

THE RELATIONSHIP BETWEEN VARIABLE REGION  
DETERMINANTS AND ANTIGEN SPECIFICITY ON MITOGEN  
REACTIVE B CELL SUBSETS\*

BY DANIELE PRIMI, FATHIA MAMI, CHRISTIAN LE GUERN AND  
PIERRE-ANDRÉ CAZENAVE

*From the Unité d'Immunochimie Analytique, Institut Pasteur, 75015 Paris, France*

Recently we determined the absolute frequencies of lipopolysaccharide (LPS) and *Nocardia*-delipidated cell mitogen (NDCM) -sensitive B lymphocytes from BALB/c mice secreting immunoglobulin (Ig) molecules bearing any one of three different idiotopes originally found on a monoclonal anti- $\beta$ -galactosidase antibody (1). These three idiotopes, 66, 137 and 395, are defined by syngeneic monoclonal antibodies against the monoclonal Ig 174 produced by a BALB/c mouse (C. Le Guern, E. Barbier, and D. Juy. Idiotypic heterogeneity of monoclonal anti- $\beta$ -galactosidase antibodies. Manuscript in preparation). Extensive studies by conventional immunization clearly demonstrated that these three determinants are not normally expressed at detectable level when BALB/c mice are immunized with  $\beta$ -galactosidase and, therefore, can be classified as nonrecurrent idiotopes (Le Guern et al., see above). Our results, however, indicate that these specificities are not only part of the idiotypic repertoire of LPS- and NDCM-sensitive BALB/c B cells, but that they can be induced to expression with a frequency similar to that of a recurrent idiope, i.e., M-460 (1, 2). Because the frequency of a given specificity (either antibody or idiotypic specificity) determined by mitogenic stimulation does not require the presence of antigen, this analysis was not dependent on a particular protocol of immunization, but rather reflected the absolute frequencies of competent B cells in a steady state. Thus the discrepancy found between antigenic and polyclonal activation with regard to idiope(s) expression may very well reflect independent expression of these V region markers and antigen-binding sites. The existence of defined idiotopes on immunoglobulins without known antigen specificity has been fully documented for some considerable time (3-6) but, up until now, it has been difficult to establish the possible existence of well-defined rules that govern the relationship between idiotopes and antigen-binding sites. Such studies have been hampered by the difficulty of selecting B cell clones or hybridomas that are positive or negative for any possible combination of a number of well-defined sets of V regions. Here, however, we were able to address this problem, because we knew the absolute frequencies of each of our three idiotopes and therefore could select and study individual clones of mitogen-reactive B cells positive for any possible combination of these determinants and correlate them to anti- $\beta$ -galactosidase activity. The results presented here indicate

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that independence exists between the expression of each of the three V region determinants studied and the antigen-binding site in the pool of LPS- and NDCM-sensitive B cells. The exceptions to this are those clones simultaneously positive for the three idiotopes, which all displayed antigen-binding activity.

### Materials and Methods

*Animals.* BALB/c mice were obtained from the Institut Pasteur (Unité de Génétique des Mammifères).

*Mitogens and Antigen.* LPS from *S. typhimurium* was obtained from Difco Laboratories (Detroit, MI). NDCM was a kind gift from Dr. J.-F. Petit and Dr. R. Ciorbaru.  $\beta$ -galactosidase from *Escherichia coli* was obtained from Sigma Chemical Co. (St. Louis, MO).

*Culture Conditions.* Spleen cell suspensions were cultured in RPMI 1640 supplemented with glutamine, antibiotics, 10% fetal calf serum (FCS), and  $2 \times 10^{-5}$  M 2-mercaptoethanol in the presence of rat- or BALB/c-irradiated thymus filler cells at a concentration of  $3 \times 10^6$ /ml. Cultures were continued to days 10–14, and the supernatants were assayed for the presence of idiotopes and  $\beta$ -galactosidase activity.

*Antibody Preparation.* The anti- $\beta$ -galactosidase 174 monoclonal antibody was obtained by the fusion of BALB/c anti- $\beta$ -galactosidase spleen cells and Sp2/0. 137-, 66-, and 395-anti-174 monoclonal antibodies were obtained as described (Le Guern et al., manuscript in preparation). Briefly, BALB/c mice were immunized with 100  $\mu$ g of 174-protein copolymerized with keyhole limpet hemocyanin (KLH) in complete Freund's adjuvant. This was followed 15 d later by a second injection of the same dose of antigen in complete Freund's adjuvant and by one injection of copolymer in saline. Spleen cells from these animals were then fused with Sp2/0, and anti-174 hybrids were screened and cloned. The products of three of these clones (137, 66, and 395) were purified by absorption on a Sepharose cyanogen bromide (CNBr) 174 column followed by elution with a glycine-HCL buffer (0.2 M, pH 2.2).

*Idiotope Determination.* Sheep erythrocytes (SRBC) were coupled by the CrC13 method with  $\beta$ -galactosidase or 137-, 66-, and 395-purified antibodies and mixed in V-shaped microtiter plates with culture supernatant at a final concentration of 0, 5%. After 2 h incubation at room temperature, the number of positive (agglutinated) wells was recorded. The hemagglutination (HA) assay for idiotopes and antigen specificity detection was the method of choice, as we previously demonstrated (1) that this assay can detect at least 0.1 ng of ligand. The specificity of the assay has been previously described (1).

### Results and Discussion

For the present study it was essential to examine a large number of cultures, each containing less than one competent B cell clone positive for each of the three idiotopes (Id-137, Id-66, and Id-395) present on the 174 molecule or with anti- $\beta$ -galactosidase activity. Accordingly, we took advantage of our previous studies (1) in which we determined the absolute frequencies of anti- $\beta$ -galactosidase, Id 137, Id 66, and Id 395 positive clones of BALB/c B lymphocytes activated either by LPS or NDCM. These frequencies are, respectively,  $1/5$ ,  $8 \times 10^4$ ,  $1/6$ ,  $2 \times 10^4$ ,  $>1/6 \times 10^5$ , and  $>1/5 \times 10^5$  for LPS and  $1/6 \times 10^4$ ,  $1/4$ ,  $8 \times 10^4$ ,  $1/6$ ,  $2 \times 10^4$ , and  $>1/5 \times 10^5$  for NDCM-sensitive cells. On the basis of these results, and to have less than one Id-positive clone per well, we decided to set up two groups of 864 cultures each containing  $2 \times 10^4$  B lymphocytes. One group received 50  $\mu$ g/ml of LPS and the other 50  $\mu$ g/ml of NDCM. After 12 d, the supernatant of each culture was individually tested for HA activity with SRBC coupled with 66, 137, 394 antibody or  $\beta$ -galactosidase. The results of these studies are shown in Table I.

It is immediately evident that in both groups a large number of cultures produced anti- $\beta$ -galactosidase antibodies which did not bear any of the three idiotopes under

TABLE I  
*HA Activity of Supernatants of LPS- and NDCM-activated Cultures*

$\beta$ -gal* SRBC	Agglutinated erythrocytes			Numbers of positive cultures activated with	
	137-SRBC	395-SRBC	66-SRBC	LPS	NDCM
+	+	+	+	23	26
+	+	-	-	40	10
+	-	+	-	4	3
+	-	-	+	3	4
+	+	+	-	3	1
+	+	-	+	0	5
+	-	+	+	1	3
+	-	-	-	119	139
-	-	-	-	547	544
-	+	-	-	97	80
-	-	+	-	9	12
-	-	-	+	10	21
-	+	+	-	3	4
-	+	-	+	2	9
-	-	+	+	3	1
-	+	+	+	0	2

Cultures (864/group) contained  $2 \times 10^4$  anti-Thy-1.2 and complement-treated BALB/c splenocytes and 50  $\mu$ g/ml of LPS or NDCM. Supernatants were tested for HA activity on day 12.  
 \*  $\beta$ -galactosidase.

study. These results are therefore in agreement with the observation that these determinants are not detected during the anti- $\beta$ -galactosidase antibody response of BALB/c mice. Among those clones positive only for one idiotype determinant and without antigen activity, we found that both with LPS and NDCM the 137 idiotype-positive cultures were by far the most represented. This observation, compatible with our previous frequency determinations, may very well indicate that, although this specificity is not detected in BALB/c anti- $\beta$ -galactosidase antibodies response, it may also be expressed in a recurrent fashion on BALB/c antibodies specific for an unknown antigen. Thus it should be stressed that the classical definition of private idiotypes only concerns the subset of antigen specific B cells and can by no means be extrapolated to the whole B cell repertoire of a given animal. Fig. 1 summarizes the percentage of anti- $\beta$ -galactosidase clones among those cultures positive for one, two, or all three idiotypic determinants. In both groups it is evident that only a minority of those clones positive for just one idiotype had activity against the antigen. These data indicate the existence of independence of idiotope expression and antibody specificity for  $\beta$ -galactosidase in our system and should therefore caution us against considering idiotopes as clonal markers. These findings may very well be related to the assay used in this study. It should be borne in mind that idiotopes are usually studied by site specific assay and therefore these studies can only be of value for a limited number of clones. Our studies are based on both nonspecific activation and a nonspecific antigen-binding assay and are therefore more valuable with regard to the total repertoire of a determinate animal. Thus, the association of a determinate idiotope with a defined binding site may only be fortuitous; indeed, these determinants may normally be associated preferentially with unknown antibody specificities.

Similar results were obtained with culture supernatants positive for any combination of two idiotopes. The existence of clones positive for two idiotopes only, however, should be treated cautiously, as we cannot exclude the possibility that these wells

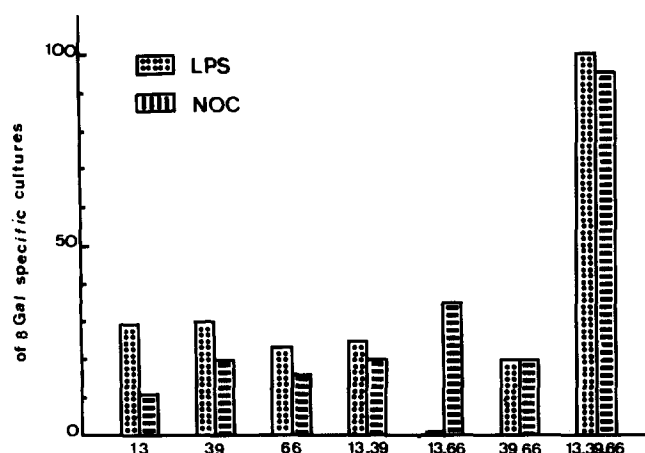


FIG. 1. Percentage of anti- $\beta$ -galactosidase clones among those cultures positive for one, two, or three idiotypic determinants.

contained two clones, each positive for one idiotope, owing to the small number of these cultures observed. Indeed the expected frequency for the presence of two clones in the same well was never significantly different at the  $\chi^2$  test than the frequency of observed cultures positive for two idiotopes.

The most striking result obtained, however, was the strong correlation between the anti- $\beta$ -galactosidase activity and the simultaneous presence of the three idiotopes observed in almost all the cultures. Because these findings cannot be statistically accounted for by the presence of three clones, each positive for a single idiotope in a single well ( $P < 0.01$ ), this observation can be interpreted in one of two ways; i.e., either these clones are all identical to the original, 174, or the spatial conformation of the three idiotopes suffices to define the antigen-binding site. Although not mutually exclusive, these two hypotheses are different, inasmuch as the first implies that all the clones positive for the three idiotopes are identical, whereas the second allows the existence of different clonotypes. At present we have no arguments to support either of these two hypotheses; however, both these models imply that the repertoire expressed after immunization by an antigen is not representative of the complete repertoire of BALB/c mice. The strict correlation between antigen specificity and simultaneous presence of three idiotypic determinants raises the question of the generality of the phenomenon, and we are currently studying, in the same system, the contribution to the antigen-binding site of a fourth idiotope recently identified on the 174 molecule, which is different from the other three. These studies might also allow us to discriminate between the two hypotheses formulated above.

The results presented here have some important implications for our understanding of certain immunological phenomena. We recently demonstrated that the idiotypic repertoires revealed by LPS and NDCM are not identical. In particular, we found that the frequency of Id-66-positive precursors was much higher in NDCM- than in LPS-activated cultures. The data presented here confirm and extend these observations. It is evident from the data in Table I that both the 66 and 13 idiotopes are more frequently associated to anti- $\beta$ -galactosidase antibody on the LPS-sensitive cells than on those responsive to NDCM. Although at the present time we do not have a

clear-cut explanation for these findings, our data clearly indicate that both quantitative and qualitative differences of idiotypic repertoire can not only be found between animals of the same species but also at the level of different B cell subsets of an individual animal.

For several years the existence of idiotype-positive immunoglobulins without detectable antibody function has excited considerable speculation (reviewed in 6). The antibody response (Ab3) of animals immunized with anti-idiotypic antibodies has particularly been studied (4, 6-14). These responses are generally characterized by antibodies idiotypically similar to Ab1 but they may not recognize antigen. Those molecules that do not possess the antigen-binding site have generally been termed Ab3, and the current interpretation is that they recognize V region determinants on Ab2 molecules. Although our data cannot exclude this interpretation, we would like to propose that the difference between Ab1 and Ab3 antibodies may simply reside in the number of common V region determinants they share, which, as shown here, may greatly influence the antigen-binding site. Thus, as recently pointed out (15), Ab3 molecules can be classified as Ab3 $\alpha$ , idiotope-induced antibody, and Ab3 $\beta$  paratope-induced molecules. The latter, however are not necessarily antigen specific.

Finally we would like to stress that, because our experiments were expressly designed to have less than one clone positive for each idiotope in each culture, we can formally exclude the presence of Ab3 $\alpha$  antibodies in those supernatants positive for two or three idiotopes. We believe, therefore, that our protocol could represent a useful tool for determining the way in which two immunoglobulins interact with one another.

### Summary

On the basis of previous frequency determinations we could set up large numbers of cultures, each containing less than one competent precursor B cell specific for  $\beta$ -galactosidase or for each of three idiotopes previously found on a monoclonal anti- $\beta$ -galactosidase antibody. Cultures were polyclonally activated by either lipopolysaccharide or *Nocardia*-delipidated cell mitogen. Each culture supernatant was individually tested for hemagglutination activity against sheep erythrocytes coupled with  $\beta$ -galactosidase or with each of the three purified monoclonal anti-idiotypic antibodies. The results showed that only a minority of those clones positive for only one or two idiotopes recognized antigen. However, all those clones simultaneously positive for the three V region determinants recognized  $\beta$ -galactosidase. The implications of these results for our understanding of the relationship between the antigen-binding site and idiotope expression are discussed.

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