing that antiidiotypic antibodies, specific for recurrent idiotypes, can induce antivirus antibodies and also protective immunity.

Summary

Conventional rabbit antiidiotypic antibodies were raised against a private rabbit anti-tobacco mosaic virus (TMV) idiotype. These rabbit antiidiotypic antibodies were covalently coupled to lipopolysaccharide and then injected into BALB/c mice. As compared with controls, these mice, which have never seen the antigen, synthesized anti-TMV antibodies that are strongly idiotypically cross-reactive with the starting rabbit idiotype. Monoclonal anti-TMV antibodies were prepared from these mice. Furthermore, xenogeneic or syngeneic antiidiotypic antibodies, raised against these monoclonal anti-TMV antibodies, recognized specifically the rabbit idiotype. Rabbit antiidiotypic antibodies alone can induce the same effects, but the concentration of anti-TMV antibodies is lower.

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