ABPC 48 CROSS-REACTIVE IDIOTOPES IN BALB/c MICE

Natural and Levan-induced Expression

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The existence of a functional idiotypic network was postulated by Jerne (1) nearly one decade ago. Many experiments do indeed prove the feasibility of idiotypic manipulations of the immune system (see reference 2 for a review of this topic). It still remains to be established however, that idiotype-antiidiotype interactions play a major role in the ontogenesis of the immune system and that such interactions control its steady state. Information on these points may be gained from the characterization of idiotypes expressed in mice during the natural immunization event, as compared to those triggered by deliberate immunization with known antigens.

Anti-levan response in BALB/c mice allows such an analysis. Bacterial levan is considered one of the most common natural antigens and the anti-levan immune response has been extensively analyzed by Lieberman et al. (3). Bacterial levan is a polyfructosan with β2–1 and β2–6 linkages. Bona et al. (4) have shown that the anti-β2–6 polyfructosan response may be elicited by immunization of young mice (from age 1 or 2 wk up) in contrast to the anti-β2–1 polyfructosan (anti-inulin) response, which can be elicited later in ontogeny. The idiotypes associated with anti-β2–6 antibodies in BALB/c include two myeloma proteins, ABPC48 and UPC10 which we have shown in a previous study (5) to share idiotypic determinants. The obtaining of a battery of monoclonal syngeneic antiidiotypic antibodies permits us to perform very sensitive binding assays specific for one chosen idiotope.[1] As shown here, the analysis of the immune response in adult BALB/c mice injected with levan reveals that ABPC48 idiotopes contribute to this response. Information on the idiotypic pattern of a natural immunization was also gained when ABPC48 idiotype expression and anti-levan titer were compared in young BALB/c mice immunized with bacterial levan and in nonimmunized mice.

Material and Methods

Mice. BALB/c mice were purchased at the breeding Center of the Centre National de la Recherche Scientifique (CNRS, Orleans, France) and grown in our own animal facilities.

Antigens. Bacterial levan of *Bacillus subtilis* origin (polyfructosan with β2–1 and β2–6 linkages) of several molecular weights (2×10^7, 2×10^5 and 5×10^4), plant levan (mol wt

[1] We shall refer to an idiotope as the structural element that participates in the binding of one given idiotype to one given monoclonal antiidiotypic antibody. Idiotopes are likely to be overlapping regions on the variable domain of an immunoglobulin rather than ponctual determinants (6).

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/83/09/0872/13 $1.00
Volume 158 September 1983 872–884

7,500, β2–6 linkages), and inulin (mol wt 2,500, β2–1 linkages) were generous gifts from Dr. Rappoport (Institut Pasteur, Paris). The immunization procedure was 50 μg intraperitoneally of bacterial levan (mol wt 2×10^7). Animals were bled through the retroorbital sinus.

Monoclonal Immunoglobulins. ABPC48 (IgA, κ) and UPC10 (IgG2a, κ) myeloma proteins were kindly given to us by Pr Cazenave. Both immunoglobulins bind β2–6 polyfructosans. IDAs[2] are monoclonal antibodies produced by hybridomas derived from a BALB/c mouse immunized with ABPC48 (5). They are all IgG_1, κ, with the exception of IDA23 (IgG2a, κ). These monoclonal antibodies were purified on a Protein A Sepharose column (7). Fab fragment of IDA10 was prepared as described by Eisen et al. (8).

ELISA Reagents and Buffers. β-Galactosidase was a generous gift from Dr. Nicole Guiso (Institut Pasteur, Paris). Monoclonal antibodies and sheep anti-mouse immunoglobulins were coupled to the enzyme by ourselves according to Avrameas et al. (9). ELISA buffer was the same as described previously (5) except for the coating of microtiter plates (Nunc immunoplates I Nunc, Denmark): phosphate buffer 50 mM, pH 8. The *p*-nitrophenyl release was measured at 405 nm after 18 h incubation at 37°C in a Titertek multiskan (Flow).

Levan Binding Assay. The capacity of sera or monoclonal antibodies to bind to bacterial levan was studied as follows: microtiter plates were coated with bacterial levan (mol wt 2×10^5, 10 μg/ml) for 18 h at 4°C. Plates were washed and then incubated for 4 h at 4°C with various dilutions of sera or monoclonal antibodies, washed three times and then incubated for 3 h with β-galactosidase labeled-sheep anti-mouse Ig antibodies (20 ng per assay). After three washings, the plates were incubated for 18 h at 37°C with *p*-nitrophenylgalactopyranoside.

Idiotype Binding Assay. Microtiter plates were coated with purified monoclonal antiidiotypic antibodies (1 or 2 μg/ml) overnight at 4°C. Plates were washed and sera or monoclonal antibodies were then incubated (4 h, 4°C); after three washings, plates were incubated for 3 h with β-galactosidase labeled monoclonal antiidiotypic antibodies (5 ng per assay). Finally, plates were incubated for 18 h at 37°C with *p*-nitrophenylgalactopyranoside. By such sandwich assays, we are able to detect the presence of one (homologous sandwich) or two (heterologous sandwich with two different antiidiotypic antibodies) idiotopes. Results are presented in micrograms equivalent of ABPC48 protein per milliliter. This calculation was made from a standard binding curve obtained with ABPC48 and done in each experiment to control fluctuations from one experiment to another.

Inhibition of Binding Assays. Inhibition of levan and idiotype binding assays was performed with a preincubation of sera or monoclonal antibodies with the inhibitor (levan, monoclonal antiidiotypic antibodies . . .) either 1 h at room temperature or overnight at 4°C before incubation in ELISA plates.

Results

Quantitative Idiotype Binding Assays with Monoclonal Antibodies. The presence of a given idiotope may be detected and quantified by a sandwich binding assay involving a monoclonal antiidiotypic antibody adsorbed on a plastic plate and the same antiidiotypic antibody conjugated to an enzyme. Fig. 1 *a* shows the binding of ABPC48 myeloma protein to different monoclonal antiidiotypic antibodies in such a sandwich assay. In a given range of concentration of idiotype, this assay is quantitative (for example, up to 500 ng/ml for IDA23 or 1 μg/ml for IDA16 binding assay). The slopes reflect the different affinities of IDAs antibodies for ABPC48. These results are in good agreement with the characteristics of IDAs already described (5). Also represented on Fig. 1 *a* the binding of ABPC48 antibody to levan, as measured in an ELISA assay with levan adsorbed

[2] *Abbreviations used in this paper:* ELISA, Enzyme-linked immunoassay; βGal, β-galactosidase; Id, idiotope; IDAs, monoclonal antiidiotypic anti ABPC48 antibodies; Ig, immunoglobulin.

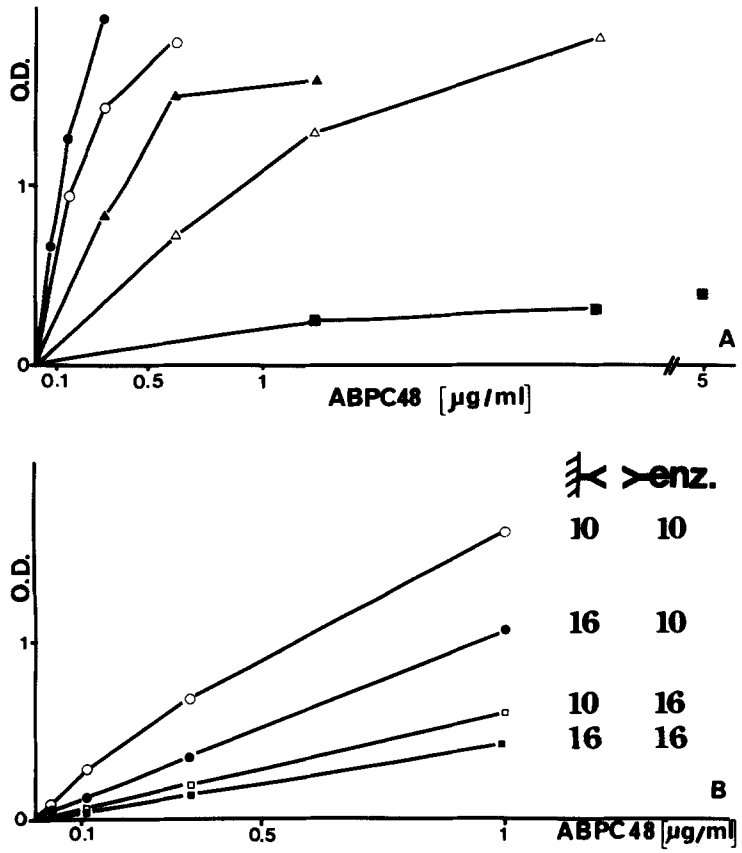

FIGURE 1. Binding assays for ABPC48 idiotype and anti-levan activity. (*a*) The idiotypic pattern of ABPC48 is revealed by sandwich assays using homologous monoclonal antibody adsorbed on microtiter plates (1 μg/ml) and the same monoclonal antibody conjugated to β-galactosidase (5 ng conjugate per assay) respectively IDA10 (○), IDA16 (△), IDA17 (●), and IDA23 (▲). For anti-levan activity (■) microtiter plates are coated with 50 μl of bacterial levan (mol wt 2×10^5; 10 μg/ml). The binding of ABPC48 is revealed by sheep anti-mouse immunoglobulin antibodies conjugated to β-galactosidase (20 ng per assay). (*b*) The idiotypic pattern of ABPC48 is analyzed by a heterologous sandwich assay. Adsorbed antibodies are IDA10 (open symbols) or IDA16 (closed symbols). β-galactosidase conjugates are IDA10 (circles) or IDA16 (squares).

on microtiter plates. The low values obtained may reflect the low affinity of ABPC48 for levan but also the isotype specificity of the β-galactosidase labeled sheep anti-mouse Ig antibodies. The binding of ABPC48 to levan is used in this study as a standard binding for the anti-levan activity. Therefore, very high values in equivalent ABPC48 are obtained for anti-levan titer in sera. In the analysis reported below, we always studied sera at dilutions for which binding is proportional to the concentration of idiotype; several dilutions were assayed to control that this was indeed the case. In Fig. 1 *b*, homologous and heterologous binding assays with IDA10 and IDA16 are represented. The specificity of heterologous assays is established by the fact that both monoclonal or polyclonal antibodies raised against either IDA10 or IDA16—two antibodies shown to present no cross reactivities (5, 10)—are not detected in such assays. Anti-IDA10

and anti-IDA16 antibodies, even when mixed together are not detected in heterologous assays, whereas they are revealed in homologous assays (results not shown). The two heterologous binding assays have intermediate relative sensitivities as compared to the homologous ones. So, they allow us to prove the existence of two different idiotopes on the same molecule.

UPC10 as ABPC48 also binds to β2–6 fructosan linkages. In a previous study, we have shown that UPC10 shares some A48 idiotopes (5). The data depicted in Fig. 2 indicate that two monoclonal antiidiotypic antibodies (i.e. IDA10 and IDA16) recognize A48 idiotopes expressed on UPC10. In contrast, IDA17 and IDA23 monoclonal antibodies do not bind to UPC10. Sandwich binding assays for UPC10 performed with IDA10 and IDA16 are respectively 15 and 5 times less sensitive than with ABPC48. This is a confirmation of a weaker interaction between UPC10 and these two monoclonal antibodies (5). These data are in agreement with the previous results which showed that UPC10 was a poor inhibitor of the binding of ABPC48 to IDAs antibodies (50 μg/ml vs. 50 ng/ml). So it must be stressed that the sandwich binding assays used for the analysis of unknown heterogenous populations of antibodies allow detection of idiotypic determinants related to those of ABPC48 even when the interactions are weaker. By classical inhibition binding assays, such structures could have been missed.

Analysis of Anti-levan Titer and Idiotype Expression in BALB/c Mice. Bacterial levan is an environmental antigen that stimulates continuously the specific clones as assessed by significant hemagglutination titers in the sera of animals of various species (mouse, horse, guinea pig, etc.) that have not been deliberately immunized. Therefore, we have studied the age-dependent variation of the concentration of anti-levan antibodies and A48 idiotopes in BALB/c sera. The results are depicted in Fig. 3 for sera of groups of BALB/c mice bled at different ages before any intentional immunization. The anti-levan titer increases with the age

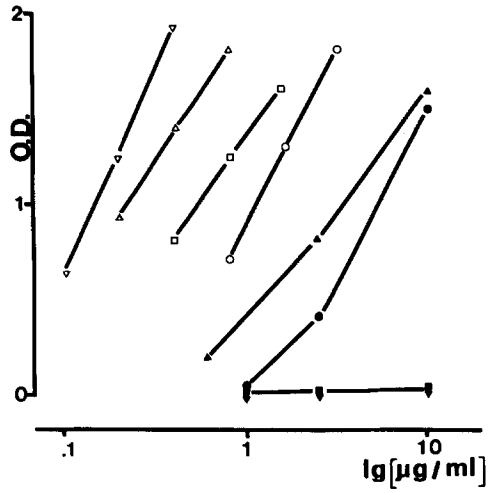

FIGURE 2. Relative sensitivity of several idiotypic binding assays. Idiotypic pattern of ABPC48 (open symbols) or UPC10 (closed symbols) myeloma proteins are analyzed with several monoclonal antibodies: IDA10 (△); IDA16 (○); IDA17 (▽); and IDA23 (□).

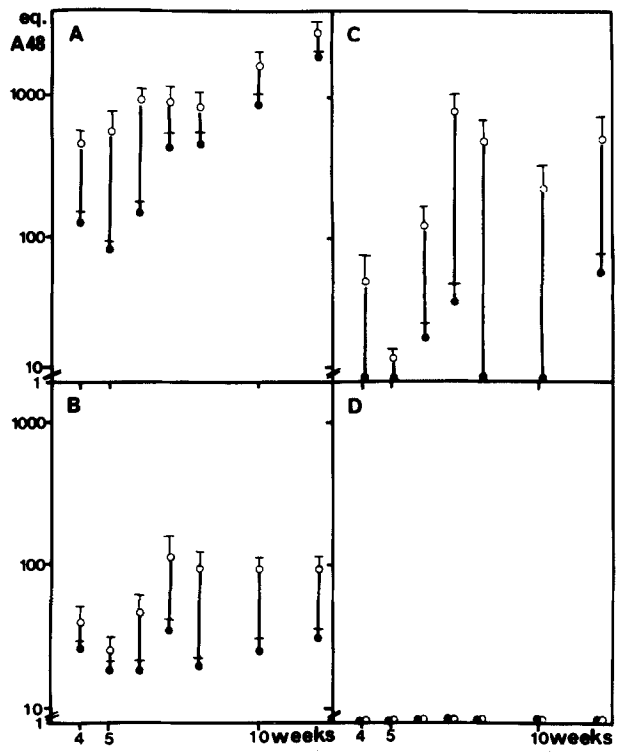

FIGURE 3. Idiotypic expression and anti-levan activity in sera of BALB/c mice at different ages. Mice were bled at the indicated age, injected with 50 μg levan (mol wt 2×10^7) and bled again 7 d later. Individual analysis was made for the anti-levan activity (*A*) and the presence of idiotypic determinants: Id10 (*B*), Id16 (*C*), and Id17 (*D*). The equivalent amount of ABPC48 is calculated from a standard binding assay of ABPC48 protein. Geometric means of values and standard deviation for preimmune (●) and immune (○) sera are represented. Four to six animals were analyzed at each age. The black thick stroke shows the difference between preimmune and immune sera. Values located at zero level are background.

of the mice (panel *A*). Id10 is detected at any age and this expression does not increase with age (panel *B*). Id16 is detected for some mice and not for others (panel *C*) and Id17 is undetectable (panel *D*). The limit of the detection for the different assays was ~1–5 μg equivalent ABPC48 per milliliter of serum (sera were diluted at least 50 times to avoid any background effect by nonspecific adsorption on plastic plates). After bleeding, mice were injected with bacterial levan and bled again 7 d later. These results are represented in Fig. 3. The anti-levan titer, the expression of Id10 and Id16 are increased; Id17 level is still background. The magnitude of the response is different from one mouse to another (see individual results for several mice in Table II) and the expression of Id16 is particularly enhanced (10 to 100 times).

At this point, it appears clearly that Id10 and Id16 expression is correlated with the anti-levan response, whereas Id17 expression could not be induced at a detectable level by this antigen. Lieberman et al. had shown (3) that the immunization with levan elicits two kinds of antibodies: a first set is specific for β2–1 fructosan linkages which cross-reacts with β2–6 fructosan linkages and the second

one binds specifically β2–6 polyfructosans (ABPC48 binds β2–6 polyfructosans). We measured the inhibition of the levan binding assay by several polyfructosan molecules (Table I). Inulin inhibits only partially the levan binding activity of preimmune and immune sera, plant levan (β2–6 polyfructosan) does inhibit nearly complete anti-levan activity, whereas bacterial levan (mol wt 5×10^4, carrying both antigenic determinants of β2–1 and β2–6 polyfructosans) completely inhibits this binding. We concluded from that study that the majority of the anti-levan antibodies detected have a β2–6 polyfructosan binding site. Inhibition of binding to idiotype by bacterial levan is also represented in Table I. This inhibition is complete, either for preimmune or immune sera analysis except for one mouse (group 8 wk, number 1) that presents a weak percentage of $Id10^+$ immunoglobulins without detectable anti-levan activity. After immunization with levan, this mouse increases considerably its levan-inhibitable Id10 expression but still keeps the same expression of non-levan-inhibitable Id10 molecules. With this exception and from these inhibition experiments we conclude that nearly every $Id10^+$ and $Id16^+$ molecules that are present in these preimmune and immune sera have a levan binding site.

Molecular Linkage Between Id10 and Id16. To establish a possible distribution of Id10 and Id16 idiotopes on the same molecule ($Id10^+$-$Id16^+$ molecule), we used the heterologous binding assays depicted in Fig. 1*b*. Only molecules that interact with both IDA10 and IDA16 are revealed by these assays. The results obtained with several sera are presented in Table II. Values are expressed in micrograms equivalent of ABPC48 per milliliter. Immunoglobulins with both

TABLE I
Inhibition of Binding Assays with Polyfructosan

Mice	Inhibition (%) of levan binding assay by:						Inhibition (%) of idiotype binding assay	
	Inulin (μM)		Plant levan (μM)		Bacterial levan (μM)		Id 10	Id 16
	1.5	30	6×10^{-2}	6	2×10^{-3}	0.2		
4 wk no. 2								
p.s.	18	31	63	82	88	100	100	100
i.s.	15	23	43	86	79	100	100	100
6 wk no. 3								
p.s.	13	33	43	100	77	100	100	100
i.s.	3	16	60	98	80	100	100	100
8 wk no. 1								
p.s.	12	12	50	100	73	100	62(2.4)*	100
i.s.	18	28	56	100	100	100	98(2.8)	100
12 wk no. 2								
p.s.	9	27	53	88	81	100	100	100
i.s.	17	37	27	85	82	100	98(2.6)	100

The inhibition of levan binding assay is performed with inulin (β2–1; mol wt 2,500), plant levan (β2–6; mol wt 7,500) and bacterial levan (β2–1 and β2–6; mol wt 5×10^4). The inhibition of idiotype binding assay is performed with bacterial levan (mol wt 5×10^4; 0.2 μM).

* Levan noninhibitable Id10 molecules (μg/ml). *p.s.*, preimmune serum; *i.s.*, immune serum 7 d after injection of 50 μg bacterial levan (mol wt 2×10^7).

TABLE II

Binding Assays for Id10$^+$–Id16$^+$ Molecules

Mice	Before immunization				After immunization (7 d)			
	*IDA10/IDA10	IDA16/IDA10	IDA10/IDA16	IDA16/IDA16	IDA10/IDA10	IDA16/IDA10	IDA10/IDA16	IDA16/IDA16
4 wk								
no. 1	6.8	7.1	0	0	11	13.1	6.4	5
6 wk								
no. 1	7.5	11.7	7.2	6	35	36	22.4	27
no. 2	17.5	7.8	0	0	91	21	1.6	0
no. 3	7.3	4.7	4	1	80	91	57.6	65
7 wk								
no. 1	23	18	19.2	19	175	250	130	148
8 wk								
no. 1	4.3	7.9	3.2	3	150	200	375	450
10 wk								
no. 1	4.2	4	2.8	1	75	74	12	10
no. 2	7.3	3.7	0	0	79	43	2	1
12 wk								
no. 1	2.6	2.5	0	0	8.8	9	4.4	11.5
no. 2	35	28	28.4	25	93	120	60.8	83

Homologous and heterologous binding assays with IDA10 and IDA16 are represented (for details, see Fig. 1 *b*). Values are given in equivalent micrograms of ABPC48 per milliliter, calculated from a standard binding assay with ABPC48 (Fig. 1 *b*). Immune sera were obtained 7 d after injection of 50 μg bacterial levan (mol wt 2×10^7).

* Monoclonal antibodies $\frac{\text{adsorbed on plastic}}{\text{conjugated to } \beta\text{gal}}$.

idiotopes are present in most analyzed sera. The heterologous binding assay with IDA10 conjugated to β-galactosidase allows detection of molecules expressing Id16 that are not detected in the homologous Id16 binding assay, as expected from the better sensitivity of IDA10-βgal than IDA16-βgal binding assays (see Fig. 1 *b*). These experiments also give information on qualitative aspects of idiotype expression. Since the results are given in micrograms of ABPC48 protein equivalent, sera that give about the same results in the four assays contain mostly Id10$^+$-Id16$^+$ molecules similar to ABPC48 (for example, mice No. 1 from 6-, 7-, and 8-wk groups). On the contrary, one detects in several sera large amounts of Id10 and very low amounts of Id16 (for example, mice No. 2 and 6- and 10-wk groups). In these sera, molecules with the Id10 idiotope present structural differences with ABPC48 that do not allow the binding of IDA16 with a good affinity, even if determinants related to the Id16 idiotope are actually present on these molecules, as is proven by the positive results with the more sensitive heterologous assay.

The molecular linkage between the Id10 and Id16 idiotopes was confirmed in binding inhibition assays. IDA16 binding molecules were no longer detected in dilutions of sera preincubated with IDA10 protein (10 μg/ml) and then tested in the IDA16-βgal homologous binding assay. In the reverse test, IDA16 (10 μg/ml) is a poor inhibitor (10–30%) of the binding of molecules detected in the

IDA10-βgal homologous binding assay, when the mice have few molecules detectable by IDA-16βgal homologous binding assay (results not shown). For these mice, the results with the heterologous binding assays (Table II) prove that the detected molecules have a very low affinity for IDA16. So it is difficult to conclude that $Id10^+$ molecules without Id16 idiotope are present in the analyzed sera.

Long-lasting Idiotype Expression. The level of the natural expression of $Id10^+$-$Id16^+$ molecules detected in nonimmunized mice does not quantitatively correlate with the natural anti-levan titer (Fig. 3) although these molecules have a levan binding site and therefore contribute to the anti-levan titer. To obtain a more precise estimation of their induction after stimulation with levan, we performed a kinetic study. Results are presented on Fig. 4. The enrichment in $Id10^+$-$Id16^+$ molecules is indeed substantial: following a peak at day 6 (or slightly earlier) a drop is observed but these molecules remain during several weeks at 10–20 times above the level detected before immunization. Anti-levan activity is still high during that time; it then decreases markedly but remains noticeable for over 8 wk. To appreciate the contribution of $Id10^+$-$Id16^+$ molecules to the anti-levan titer, we attempted to inhibit the levan binding assay by preincubating

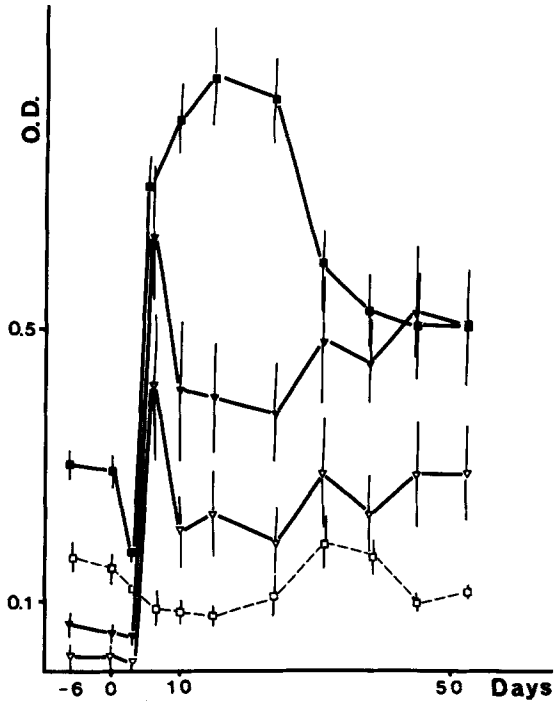

FIGURE 4. Kinetics of anti-levan and idiotypic response. 12 wk old mice were bled twice before the injection of levan (50 μg intraperitoneally; mol wt 2×10^7) and then bled again at different times. Antilevan (■), Id10 (▼), Id16 (▽), and Id17 (□) expression were measured in individual sera. Geometric means and standard deviation are represented. In this experiment, ABPC48 protein gives 0.1 unity of optical density at the following concentrations: 700, 65, 200, and 20 ng/ml for respectively antilevan, Id10, Id16, and Id17 binding assays. Sera were diluted 500 times excepted for Id17 assay (100 times).

dilutions of sera with a known concentration of Fab IDA10 (it is not possible to use IDA10 complete protein for inhibition since we use an antiimmunoglobulin conjugate to reveal anti-levan activity). We first determined that the anti-levan activity of ABPC48 is completely inhibited at a concentration of 0.250 μg/ml of Fab IDA10. We then used a 20 times greater concentration of Fab IDA10 for inhibition experiments with sera. Very few sera presented a significant inhibition of anti-levan activity (results not shown). Thus, $Id10^+$ molecules represent a minor component of the anti-levan response of BALB/c mice or, alternatively, IDA10 is a poor competitor with the levan binding site of these molecules.

On Fig. 4 we also represented the expression of the Id17 idiotope. For this assay, sera were diluted 100-fold, whereas for the Id10, Id16 and anti-levan assays they were diluted 500-fold. Natural expression of Id17 is detectable for this group of mice and corresponds to $\sim$2 μg equivalent ABPC48 per milliliter (to be compared to 20 μg/ml for Id10 and Id16). Id17 level was found constant at the different bleedings made during the anti-levan immunization.

Discussion

Most of the work published on idiotypic expression deals with idiotypic induction after immunization (2). Preimmune sera have rarely been analyzed except as negative controls. The absence of sensitive and specific assays imposed as a rule to consider preimmune sera activities as background. However, several attempts (11–14) have been made to define natural idiotypic activities by means of polyclonal antiidiotypic antibodies. In such systems, the immunoglobulins that are detected may correspond to various structures. The quantitative sandwich binding assay carried out with monoclonal antibodies allows the detection of low concentrations of idiotypic determinants. Moreover, sandwich assays and inhibition binding assays do not reveal the same set of molecules: the latter detect only molecules that, in terms of location of their binding site and in terms of their affinity, are competitors in the idiotype-antiidiotype interaction (in particular molecules homologous to either idiotype or antiidiotype). In contrast, sandwich binding assays allow detection of any immunoglobulin that binds specifically to the monoclonal antiidiotypic antibodies. A binding assay was already used for the detection of idiotypes by Reth et al. (15) but this assay was restricted to antibodies constituted with a λ_1 chain as it is the case for antiNP antibodies. The assay described here allows, in principle, the detection of antibody of any antigenic specificity. Sandwich binding assays have been already described for the study of allotypic specificities (16).

The analysis of preimmune sera from mice at different ages for the presence of ABPC48 cross reactive idiotopes leads to the remarkable conclusion that Id10 determinants are detected in all sera. By binding inhibition assays and by heterologous sandwich binding assays it is also shown that at least a fraction of the immunoglobulins with the Id10 idiotope also possess the Id16 idiotope. All $Id10^+$-$Id16^+$ molecules have a levan binding site as shown by binding inhibition assay with bacterial levan. By the homologous sandwich assay that detects Id16 idiotope, it is not possible to detect Id16 in all sera. The limitation, however, could be the sensitivity of this assay.

The anti-levan activity is measured by a binding assay to bacterial levan, a

polyfructosan with both β2–1 and β2–6 determinants. In preimmune sera, the anti-levan titer increases with the age of the mice. The binding inhibition assay with different polyfructosans shows that, whatever the age of the mice, β2–6 polyfructosan (i.e. plant levan) is far more inhibitory than β2–1 polyfructosan (i.e. inulin). Oligofructosans are not available but the molecular weights of plant levan (7,500) and insulin (2,500) are comparable enough to allow to consider that the major component of the natural anti-levan activity is directed against β2–6 polyfructosans. Actually, the weak inhibitory power of inulin can be interpreted with the existence of antibodies able to bind with different affinities, the two kinds of polyfructosans, as proposed by Lieberman et al. (3). Levan injection leads to an increase of anti-levan titer correlated with an increase of $Id10^+$-$Id16^+$ immunoglobulin expression. When mice present a low natural expression of Id17, this remains constant after immunization. Work is in progress to characterize Ig with Id17 idiotope. The magnitude of the $Id10^+$-$Id16^+$ response is variable but does not depend on the age of the mice. The fast increase of $Id10^+$-$Id16^+$ molecules (peak at 5–7 d) suggests that preactivated clones are expanded. The same analysis as for preimmune sera shows that Id16 determinants are found on $Id10^+$ immunoglobulins that are anti-levan antibodies. Kinetics analysis shows that Id10 and Id16 increased expression remains stable for at least 7 wk and parallels the anti-levan titer enhancement. In conclusion, anti-levan antibodies with two ABPC48 idiotopes are found in normal sera at constant levels. Their natural level does not increase with the age of the mice as it is observed for the natural anti-levan titer but they contribute, as minor components, to the immune response to bacterial levan. The presence of $Id10^+$-$Id16^+$ molecules with anti-levan activity may be the manifestation of early immunization with environmental levan. If so, steady level of these idiotypes during the aging of the mice requires the setting of a control mechanism. One possibility might be the production of autoantiidiotypic antibodies as it has been proposed for the anti-inulin response (17).

ABPC48 idiotype was not found in the anti-levan response of normal BALB/c mice analyzed by Lieberman et al. (3). Several hypotheses may be proposed to explain this discrepancy. First, the origin of levan molecules is not the same (*B. subtilis* in this work and *Aerobacter levanicum* for Lieberman's work). Secondly, and more important are the differences in the antiidiotype probes and assays that were used: inhibition of hemagglutination with polyclonal antibodies as used by Lieberman et al. only permits the detection of antibodies that compete for the binding between the idiotype (here ABPC48) and the more representative (and with greater affinity) antiidiotypic antibodies raised against it; in contrast, we used monoclonal syngeneic antiidiotypic antibodies in sandwich binding assays.

The demonstration that ABPC48 idiotopes are present in preimmune sera has some bearing on the interpretation of recent results obtained by Bona et al. (18). These authors propose that physiologically relevant idiotypic interactions could be mainly a two partners (+, −) regulatory system where antiepitope elicits antiidiotope antibodies and antiidiotope antibody elicits idiotope-bearing molecules. An important statement in this model is that idiotope-positive molecules with an antigen binding site (i.e. levan) may be induced by the manipulation with

antiidiotypic antibodies, and it is further suggested that this reflects the activation of silent clones (19, 20); such manipulations may indeed facilitate the emergence of ABPC48 idiotype in the anti-levan response; however, since several ABPC48 idiotopes are already present in normal sera, it is difficult to decide whether idiotypic induction follows the recruitment of silent clones rather than the expansion of preactivated ones.

By immunization with monoclonal antiABPC48 antiidiotypic antibodies (IDA), we obtained polyclonal and monoclonal antibodies, some of them with a definite albeit very low affinity for levan (21, 22). Even in this case, we cannot exclude the fusion event with a B cell derived from a clone activated before the immunization with the monoclonal IDA. However, a fine analysis of the kinetics of response to different monoclonal IDAs shows a transient increase of the expression of $Id10^+$-$Id16^+$ molecules with a levan binding site (22). This indicates that the injection of an antiidiotypic antibody permits expansion of activated clones that express idiotype-positive antigen binding immunoglobulins: such cells are likely also to act as partners in cell fusions.

Our results clearly show a discrepancy between a natural idiotypic expression—which remains constant—and the natural titer against a bacterial antigen—which rises during life time. Further experiments are needed to discover the controls that govern ABPC48 cross-reactive idiotype expression and limit the expansion of relevant B cell clones.

Summary

Using monoclonal antiidiotypic antibodies, we developed a sensitive binding assay that detects molecules with one or with two idiotopes of the ABPC48 idiotype. ABPC48 cross-reactive idiotypes were thus shown to be present in substantial amounts in sera of nonimmunized mice. Levan binding sites are found on these idiotypes. During the life time of the mice, the natural anti-levan titer increases while ABPC48 idiotypic expression remains constant, suggesting different controls for these two activities. On the other hand, ABPC48 cross-reactive idiotypes participate—as minor components—in the response that follows a deliberate immunization with bacterial levan. This induction process is likely to reflect the selection of idiotopes expressed by the B cell clones preactivated in sera of nonimmunized mice rather than the activation of silent clones. We suggest that a similar situation might explain the reported emergence of ABPC48 idiotypes in animals primed with antiidiotypic antibodies and subsequently stimulated with levan.

We thank Drs. P. A. Cazenave, A. Coutinho, and C. Transy for helpful discussions. This work was supported in part by a grant of the Ligue Nationale Française contre le Cancer and the Université Pierre et Marie Curie.

Received for publication 28 March 1983.

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