Polyclonal B cell activators (PBA) are substances that directly activate B cells to clonal growth and antibody secretion by interacting with nonclonally distributed receptors on the surface of lymphocytes (1-5). The capability of B cells to respond to various PBA divides the B cell pool into different functional subsets that might or might not overlap with each other (6-9). Because polyclonal activation does not involve the variable region of immunoglobulin, the triggering is not immunologically specific, and, therefore, it has been proposed (6, 10) that the repertoire of antibody specificity is randomly distributed or even repeated among the “subsets” of B cell clones defined by reactivity to different mitogens. This proposal has been experimentally confirmed by observing increased antibody synthesis specific for conventional antigens or haptens induced by various PBA. These studies, however, did not take into account the extreme degeneracy of the immune system or, in other words, the great lack of precision in the fit between antibodies and antigens that can vary over a range of several orders of magnitude. Consequently, because of the extreme heterogeneity of the immune response, the question of whether different B cell subpopulations, as defined by mitogen reactivity, selectively express identical clonotypes has not been answered as yet. We decided to address ourselves to this question by comparing the expression of four idiotopes in spleen cells of BALB/c mice activated by two mitogens that have been described as acting on separate B cell subsets, i.e., lipopolysaccharide (LPS) and Nocardia delipidated cell mitogen (NDCM) (11).

The 66, 137, and 395 idiotopes are defined by BALB/c monoclonal antibodies against the monoclonal immunoglobulin (Ig) 174 to βgalactosidase produced by a BALB/c mouse. None of these three determinants are normally expressed when BALB/c mice are immunized with βgalactosidase, and therefore they can be classified as nonrecurrent idiotopes. Contrary to this, the M-460 idiotype (Id) specificity defined by the monoclonal anti-M460 antibody F6(51) is present on a portion of anti-trinitrophenyl (TNP) antibodies produced by BALB/c mice after immunization with thymus-dependent or thymus-independent TNP antigens (12).

We therefore determined the approximate frequencies of LPS- and NDCM-sensitive B lymphocytes secreting Ig molecules that bear the 66, 137, 395, and M-460 idiotopes.

1 Abbreviations used in this paper: HA, hemagglutination; LPS, lipopolysaccharide; NDCM, Nocardia delipidated cell mitogen; PBA, polyclonal B cell activator; PFC, plaque-forming cell; SRBC, sheep erythrocytes; TNP, trinitrophenyl.

2 Le Guern, C., E. Barbier, and D. Juy. Idiotypic heterogeneity of monoclonal anti-βgalactosidase antibodies. Manuscript submitted for publication.

3 Le Guern, C., B. Marianié, and E. Petit Koskas. Correlations between idiotypic and antibody specificities. Manuscript submitted for publication.
The repertoire can be defined in quantitative terms as the frequency of B cells that are precursors for clones secreting Ig with a given specificity. These frequencies are determined in vitro by analyses limiting the number of specific precursors in culture to one.

The results presented here (a) confirm our previous observation demonstrating that polyclonal activation results in the phenotypic expression of normally silent clones (13) and (b), more importantly, clearly indicate that the V gene repertoire is not randomly distributed on mitogen-reactive B cell subpopulations. The implications of these findings for our understanding of the mechanisms underlying the selection of the available idiotypic repertoire are discussed.

Materials and Methods

Animals. BALB/c mice were obtained from the Pasteur Institut Paris, France.

Mitogens and Antigen. LPS W from S. typhimurium was obtained from Difco Laboratories, Detroit, MI. NDCM was a kind gift from Dr. J.-F. Petit and Dr. R. Ciorbaru. βgalactosidase from Escherichia coli was obtained from Sigma Chemical Co., St Louis, MO.

Culture Conditions and Frequency Determinations. Frequency analysis was performed with LPS and NDCM as described by Andersson et al. (14) and Barnabé et al. (15). Spleen cell suspensions were cultured in RPMI 1640 supplemented with glutamin, antibiotics, 10% fetal calf serum, and 2 × 10⁻⁵ 2-mercaptoethanol in the presence of rat or BALB/c irradiated thymus filler cells at a concentration of 3 × 10⁶ cells/ml. At each cell dose, 96 replicate cultures were set up and assayed at day 5 for Ig-secreting plaque-forming cells (PFC) by using a staphylococcal protein A plaque assay (16). Alternatively, cultures were continued to days 10-14, and the supernatants were assayed for the presence of idiotopes. Rabbit anti-mouse class-specific Ig were a kind gift of Dr. A. Coutinho.

Idiotope Determinations. Sheep erythrocytes (SRBC) were coupled by the CrCl₃ method with βgalactosidase, 137, 66, 395, and F6(51) 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.

Antibody Preparation. M-460 myeloma protein was purified on a dinitrophenyl-Lys-Sepharose-CNBr-4B column as previously described (12). F6(51) anti-M-460 was obtained and purified as previously described (17).

The anti-βgalactosidase 174 monoclonal antibody was obtained by the fusion of BALB/c anti-βgalactosidase spleen cells and Sp2/O.3 137, 66, and 395 anti-174 monoclonal antibodies were obtained as described in footnote 2. Briefly, BALB/c mice were immunized with 100 μg of 17-4 protein copolymerized with keyole limpet hemocyanin in complete Freund's adjuvant. This was followed 15 d later by a second injection of the same dose of antigen in incomplete Freund's adjuvant and by one injection of copolymer in saline. Spleen cells from these animals were then fused with Sp2/0, and anti-17-4 hybrids were screened and cloned. The products of clones 137, 66, and 395 were purified by absorption on a Sepharose CNBr column followed by elution with a glycine-HCl buffer (0.2 M, pH 2.2).

Results

Sensitivity and Specificity of the Assay. Previous immunochemical studies on the fine specificity of 137, 66, and 395 antibodies clearly demonstrated that these proteins recognize different idiotopes situated in the vicinity of the βgalactosidase-binding site of the 174 Ig. To test the sensitivity and the specificity of our assay, we measured the capability of 174 and M-460 to agglutinate SRBC coupled with βgalactosidase or with the monoclonal antibodies F6(51), 66, 137, and 395, which are all γ1 κ proteins. As shown in Table I, 174 agglutinated 66, 395, 137, as well as βgalactosidase but not F6(51) SRBC. Contrary to this, M-460 displayed agglutinating activity only against
F6(51)-SRBC. These results therefore confirm the specificity of the interactions. The data in Table I also show that the hemagglutination (HA) assay could detect at least 0.1 ng of specific antibodies. Because under our same culture conditions it has already been demonstrated that a single B cell clone secretes 30 ng of A5A-positive Ig in the culture supernatants (18), we consider the sensitivity of the HA test more than adequate for our studies.

**Frequencies of LPS- and NDCM-reactive BALB/c Spleen Cells.** Because the aim of our studies was to compare the idiotypic repertoire induced by LPS with the one revealed by NDCM, we found it necessary to determine the total number of B cells reactive to the two mitogens. This frequency determination was done in vitro at cell concentrations limiting the numbers of clones producing Ig to around one per culture. According to Poisson's distribution, one B cell precursor for a clone of cells secreting molecules is present in that number of cells plated in individual cultures that let 63% of all cultures appear positive in the assay (19). First, we screened spleen cells of normal BALB/c mice cultured in the presence of either LPS or NDCM for the approximate frequency of total Ig PFC-secreting clones in twofold dilutions from 100 to 6 cells per culture. For these determinations, 48 cultures were tested at each cell concentration. The data shown in Fig. 1 shows the frequency of LPS- and NDCM-reactive B cells in a given spleen cell preparation. These frequencies are quite similar, although the number of NDCM B cell precursors seems slightly larger than the one of the LPS-sensitive B cells.

**Table 1**

**HA Titers of M-460 and 17-4 Monoclonal Antibodies**

| Monoclonal antibody | βgalactosidase-SRBC | F6(51) SRBC | 66-SRBC | 137-SRBC | 395-SRBC |
|---------------------|---------------------|-------------|---------|-----------|----------|
| M-460               | 0                   | 18          | 0       | 0         | 0        |
| 17-4                | 19                  | 0           | 17      | 18        | 17       |

25-μl of a solution of 100 μg/ml of purified monoclonal antibodies was serially diluted and tested for HA activity using SRBC coupled with the indicated ligands.

![Figure 1](image.png)

**Fig. 1.** The indicated numbers of spleen cells from normal BALB/c mice were cultured in the presence of either LPS (●) or NDCM (■) in groups of 48 replicates and assayed on day 5 for Ig-secreting cells with protein-A SRBC.
Frequencies of Idiotopes Producing B Cells. Having established the frequencies of B cells reactive to the two mitogens, we proceeded to determine the absolute numbers of NDCM- or LPS B-sensitive lymphocytes secreting Ig with βgalactosidase activity or positive for the idiotopes under study. For this, supernatants of each individual culture (96 cultures for each cell concentration) were tested for HA activity against SRBC substituted with the relevant ligand. The approximate numbers of anti-βgalactosidase B cell clones induced by the two mitogens is shown in Fig. 2. Thus, apparently, the number of antigen-specific B cell precursors is similar, if not identical, in the LPS- and NDCM-responsive B cell pool, confirming, therefore, the notion that the repertoire of antibody specificities seems to be repeated among the subsets of B cells.

![Graph showing frequencies of idiotopes producing B cells.](image)

Fig. 2. Spleen cells from BALB/c mice were treated with anti-Thy-1.2 and complement and cultured at the indicated concentrations either with LPS or NDCM. Results represent groups of 96 replicates assayed for the presence of anti-βgalactosidase antibodies or for the indicated idiotopes in the supernatants after 12 d.

| Mitogen | Ig-secreting clones/total B splenocytes | Anti-βgalactosidase clones | M460 Id-positive clones | 66 Id-positive clones | 137 Id-positive clones | 393 Id-positive clones |
|---------|-----------------------------------------|---------------------------|------------------------|----------------------|-----------------------|-----------------------|
| LPS     | 1/30                                     | 1/2,000                   | 1/1,800                | <1/10,000            | 1/2,000               | <1/5,000               |
| NDCM    | 1/25                                     | 1/2,400                   | 1/2,200                | 1/2,400              | 1/2,000               | <1/5,000               |

Table II: Absolute Frequencies of Anti-βgalactosidase and Idiotope-secreting Clones among LPS- and NDCM-reactive B Cells in Normal BALB/c Mice.
cell clones defined by reactivity to different mitogens. The analysis of the frequency of idiotope-positive B cell precursors, however, gave different results. Although the numbers of F6(51), 137, and 395 idiotope-positive precursors were found similar in both the LPS- and NDCM-sensitive B cell populations, the absolute numbers of 66 positive cells varied dramatically, depending on the mitogen added to the cultures. Because we can detect all clones stimulated by LPS or NDCM, we can correlate the frequencies of 66 idiotope-producing B cells to the total number of mitogen-reactive B cells and can therefore calculate the absolute frequencies of 66 idiotope-positive precursors within the pool of LPS- or Nocardia-reactive B cells (Table II). Thus, 1 in 2,000 Nocardia-reactive B cells express the 66 idiotope, whereas among the LPS B cell pool the frequency of B lymphocytes secreting this idiotope marker is almost negligible.

Of all the cell types analyzed, only the data obtained for the frequency of Ig-producing cells and for β-galactosidase activity could be completely related to a linear degression of the logarithm of the fraction of negative cultures. All other curves showed a mild leveling off in their degression line when the cell number exceeded 5 × 10^4 cells/culture. For these reasons we tested smaller differences of cell concentrations where mitogen-reactive B cell precursors became limiting. The data obtained at cell concentrations lower than 5 × 10^4 cells/culture, however, fits a linear degression line and we have, therefore, extrapolated these degression lines in a linear fashion.

It should also be mentioned that although the data expressed in Fig. 2 is representative of a single experiment, identical results were repeatedly obtained in a series of five independent frequency determinations.

Anti-LPS Antibodies Are Not Responsible for the Differences in the Numbers of 66 Id-positive B Cells. The specific preferential binding of the antigenic determinants on PBA molecules to specific Ig receptors makes the dose-response profile to PBA markedly different between specific and mitogenic response (20). Consequently, mitogenic activation occurs at high concentrations, whereas induction of specific antigen-binding
Table III

| Cultures stimulated by | IgM | IgG1 | IgG2 | IgG3 | IgG4 |
|------------------------|-----|------|------|------|------|
|                        | Day | Day  | Day  | Day  | Day  |
| LPS                    | 6   | 7    | 8    | 6    | 7    |
|                        | 12,960 | 11,610 | 2,115 | 306 | 648  |
| NDCM                   | 11,957 | 14,490 | 5,960 | 153 | 729  |

Spleen cells from BALB/c mice were treated with anti-Thy-1.2 and complement and cultured in the presence of 50 μg/ml of the indicated mitogens at a concentration of 2 × 10⁶ cells/ml. Results represent the mean of six individual cultures.

Table: Induction of IgG Subclasses by LPS and NDCM Stimulation

Discussion

Polyclonal B cell activators have the property of triggering B cells in a nonspecific fashion. Because the triggering signal is believed to be delivered through non-clonally distributed receptors, it has been repeatedly suggested that the V gene repertoire is equally distributed among functional B cells subsets as defined by their capability to respond to various PBA. This notion, however, is contradictory inasmuch as it implies that PBA always activate large populations of B cells. On the contrary, it has been demonstrated that several mitogens act on small B cell subsets (6, 22). Thus, we can very well imagine a mitogen triggering a number of B cells smaller than the total
number of V genes, and, consequently, the total repertoire can only be partially expressed. In this paper, we dealt with this problem by determining the absolute frequencies of idiotope-positive B cells sensitive to two mitogens. We chose to use LPS and NDCM because, first, they are both strong mitogens, and, second, they have been described as acting on separate B cell subsets (11). An analysis of mitogen-reactive B cell subsets provides a complete description of the immune system with regard to the specific clones expressed within a B cell subset. As the frequency of a given specificity (antibody or idiotype) is determined in the absence of antigen, this analysis has the advantage of not being dependent on a particular protocol of immunization but rather reflecting the absolute frequency of competent B cells in a steady state. We found that the numbers of LPS- and NDCM-sensitive B spleen cells are similar (1/50 and 1/60, respectively), and therefore we could easily compare the repertoire induced by the two ligands. No significant differences were found in the frequency of βgalactosidase-specific antibodies or in the absolute numbers of F6(51)-, 137-, and 395-positive Ig in the two B cell subsets. The frequency of the 66 Id, however, varied dramatically, depending on the mitogen added to the culture. It should be pointed out that our determination of the frequency of LPS-reactive B cell clones producing Ig recognized by F6(51) is similar to the one measured by Barnabé et al. (15), and, therefore, it seems unlikely that our results can be due to a faulty experimental protocol. Our determination of βgalactosidase-specific B cells is significantly different from the one measured by Accolla and Celada (23). This is not surprising, however, because we measured frequencies in LPS-sensitive B cells, whereas they measured the number of βgalactosidase-specific lymphocytes in mice primed in a thymus-dependent fashion. Because we also excluded the possibility that the low frequency of 66 Id-positive LPS-sensitive cells was only apparent because of the existence of 66 positive anti-LPS antibodies, we are left with the conclusion that the V gene repertoire is not duly repeated on mitogen-reactive B cell populations. Stated another way, the implication of our findings is that the LPS-reactive pool of all B cells is not completely representative for all B cells in the spleen (18). Eichmann et al. (18) reached the opposite conclusion by observing that the frequency of A5A-positive LPS-reacting B cells was influenced by priming with antigen and anti-idiotype. On the basis of our results, however, it is clear that the data obtained with one specificity cannot, in all certainty, be extrapolated to the total B cell pool. In addition to this, it has been repeatedly demonstrated (21) that B cell populations sensitive to LPS and T helper cells overlap considerably, and it is therefore not surprising that antigen priming should affect the LPS-sensitive B cell subpopulation.

The simplest explanation for our finding that 66 Id is not randomly distributed among mitogen-reactive B cell subpopulations could be traced to the fact that this idiotypic specificity arises as a consequence of a recombination of a V gene with a particular C gene and that LPS and NDCM would induce the selective expression of different IgG subclasses. This hypothesis, however, seems untenable because, for us, both LPS- and NDCM-driven cultures produced mainly IgM and IgG3 PFC. Thus, at the present, the mechanism responsible for the nonrandom distribution of idiotypes on mitogen-reactive subsets remains unresolved, and we can therefore only propose speculative hypothesis. During the course of differentiation, pre-B cells can respond to mitogenic signals at a time when Ig expression is not detectable. Consequently, it can be postulated that the pool of pre-B cells differentiate under the influence of
different internal mitogens and that the basis for the positive or negative selection for particular gene rearrangements could be traced to the nature of the internal mitogens. Indeed, it has already been demonstrated (24) that B cells and their precursors polyclonally express Ig variable region determinants on their membrane and that the encounter of pre-B cell precursor with these internal mitogens might have a profound influence on their subsequent expression of V genes. Among other alternatives, it can be proposed that mature B lymphocytes, regardless of their particular stage of differentiation, are multipotential in terms of the idiotopes they can express upon activation. The differential expression of idiotopes would then be explained by the ability of different stimuli to direct the genetic processes leading to the expression of a particular idiotope from the surface. This hypothesis is certainly far-reaching and needs to be tested in clonal cultures in which it can be demonstrated that the same clone of IgM-secreting cells can, in fact, produce different idiotopes, depending on the nature of the external stimuli.

Whatever the mechanism underlying the preferential expression of certain idiotopes on discrete B cell subpopulations, our findings caution not only against considering the repertoire of a mitogen-reactive B cell subpopulation as representative for the one of the complete B cell pool, but that they should also be taken into consideration when interpreting data obtained with thymus-dependent antigens. We would also like to stress that a considerable portion of 66, 137, or 395 Id-positive clones detected in our assay (60%, 70%, and 78%, respectively) do not possess anti-βgalactosidase activity (D. Primi et al., manuscript in preparation). This, therefore, is in accordance with recent data (25) showing that an important fraction of 460 Id-bearing molecules in nonimmunized mice lacks TNP-binding activity. In addition to this, in the 460 system it has also been demonstrated (15) that anti- (anti-idiotype) immunity leads to a 10-fold increase in precursor B frequencies with a comparatively lower increase in antibody-producing cells. All these evidences, therefore, indicate a great independence between Id-positive and antibody-producing precursors.

Finally, we would like to stress that the 66, 137, and 395 determinants are not expressed at detectable levels in the anti-βgalactosidase antibody response of BALB/c mice immunized against βgalactosidase and therefore can be classified as private idiotopes. Our experiments, however, clearly demonstrated that these specificities not only are part of the idiotypic repertoire of all BALB/c mice tested but also that they can be induced to expression with a frequency similar to that of a public idiotope, i.e., 460. These observations, therefore, precisely dovetail with the notion that the potential idiotypic repertoire is more or less similar in all individuals of one species.

Summary

The experiments presented here were designed to investigate whether the idiotypic repertoire is equally distributed among B cells subpopulations as defined by mitogen reactivity. To this end we used lipopolysaccharides (LPS) and Nocardia delipidated cell mitogens (NDCM), which are two mitogens that have been described to act on different B cell subsets. The repertoire can be defined in quantitative terms as the frequency of B cells that are precursors for clones secreting immunoglobulin with a given specificity or with a determine idiotype. We determined, therefore, the absolute frequency of LPS- and NDCM-sensitive B lymphocytes secreting immuno-
globulin molecules that bear three idiotopes originally found on a monoclonal antif-\beta\text{galactosidase} antibody. Because the frequencies of B cells carrying one of these idiotopes are dramatically different in the LPS- and NDCM-sensitive B cells subsets, we conclude that the idiotypic repertoire is not randomly distributed among mitogen-reactive B cell subpopulations.

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