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# The Polyclonal Expression of Immunoglobulin Variable Region Determinants on the Membrane of B Cells and Their Precursors

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## Preface

This paper is a survey of the experimental findings and ideas that we have accumulated during the last two years, on the interaction of antibodies against immunoglobulin variable regions with B cells and their precursors. This work could not have been done if several of our colleagues had not provided us with their

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carefully and laboriously prepared reagents. These colleagues include: Pierre-André Cazenave, Michael Reth, Klaus Rajewsky, Bonnie Blomberg, Humberto Cosenza, and Rose Liebermann. We wish to acknowledge their help and support without making them responsible for the experimental findings or for the ideas expressed here.

Details of some experiments described here will be reported elsewhere, as well as the general, unifying concepts developed from these and other findings. We want to acknowledge the profound influence, encouragement, and criticism of Niels K. Jerne in our attempts to make sense of the observations.

This paper contains our present views derived from the available data which are, in many cases, fragmentary. We have refrained from publishing some of these results because we are not absolutely clear about their overall meaning and the generality of the rules we can infer from them now. Consequently, this paper is more of a progress report than our final conclusions on these matters. Some of our interpretations of the results might well be wrong, and we will try to make it clear in the paper which are the solid facts and which are our speculations.

We are well aware that some of the findings reported here, as well as our interpretations, are unconventional and might appear shocking to the immunological community. We understand, however, the truth of Bertrand Russell when he advised: "do not fear to be eccentric in opinion, for every opinion now accepted was once eccentric."

## Introduction

What makes the immune system especially interesting is the diversity of its elements. How this diversity arises, is a crucial problem that has yet to be solved. As we know that mutations of immunoglobulin genes, as of all other genes, occur at certain rates, and that mistakes in the process of DNA replication are not always correctly repaired, it is a priori evident that some of that diversity arises somatically. The recent formal demonstration that the number of immunoglobulin genes is far lower than the number of polypeptide chains [75], and current estimates comparing numbers of genes with the size of the antibody repertoire, would indicate that 90 to 95% of all available specificities must in fact be derived somatically.

As mutational events are necessarily rare, as indicated by the available DNA sequences, such an impressive degree of somatic diversification would be impossible without a mechanism for the selection of variants against the overwhelming predominance of germ-line genes. Even at the exceptionally high rates of mutation that can be specially invoked for immunoglobulin genes, the immune system would be – and it is not – a germline repertoire.

From these considerations, it follows that the problem of the origin of antibody diversity cannot be considered without postulating mechanisms by which the germ-line specificities and their variants are recognized, accepted, expanded, or suppressed in the dynamic state resulting in the available antibody repertoires. This applies also to a strictly germ-line system, where no somatic variations of antibody genes are permitted, since not all genes are expressed simultaneously, and the clones vary extensively in size, both in relation to each other and to the developmental

stage of the animal. From our point of view, the understanding of the immune system and of the generation of antibody diversity does not depend on the demonstration of a few hundred more or a few hundred less germ-line antibody genes, but rather on the solution to the mechanisms by which available repertoires are set and tuned by the system itself.

The immunological theory lacks at present a coherent framework for the basic rules that preside over the selection of available repertoires from the pool of germ-line encoded or somatically derived antibody specificities. This will necessarily have to include postulates on the specificities of germ-line genes, on the evolutionary pressures that maintain them in the germ-line genome, and on the developmental pressures that select for the somatically arising variants.

In general, the basic ideas spanning through this discussion assume that the survival value of the antibody system is built on its *completeness of recognition* [22], and in addition, is directly proportional to its *diversity* and, therefore, to its abilities of detailed discrimination. We postulate, therefore, that the germ-line collection of antibodies must be complete and, in addition, contain the potentialities for somatic expansion of the degree of detailed diversity. Since all these processes must necessarily be antigen-independent, because of the Promethean characteristics of the immune system [59], we will assume that the molecular patterns operating the somatic selection (by being recognized) are carried within the germ-line system itself, by the same molecules upon which the selection operates (and which recognize). In short, we postulate: (a) that the pool of germ-line antibody genes constitutes a complete, self-recognizing repertoire (a complete network) [1, 44] and (b) it somatic expansion via mechanisms involving idiotypic-anti-idiotypic recognition. In this recognition are included the reactions amongst soluble and membrane bound immunoglobulin molecules, as well as of antibodies with other non-immunoglobulin structures expressed within the B cell system, which "share" determinants with germ-line antibodies, leading to the positive selection for variant clones without necessarily selecting against germ-line gene expression [22]. In this paper we shall review the experimental evidence that provided the basis for these ideas.

## Idiotypes

An idiotype, as any antigenic determinant, is indirectly defined by the specificity of the antibodies that recognize it. In the original description of the "individual antigenic specificities" of myeloma proteins, monoclonal idiotypes were defined by mixtures of specific antibodies in antisera [74]. In the idiotypic systems available today which use myeloma proteins, the "idiotype", although monoclonal, is a complex entity, composed of distinct antigenic determinants or "idiotopes" displayed by the same molecule. In these cases, "idiotype" aims at defining a single antibody species on the basis of the various idiotopes it expresses.

In contrast, "antibody idiotypes" were first defined by mixtures of specific antibodies to mixtures of determinants expressed on pools of different antibodies present in immune sera [47, 61, 62]. In these cases, "idiotype" is again an operational definition which is based on serologic specificities, and which aims at defining as a unique population, several (often many) different antibody species

related in terms of reaction with a given antigen, by the common or prevalent idiotopes present in the mixture.

Obviously, the specificity of these reactions is provided by the "average" molecular patterns of the idiotopes in the mixture and the "average" combining sites in the anti-idiotypic antisera. Even if any particular idiotope present in the pool of specific antibodies were to be shared by other immunoglobulin molecules, and if some of the anti-idiotypic antibodies could also recognize other determinants, their dilution in the mixtures would account for the observed specificity of idiotype-anti-idiotype reactions. It should be expected, therefore, that a given anti-idiotypic reagent might react with molecules other than those defined as to carry the idiotype. This has been classically demonstrated by the finding of a given antibody idiotype in immunoglobulin molecules with unknown or with a different antibody specificity [18, 29, 61]. On the other hand, specific anti-idiotypic antisera, operationally defining a given antibody, can be made by extensive absorption with normal immunoglobulins [48]. Again, in this case, the absorption is likely to leave behind the specific constellation of antibodies to the complete set of idiotopes expressed on that antibody molecule. The expression of this very combination of idiotopes on the same molecule is likely to be unique, but each one of the idiotopes is probably expressed in many other different antibody molecules. Since the methods available today have a threshold detection of $10^9$ or $10^{10}$ immunoglobulin molecules per ml, these idiotopes would be expected to pass undetected. Several reviews have recently concentrated on the immunochemical and structural basis of idiotypy [15, 41], and our considerations only pretend to point out that "idiotypes" are defined by the specificity of antibodies, which is degenerated [43].

In view of all this, "idiotypes" can hardly be considered "clonal" markers of immunoglobulin molecules. It is surprising that while combining site specificity of antibodies is generally agreed *not* to be a clonal marker, the combining site specificity of a second set of antibodies (anti-idiotypes) is generally agreed to provide a reliable clonal marker. During the last few years, idiotypes have been extensively used as tools in the study of antibody genes [see for reviews 27, 28, 55, 78]. Although it is clear that much progress in the solution to these questions has been achieved by these approaches, it should be remembered that idiotypes cannot be considered as phenotypes of structural antibody genes. As pointed out by Eichmann [28], "in this area, the term 'idiotype' is used not only to define a given set of antigenic determinants recognized by a particular antiserum, but also to describe a population of antibody molecules reactive with such an antiserum, and in addition, to describe populations of antibody molecules that share certain specific characteristics without necessarily being defined by an anti-idiotypic reagent". This new trend in idiotypic research is based on the recurrence of "idiotypes" in different (all?) individuals of the same strain, or species, or even in various species [45]. In many cases, and as should be expected [43] the idiotype has been detected in the serum of normal animals in considerable amounts [52, 54]. It has certainly been a long way from the original, very exclusive definition of idiotypes by their expression in single individuals and absence in the normal serum, to the present "massification" of the definition now applied to not so rare antibody molecules.

The introduction of the hybridoma technique into the field of idiotypy, making monoclonal idiotypes and anti-idiotypes available, has constituted a large progress

in the clarification of these issues. On the one hand, each of the previously defined "single" idiotypes of the mouse which has been analyzed so far, has been split into a very large number of distinct antibody species, all recognized by the conventional anti-idiotypic reagent [6, 30, 35, 68]. These findings provide the direct demonstration that an idiotype is *not* a clonal marker, but rather an operationally defined characteristic of a large number of distinct antibody molecules. Moreover, these experiments dealt only with the analysis of monoclonal idiotypes that had been selected from the available repertoires in terms of combining site specificity, by immunization with the antigen. The collection of different idiotypic antibodies thus obtained is probably, therefore, a gross underestimate of the total pool of immunoglobulin molecules which "share" the same idiotype, as defined by conventional reagents, some of which would fail to bind the antigen.

The availability of monoclonal anti-idiotypes, on the other hand, made it possible to study "idiotopes" rather than "idiotypes" [14, 69]. The analysis of the representation of idiotopes in various antibody populations, is likely to show that different "idiotypes" may include identical idiotope(s), and that different "idiotopes" may be represented in similarly identical "idiotypes". It should be noted, however, that although monoclonal, these reagents are still antibodies, and therefore, it should be expected that the same monoclonal anti-idiotope might react with several similar but distinct *determinants* on antibody molecules. Here again, the observations have to be taken under the general rules of antigen-antibody reactions, indicating that the same reagent might identify not only one, but several different "idiotopes", which may obviously occur on structures other than immunoglobulin molecules.

The value of a definition is perhaps not intrinsic, but it depends on how it is used. Clearly, one can define as an "idiotype" any molecule that is recognized by an established anti-idiotypic reagent, even if that molecule is also characterized as *not* being immunoglobulin. This has been the case in experiments dealing the "elusive" [26] T cell receptors. Immunologists, idiotypists in particular, are however very keen on specificity, and in this case the issue was rescued by assuming that the anti-idiotypic reagents were still recognizing the same gene products. This is obviously a purely tentative assumption, because even the very limited data on the genetic linkage of these "idiotypes" to immunoglobulin constant region genes can only provide indications on the control of their *expression* and not on the structural genes which encode them.

In this paper, we will proceed a step further, by showing that what is currently defined as "idiotype" on the basis of the reaction with an anti-idiotypic reagent, includes a set of molecules which are *not* immunoglobulins, and in addition, we assume that we are probably *not* dealing with V-gene products. Although fully accepting Jerne's postulate that anything else will always "look like" existing "idiotypes" [44], we will focus on the cross-reactivity between "common", "major", or "public" idiotypes and other surface structures expressed on cells of the B lymphocyte lineages. We will argue that these observations are not a mere detail in the idiotypic mimicry postulated in the network theory, but rather that they represent fundamental components in the evolution of the immune system.

## The Reaction of Anti-Idiotypic Antisera with Lymphocytes from Normal Mice

### *Anti-Idiotypes to Antibodies Specific for Thymus-Independent Mitogens*

These investigations were initiated for the practical purpose of obtaining reagents to mitogen receptors on B cells, and not until much later did we become aware of the greater significance of these findings. The rationale behind the initial experiments was as follows: (1) thymus-independent antigens are often polysaccharides of very simple structure. They interact specifically with at least two types of molecules in the immune system, in particular on B cell sufaces, namely with clonally distributed combining sites of antibody receptors and with polyclonally distributed "mitogen receptors". (2) From the structure of these antigens it would appear plausible that similar sites on the molecules were specifically recognized by these two types of receptors. (3) If this were the case, it could be expected that the combining sites of these receptors were sufficiently similar to allow for specific antibodies against the antibody receptor (anti-idiotypic antibodies) to recognize also the combining sites of the polyclonally distributed mitogen receptors.

These ideas were encouraged by the report on the activity of anti-idiotypic antisera against anti-insulin antibodies [71]. In this case, the anti-insulin antibodies were shown to display combining sites sufficiently similar to cellular insulin receptors, for the latter to be recognized by anti-idiotypic antibodies against the former and for these anti-Id antibodies to mimic the effects of insulin. In our case the possibilities appeared even more favorable, as many of the antibody responses to thymus-independent antigens show restricted heterogeneity and are often dominated by one or a few clones. In the initial experiments, we chose to work on the idiotypic system described in BALB/c mice on the responses to α-1, 3 dextran, as it offered several advantages. First, the IgM antibody responses to dextran had been characterized as thymus-independent [7, 79], indicating the direct mitogenicity of these molecules for B lymphocytes [25]. We could in fact show that dextran B1355, containing α-1, 3 linkages, was a polyclonal B cell mitogen, and although the quantitative determinations of the numbers of mitogen-reactive B cells were not performed, we could infer, by comparison of mass culture responses with those induced by lipopolysaccharide, that dextran stimulated in the order of 10% of all splenic B cells [23]. We have also shown that all mouse strains tested responded equally well to dextran as a polyclonal mitogen, indicating that all of them express polyclonally distributed "mitogen receptors". The second advantage with this system was offered by the "clonal dominance" of the antibody responses to dextran in mice, a condition that we thought was a prerequisite for our experiments to work. It has been shown that the anti-dextran response in BALB/c mice was "dominated" by antibodies bearing an idiotype identical or similar to that characteristic of the α-1, 3 linked dextran-binding myeloma protein of BALB/c origin – J558 [8]. In addition, the high responsiveness to dextran was associated with expression of that idiotype and such an expression was linked to the Ig-1 complex [8, 16]. Several Ig haplotypes have therefore been characterized as dextran high responders expressing the idiotype, and low responders which did not express J558 idiotype on antibodies to dextran [16]. The possibility of following the genetic control of antibodies in this

system appeared fundamental, as we were interested in cross-reactive structures that were probably not linked to the Ig-1 locus, as all mouse strains appeared to express mitogen receptors to dextran.

With an anti-idiotypic antiserum identical to that used in the characterization of the antibody responses described above, we probed using indirect immunofluorescence, whether the anti-idiotypic antibodies could specifically recognize polyclonally distributed surface structures on B lymphocytes. We found that 10 to 15% of all splenic B cells could in fact be stained by this reagent [23]. Interestingly, practically the same numbers of cells were stained to the same intensity in mice characterized as high- and low-responders for the expression of idiotype on antibodies. The staining could be nearly totally inhibited by α-1, 3 linked dextran added to the reaction mixture, but not by α-1, 6-linked dextran [23]. This points, on the one hand, in favor of the specificity of the staining reaction and indicates, on the other hand, that the antibodies giving the positive reaction were specific for surface structures which either could bind dextran or were sterically hindered by dextran binding. Finally, all the staining was inhibited by absorbing the antiserum with J558 myeloma protein, but not by absorption with other α or λ BALB/c proteins, demonstrating the idiotypic specificity of the polyclonal reaction. Other workers have found that the staining is also largely inhibited if the reaction is performed in the presence of large amounts of normal BALB/c serum (B. Pernis, personal communication), indicating that we are dealing with idiotypic antigenic specificities present on immunoglobulin molecules, but expressed on 15% of all B cells.

The conclusive demonstration of these assumptions was achieved by repeating these experiments using *affinity purified syngeneic* anti-J558 antibodies. These behaved exactly as the total IgG fraction of the immune serum used in the above experiments. In these conditions, we could exclude any other contaminants in the antiserum, such as antibodies to the few α-chain allotypic determinants which differ in A/He and BALB/c mice, and directly show that the anti-idiotypic antibodies were responsible for our observation. The binding of both the antisera and the purified antibodies to lymphocytes was not the result of a nonspecific anti-membrane or anti-lymphocytic activity, as we could show that all cells stained in the adult spleen were also stained by anti-μ antibodies. In addition, among all B cells, only a fraction was positive, in which the $\mu+$ $\delta-$ phenotype was largely predominant.

We had shown before that antibodies to a "mitogen receptor" specific for lipopolysaccharide were mitogenic for the B cell subset expressing such receptors [24, 33]. As the only formal demonstration that the dextran-binding structure recognized by the anti-idiotypic antibodies was in fact a "mitogen receptor", we probed the mitogenic activity of anti-idiotypic antisera and of purified anti-idiotypic antibodies. As shown in Figs. 1A and B, these antibodies proved to be mitogenic for a fraction of B cells, comparable to that stimulated by dextran, in both antibody-idiotype positive and negative mouse strains. We have shown, in addition, that the fraction of splenic B cells which responds to dextran as a mitogen is superimposable on that expressing the surface structures recognized by the anti-idiotypic antibodies [23]. Taken together, these results provide the formal demonstration of the reaction between anti-idiotypic antibodies to the dextran-binding myeloma J558 and polyclonally distributed "mitogen receptors" specific for

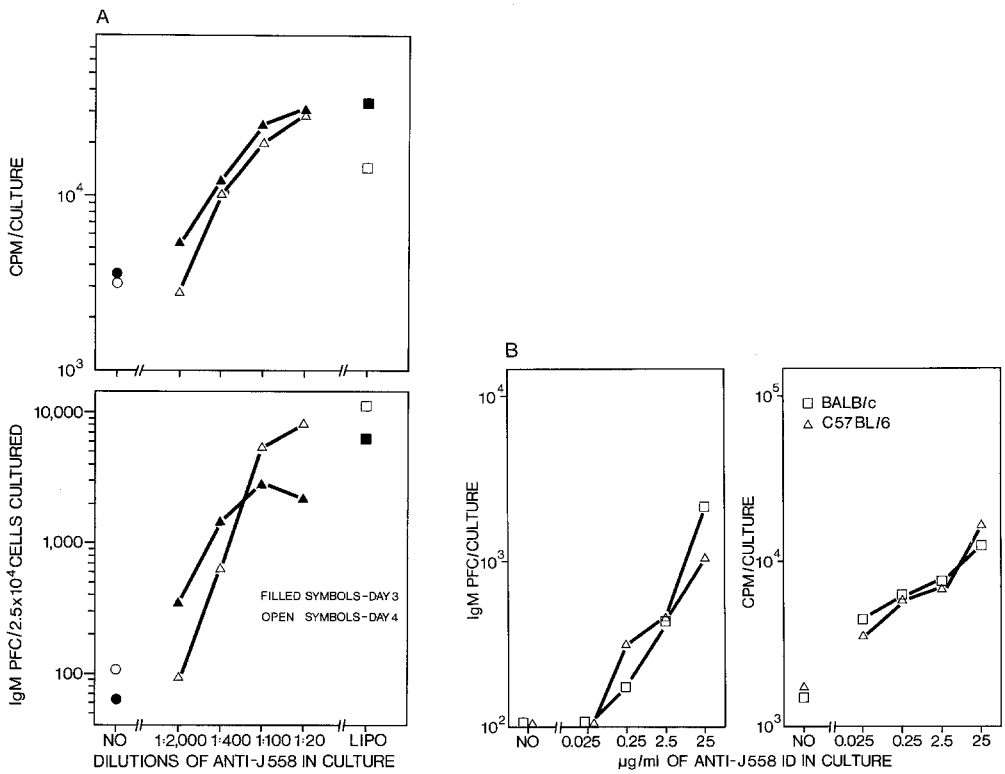


**Fig. 1 A.** Spleen cells from "nude" C57BL/6 mice were cultured ($5 \times 10^5$ cells/culture) in the presence of the indicated dilutions of the IgG fraction of a BALB/c anti-J558 anti-idiotypic antisera. The proliferative responses were assayed by 2 h pulses of tritiated thymidine and polyclonal Ig-secretion in the protein A plaque assay at the indicated days. The control responses to 25 μg/ml of lipoprotein are also shown. **B** Normal spleen cells from the indicated strains were treated with carbonyl-iron and thereafter cultured ($8 \times 10^4$ cells/culture in 0.2 ml) with the indicated concentrations of A/J anti-idiotypic antibodies eluted with low pH buffer from an immunoabsorbent column of Sepharose-J558. Proliferation and Ig-secretion were assayed after four and five days of culture, respectively. The control responses induced in the same experiment by 50 μg/ml of LPS were 17,162 and 37,282 CPM/culture and 12,800 and 19,400 IgM PFC/culture, for BALB/c and C57BL/6 cells, respectively

dextran. A number of reasons, to be discussed below, forces us to conclude that such "mitogen receptors" are not immunoglobulin molecules.

After the initial experiments, we extended these studies to other anti-idiotypic antisera to antibodies with specificity for thymus-independent antigens (mitogens). They included anti-idiotypes to the myeloma proteins W3129, binding α-1, 6 linked dextran, MOPC 104E and J558, binding α-1, 3 linked dextran and EPC109, and W3082, binding levan. In addition, we have also tested anti-idiotypic antisera to normal antibodies, such as anti-polyvinylpyrrolidone and streptococcal A-carbohydrate. These antisera had been produced in a variety of hosts, either syngeneic, allogeneic, or xenogeneic. In all cases, however, very similar findings to those described above were obtained. With most of the reagents, several mouse strains were investigated and some of these results are shown in Table 1. The

**Table 1.** Expression by splenic B cells of membrane determinants cross-reactive with idiotypes of antibodies to TI antigens

| | | % splenic IgM$^+$ cells bearing "idiotypic" markers | | | | |
|---|---|---|---|---|---|---|
| Anti-idiotype donor | Idiotypic antibody | BALB/c | C57BL/6 | A/J | CBA/J | DBA/2 |
| A/He[a] | J558 | 20.2 | 16.4 | 12.8 | 20.6 | 23.1 |
| A/J | J558 | 25.3 | | | | |
| BALB/c[b] | J558 | 24.7 | 16.7 | 25.1 | | 22.7 |
| A/He[a] | W3129 | 18.4 | 15.8 | | 16.1 | 25.1 |
| A/He[c] | EPC-109 | 25.5 | 25.4 | 15.7 | | 14.2 |
| CB20[b] | EPC-109 | 17.6 | 19.2 | | | |
| A/J[b] | W3082 | 16.4 | 18.3 | | | 19.3 |
| BALB/c[b] | W3082 | 21.5 | 20.9 | | | |
| guinea pig[d] | MOPC 104E | 12.4 | | | 14.9 | |
| rabbit | MOPC 104E | 24.5 | 33.3 | | | |
| guinea pig[e] | anti-PVP | 10.1 | 7.5 | | | |
| guinea pig[f] | A5A | | 13.9 | 19.4 | | |
| guinea pig[f] | A5A | | 5.0 | 6.4 | | |
| BALB/c | normal IgG | 0.35 | | | | |
| CBA/J | IgG anti-SRBC | 0.38 | | | | 0.7 |
| BALB/c | IgG2b anti-SRBC | $<0.1$ | $<0.1$ | | | |

The percentages reported are average values of at least five individual animals tested separately: in some cases the staining was performed on pooled spleens from 5–10 mice of a given strain. All antisera were IgG fractions prepared by either Sepharose-protein A immunoadsorbants or DEAE-cellulose chromatography. Mouse antibodies were labeled with trinitrophenyl and detected by fluorescein-labeled goat anti-TNP antibodies. Guinea pig and rabbit antibodies were used unlabeled and detected by fluorescein-coupled antisera against guinea pig or rabbit IgG. All samples were counterstained with rhodamine-coupled anti-mouse μ antisera.

As indicated, the antisera were kind gifts of the following colleagues: [a] Dr. B. Blomberg, [b] Dr. P.-A. Cazenave, [c] Dr. R. Liebermann, [d] Dr. K. Forsbeck, [e] Dr. B. Andersson, [f] Dr. K. Eichmann

specificity of the B cell structures identified by the anti-idiotypic reagents was assessed by performing the staining in the presence of the corresponding "antigen" binding to the idiotypic antibodies. In all cases where competition experiments were performed, namely with the anti-idiotypes to anti-levan and anti-dextran myeloma proteins, the staining could be largely inhibited by the corresponding "antigen".

These experiments only show that anti-idiotypic antisera, proven to be idiotype-specific by a number of different serologic techniques when tested on immunoglobulin molecules, do contain antibodies capable of reacting with large number of B lymphocytes. This would indicate either that the technique of indirect immunofluorescence is far more sensitive than available immunochemical or serologic tests, or that the reaction with cell surface is not due to recognition of

immunoglobulin molecules. In either case, the generality of these observations poses serious constraints upon the current interpretations of the functional effects in vivo or in vitro of anti-idiotypic reagents on the immune responses. Results of this kind have been interpreted on the assumption of the exclusive reaction of the anti-idiotypic antisera with clonally distributed idiotypes on immunoglobulin molecules. However, in all the experiments, there is no indication on the reactivities of the antisera with other cell surface structures. We must point out that we have tested in most cases the same reagents used to obtain results interpreted on the basis of exclusive clonal reactivities. Regardless of how general these properties of anti-idiotypic antibodies are, our findings necessarily force the re-evaluation of those results, as well as the inclusion of appropriate controls, if anything at all is to be firmly concluded. This is even more so, for in most cases we could demonstrate that the interaction of anti-idiotypic reagents with B cells has overt functional consequences, namely mitogenicity and induction of polyclonal antibody secretion. Table 2 shows a few examples with antisera of the type described above.

*Which Type of Anti-Idiotypic Reagents Recognize B Cells Polyclonally?*

Since antibodies to conventional antigens, or normal immunoglobulins, did not show polyclonal reactivity with B cells (Table 1), it appeared that our original

**Table 2.** Polyclonal B cell mitogenicity of anti-idiotypic antisera

| Specificity of antisera | Mouse strain | cpm $\times 10^{-3}$/culture | IgM PFC/culture |
|---|---|---|---|
| Expt. 1[a] – med | C57BL/6 | 3.7 | < 10 |
| – LPS (50 μg/ml) | C57BL6 | 33.2 | 25,866 |
| A/He IgG anti-E109 50 μg/ml | C57BL/6 | 25.1 | 29,733 |
| – med | BALB/c | N. D. | 17 |
| – LPS (50 μg/ml) | BALB/c | | 6,833 |
| A/He IgG anti-E109 50 μg/ml | BALB/c | | 7,667 |
| Expt. 2[a] – med | C3H/Tif | 3.4 | 200 |
| – LPS (50 μg/ml) | C3H/Tif | 19.6 | 7,567 |
| A/He IgG anti-E109 50 μg/ml | C3H/Tif | 25.5 | 17,333 |
| A/J IgG anti-A48 50 μg/ml | C3H/Tif | 8.8 | 663 |
| Expt. 3[a] – med | C57BL/6 | N. D. | 246 |
| – LPS (50 μg/ml) | C57BL/6 | | 17,840 |
| A/He anti-W3129 1 : 10, 240 | C57BL/6 | | 15,680 |
| – med | BALB/c | N. D. | 536 |
| – LPS (50 μg/ml) | BALB/c | | 12,280 |
| A/He anti-W3129 1 : 2, 500 | BALB/c | | 16,160 |
| Expt. 4[b] – med | C57BL/6 | 33.0 | 485 |
| – LPS (50 μg/ml) | C57BL/6 | 173.2 | 19,200 |
| A/He anti-J558 1 : 100 | C57BL/6 | 340.7 | 9,867 |
| – med | BALB/c | 17.5 | 368 |
| LPS | BALB/c | 201.2 | 21,973 |
| A/He anti-J558 1 : 100 | BALB/c | 157.2 | 8,213 |

Normal (Expt. 4) or iron-treated spleen cells from the various strains were incubated with the indicated concentrations of the reagents in 0.2 ml cultures, as described [22]

[a] $10^5$ cells/culture in 0.2 ml; TdR uptake measured on day 3 and PFC on day 5

[b] $2 \times 10^5$ cells/culture in 0.2 ml; TdR uptake measured on day 2 and PFC on day 3

hypothesis was correct, namely that our findings were due to the fact that the combining sites of antibodies and mitogen receptors to the same thymus-independent polysaccharide were similar and shared idiotypic determinants. It would necessarily follow from that assumption that anti-idiotypes to antibodies directed against non-mitogenic antigens should not be expected to react with mitogen receptors. For this hypothesis we tested anti-idiotypes to antibodies specific for haptenic determinants which show no intrinsic mitogenic properties, such as phosphorylcholine (PC) and trinitrophenyl (TNP), namely TEPC 15, MOPC 315, and MOPC 460 myeloma proteins, respectively. Both PC and TNP induce antibody responses only when coupled to immunogenic carriers, and the thymus-dependence of these responses is determined by the mitogenicity of the carrier [42, 65], clearly indicating that the haptenic groups by themselves do not interact polyclonally with B cells in a functionally relevant manner. Also in this case, the anti-idiotypic reagents had been shown to react at the antibody level only with the relevant myeloma proteins and PC-binding antibodies, or with anti-TNP antibodies produced in normal BALB/c mice [9, 20]. Surprisingly, we also found that anti-idiotypes of this category had the property of recognizing polyclonally distributed surface structures on splenic B cells (Table 3) and were mitogenic. These observations invalidated our initial, simplistic interpretations based on presumed structural similarities of mitogen receptors and antibodies to the same *mitogenic* moiety.

One alternative interpretation for these findings, which would also include those using the anti-idiotypic reagents analysed above, is suggested by a particular characteristic of all these idiotypes, namely their recurrence in the antibody responses of normal mice. Thus, all the idiotypes that we have discussed so far have been defined as "major" or "public", due to the fact that immunization of normal mice – at least of mice with the BALB/c Ig-1 haplotype – results in the production of antibodies bearing idiotypes similar to that of the corresponding myeloma protein. In some cases, this particular "idiotype" largely dominates the response, while in other cases, although "public", the idiotype is not "major". The finding that every mouse in the strain produces the idiotype has led to the assumption that these antibodies represent the expression of germ-line genes, as opposed to "private" idiotypes, that are considered to be the result of the random events determined by the somatic (individual) generation of diversity.

**Table 3.** Expression by splenic B cells of membrane determinants cross-reactive with idiotypes of anti-hapten antibodies

| Donor of anti-idiotype | Idiotypic antibody | % $IgM^+$ cells stained | | | | |
|---|---|---|---|---|---|---|
| | | BALB/c | C57BL/6 | A/J | SJL | C3H |
| A/J | TEPC-15 | N. D. | 13.1 | 22.3 | 28.6 | 19.6 |
| BALB/c | MOPC-460 | 19.1 | 12.3 | 26.5 | 22.2 | N. D. |
| BALB/c | MOPC-315 | 23.1 | N. D. | N. D. | N. D. | N. D. |

See legend to Table 1

Since this was the only property in common to all the anti-idiotypes tested, we hypothesized, therefore, that the polyclonal reactivity of anti-idiotypic reagents was associated with idiotypes representing germ-line V-region genes. In order to test for these assumptions, we have used anti-idiotypic reagents specific for BALB/c myeloma proteins of the same binding specificity to cover the first alternative, but different in regard to the representation of the corresponding idiotypes in normal antibody populations to account for the second possibility. EPC109 and ABPC48 are two BALB/c myeloma proteins with levan-specific antibody activity [53]. Immunization of BALB/c mice with levan, however, results in the production of antibodies bearing idiotypic determinants similar to those of EPC109, but not to those of ABPC48 [10, 11]. When we assayed the ability of anti-EPC109 and anti-ABPC48 to react polyclonally with B cells, we found that only the former was active, while the latter was a good negative control to our experiments. In parallel with the staining results, anti-EPC109 was shown to be a potent polyclonal B cell mitogen, while anti-ABPC48 had no functional effect on splenic B cells (Table 2).

These results therefore exclude the possibility that the polyclonal properties of anti-idiotypic reagents are determined by the antigen-binding specificity of the "idiotypic" antibodies, as we have found anti-idiotypes to antibodies directed against non-mitogenic determinants which are positive, as well as anti-idiotypes to antibodies against mitogenic determinants which are negative. It is likely, therefore, that our initial observations leading to the identification of a dextran-specific mitogen receptor were due to the fact that the idiotype was "public", rather than to its binding site specificity for a mitogen. Clearly, however, although the combining site specificity of the "idiotype" might not be a mitogen, the anti-idiotypic antibodies appear to recognize mitogen receptors on B cells, as shown by their functional properties. Since antibodies are made independently of antigens and recognition is degenerate it could as well be argued that the "true" binding specificity of those anti-PC and anti-TNP idiotypes is in fact a mitogen, and that the anti-hapten specificity is a mere cross-reaction. It can be concluded, however, that even if all antibodies sharing idiotypes with mitogen receptors have combining site specificities for mitogens, the converse is not true. That is, only a fraction of all anti-mitogen antibodies "looks like" mitogen receptors. This fraction appears to include only the "major" or "public" idiotypes of presumed germ-line origin. These arguments will be considered below in further detail.

*Direct, Functional Demonstration that the Determinants Recognized by the Anti-Idiotypic Reagents are not Surface Immunoglobulin*

The demonstration of polyclonal reactivity of antibodies against "major", but not against "minor", idiotypes brought in the reservation that our experiments could possibly be explained by low affinity cross-reactions of the anti-idiotypic antibodies with very large sets of immunoglobulin molecules. First, it was likely that the reagents tested in our systems were not specific to "individual antigenic determinants" of the myeloma proteins, but that they had a broader specificity (for V-region subgroups, for example). This was suggested by the following findings of Cammisuli and Cosenza (personal communication). Anti-TEPC15 antisera which are anti-idiotypic inasmuch as they react only with PC-binding antibodies or

myeloma proteins, and not with other α, k BALB/c myelomas, display polyclonal reactivity, as shown here. This reactivity, however, was lost after the antisera had been made specific for the "individual determinants" of TEPC15, by absorption with other PC-binding myelomas. The possibility that such reagents have a subgroup rather than an individual specificity, however, would not argue against the fact that the antibodies detected in our assays were specific for variable regions of Ig molecules. Furthermore, subgroup specificity of these reagents would be expected to give results falling at least one order of magnitude short, as compared to the numbers of cells we could label. On the other hand, it could be argued that our methods were sensitive enough to detect with very low affinity a pool of immunoglobulins much larger than the already considerable fraction of all Ig molecules (0.1 to 1%), detectable by serologic assays.

We decided to investigate this question by using the same reagents and the same techniques to determine the frequency of immunoglobulin species detectable under these conditions in the cytoplasm of normal background plasma cells. As shown in Table 4, only a small minority of these cells are stained, indicating that the frequency of normal IgM molecules reacting with these anti-idiotypes does not exceed 1–3%, that is roughly one order of magnitude lower than the frequency of normal B cell bearing surface determinants reacting with the same anti-idiotypes. Since the technique of indirect immunofluorescence used here is far more sensitive for detection of intracytoplasmic immunoglobulins than for membrane-bound molecules, this observation excludes that the surface staining of a very large set of B lymphocytes is due to the high sensitivity of this technique, leading to the detection of anti-Ig antibodies of very low affinity. On the other hand, these results indicate that the anti-idiotypic reagents we have used above do react, under these conditions, with a considerable fraction of all natural IgM molecules and, consequently, that they are probably not specific for individual antigenic specificities of antibodies.

In another experiment, we have addressed the same basic question, but in addition we correlated the fluorescence results with the functional ability of the anti-idiotypic antibodies to induce large numbers of B cells to divide and secrete Ig. Resting, small splenic cells were cultured with mitogenic concentrations of several anti-idiotypic antisera or purified antibodies, or, as a control, with LPS. Two days

**Table 4.** Comparison between the frequencies of lymphocytes bearing membrane "idiotype"—cross-reactive determinants and of plasma cells containing "idiotype" positive immunoglobulin in the same spleen cell samples

| Anti-idiotypic antiserum | Spleen cells tested | % $IgM^+$ lymphocytes | % $IgM^+$ plasma cells |
|---|---|---|---|
| BALB/c anti-MOPC 460 | Balb/c | 23.9 | 1.7 |
| A/He anti-J558 | Balb/c | 20.1 | 2.8 |
| | C57 BL/6 | 15.6 | 0.6 |
| BALB/c anti-J588 | C57 BL/6 | 16.7 | 1.01 |
| A/He anti-EPC 109 | Balb/c | 25.5 | 4.3 |
| A/J anti-ABPC 48 | Balb/c | 4.0 | 4.9 |

For membrane staining, see footnote to Table 1. Intracytoplasmic staining was performed with the same reagents on fixed cytocentrifuge smears

later, some cultures were harvested and the cells were stained with the same anti-idiotypic reagents used in culture in order to assess the distribution of "idiotype-positive" cells among resting, small lymphocytes, and among lymphoblasts. Since the development of blasts and the mitotic responses measured by the uptake of tritiated thymidine in these cultures was dependent upon stimulation by the anti-idiotypic reagents, we wanted to determine whether or not the cells we could detect by immunofluorescence were those initiating proliferation in cultures stimulated by the same antibodies. As shown in Table 5, by comparing the frequencies of labeled cells with four different anti-idiotypic antisera, among unstimulated spleen cells and in cultures stimulated by each of these reagents, we found that practically all the cells stimulated by the anti-idiotypes which had undergone blast formation are labeled by the corresponding antibodies, while there was a clearcut depletion of positive cells among the cells that remained resting in the same cultures. The relatively few cells expressing "idiotypes" which remained unstimulated (small) in cultures stimulated by the corresponding antibodies could probably have been activated at other antibody concentrations, and therefore, their presence in the cultures does not necessarily indicate expression of the relevant determinants on structures other than mitogen receptors. The selection for idiotype-positive cells in the blast cell population is not observed in cultures stimulated by lipopolysaccharide (LPS). Discrepancies between the frequencies of idiotype-positive cells among unstimulated cells and those found in LPS-stimulated cultures probably reflects the extent of overlap between the subsets of B cells which respond to LPS or to the anti-idiotypic reagents that are obviously different for each of these. We conclude from this experiment that *there is a good correlation between the detection of positive cells in immunofluorescence and the ability of the anti-idiotypic antisera to stimulate the*

**Table 5.** All the B lymphocytes activated by each anti-idiotypic antiserum express the corresponding idiotype-cross-reactive determinants on the membrane.[b]

| Mitogen in culture | Staining antibody | Membrane positive cells % | | $IgM^+$ small lymphocytes in unstimulated spleen |
|---|---|---|---|---|
| | | $IgM^+$ blasts | $IgM^+$ small lymphocytes | |
| LPS | anti-LPS-R[a] | 87.0 | 11.7 | |
| LPS | anti-MOPC 460 | 9.6 | 20.8 | 12.3 |
| BALB/c anti-MOPC 460 | | *100.0* | 2.8 | |
| LPS | anti-W3129 | 13.0 | 9.6 | 15.8 |
| A/He anti-W3129 | | *98.7* | 9.6 | |
| LPS | anti-J558 | 34.8 | 13.4 | 16.4 |
| BALB/c anti-J558 | | *98.2* | 16.3 | |
| LPS | anti-EPC 109 | 33.0 | 26.4 | 25.4 |
| A/He anti-EPC 109 | | *100.0* | 11.2 | |

[a] See Ref. 24 and 33
[b] C57BL/6 spleen cells. See footnote to Table 2 for culture conditions

*same set of cells into blast transformation.* It is particularly important to point out that *all* activated cells expressed membrane structures bearing determinants recognized by the anti-idiotypic antibodies.

The complement of this experiment was performed by letting the activated cells in parallel cultures mature to immunoglobulin secretion, and by assessing the reactivity of the inducing antisera with the antibodies produced by the activated cells. This was done by staining the cells intracytoplasmically both with the anti-idiotypic reagents and anti-μ antibodies. As shown in Table 6, only a very small minority of the activated cells produced antibodies that could be recognized by the activating antisera, using the same reagents and technique which had previously detected cross-reacting determinants on the membrane of *all* activated cells. Since the second assay detects, undoubtedly, intracellular immunoglobulins, we must conclude, *keeping to the rules of the clonal selection theory, that the membrane determinants detected by these anti-idiotypic reagents are not immunoglobulin molecules.*

In order to formally exclude that the surface staining could be due to passively absorbed Ig produced by contaminating plasma cells in vivo or in vitro, we have performed these experiments with C57BL/6 spleen cells, that is in a strain which does not produce Ig molecules bearing any of the four idiotypes tested in this experiment upon immunization with the corresponding antigens. We would therefore be confident that the surface staining was in fact specific for molecules other than Ig. On the other hand, the presence of idiotype positive immunoglobulins both in normal spleen plasma cells or upon polyclonal stimulation would suggest that also strains previously typed as "idiotype-negative" can be induced to produce such idiotypes. Other results obtained by serologic identification of idiotype-secreting clones in LPS-stimulated cultures also support this conclusion (Bernabé, Coutinho, and Cazenave, unpublished work). Furthermore, recent experiments would indicate that "network" manipulations of the immune system in "negative"

**Table 6.** Only a minority of the plasma cells induced by each anti-idiotypic antisera contain idiotype-positive IgM

| Staining Reagent | Mitogen in culture | | | | |
|---|---|---|---|---|---|
| | LPS | BALB/c anti-MOPC 460 | A/He anti-W3129 | BALB/c anti-J558 | A/He anti-EPC109 |
| BALB/c anti-MOPC 460 | 1.7 | *1.9* | 3.2 | 2.6 | 3.5 |
| A/He anti-W3129 | 0.6 | 0.4 | *0.4* | N.D. | N.D. |
| BALB/c anti-J558 | 1.6 | 2.0 | 2.2 | *1.0* | N.D. |
| A/He anti-EPC109 | 1.2 | 0.9 | 1.1 | N.D. | *2.0* |

strains may lead to the production of antibodies bearing cross-reactive idiotypes upon specific immunization with the corresponding antigens [17, 51, 76].

It should be pointed out again that the frequency of idiotype-containing cells detected in these assays (Tables 4 and 6) suggests a broader specificity of these reagents then a "pure" anti-idiotypic reactivity. On the other hand, serologically defined idiotypes have been detected in up to 0.6% of all natural serum Ig, figures which are not much lower than those obtained by staining background plasma cells in normal mice. This slight discrepancy could be accounted for either by a difference in sensitivity of the detection method or by the fact that our data refer to the representation of these idiotypes in the IgM population, where germ-line-type, "natural" idiotype-positive antibodies are mostly concentrated [52, 54] and which is only 10–15% of the total immunoglobulins in normal sera. We definitely prefer the last alternative.

The frequency of activated cells producing antibodies recognized by the stimulatory anti-idiotypes, while firmly excluding the surface representation of the "idiotypes" on immunoglobulin receptors, is too low to indicate whether or not the same mature, immunocompetent cell can express cross-reactive idiotypic determinants on both Ig molecules and non-Ig mitogen receptors. This question will be further discussed below.

*Polyclonal Reactivity of Monoclonal Anti-Idiotypic Antibodies*

As pointed out above, the introduction of monoclonal reagents into the studies on idiotypes made it possible to analyze single idiotypic determinants or "idiotopes" instead of dealing with mixtures of antibodies to mixtures of determinants. For our experiments, these reagents appeared of primary importance. Thus, our studies concerned surface molecules on B cells which *cross-reacted* with Ig variable region determinants and therefore are considered to express *some but not all* of the idiotypic determinants of antibodies. It would be extremely important to determine which types of idiotopes are expressed by these non-Ig molecules (and which types are not) in order to: (1) clearly demonstrate whether we were looking at "real" idiotypic determinants on hypervariable segments of V-regions or whether our results were due to broad reactivities of the reagents including subgroup specificities or other anti-framework antibodies; (2) attempt to find the general rules for our findings which we had partially failed to conclude from experiments using conventional antisera; (3) dissect the relationships between single determinants on antibody receptors and non-Ig mitogen receptors expressed by individual B lymphocyte clones; (4) exclude beyond doubt that our results were due to reactivity of the anti-idiotypic reagents with surface Ig on B lymphocytes. Part of these studies are still in progress and we will present here only the experiments necessary to substantiate the basic points.

We had expected that amongst all distinct idiotopes present on a given myeloma protein, only some would also be expressed on the non-Ig molecules we were detecting; that is, amongst all distinct anti-idiotopic antibody species present in a conventional anti-idiotype antiserum, only some would display polyclonal properties. Consequently, the extensive testing of monoclonal reagents would certainly reveal "positive" and "negative" antibodies in our tests. This prediction was confirmed by the testing of not more than half a dozen of such reagents. Thus, we

have so far found several monoclonal antibodies which fail to bind to detectable numbers of B cells and to display polyclonal mitogenicity, while the corresponding conventional reagents did contain antibodies with these properties. This is the case with a monoclonal antibody against an idiotope present on J558 myeloma protein (a gift from J. Kearney) and another specific for a TEPC15 idiotope (a gift from C. Heusser). In contrast, we have also found one monoclonal antibody with specificity to determinant(s) expressed by the MOPC460 myeloma protein [14], which has all the polyclonal properties previously detected with the conventional syngeneic anti-idiotypic antisera; namely it "stains" up to 30% of all splenic B cells and it stimulates large numbers of these into proliferation and polyclonal antibody secretion.

This type of analysis was pursued one step further by comparing the polyclonal properties of several monoclonal antibodies, all specific for idiotopes composing a "major" idiotype produced in CBA or C57BL mice upon immunization with the hapten NP. This "idiotype" had previously been treated as a single entity [54, 55], but it has now been shown to be composed of a variety of antibody species, as demonstrated by preparing monoclonal idiotypic antibodies [68] and by the analysis of these and of the immune sera with monoclonal anti-idiotype antibodies [69]. We have tested three such monoclonal anti-NP idiotype antibodies, and have found only one of them to be positive in our assays. Tables 7 and 8 show some of these results obtained with monoclonal anti-idiotypic antibodies.

From the pattern of reactivities observed with this very limited set of reagents, several conclusions can already be derived:

1) Polyclonal reactivity is not a general property of all anti-idiotypic antibodies. *The observation that several of these monoclonal reagents fail to bind and to be mitogenic for large sets of B cells in the same assays as we find others to be positive, provides the negative control of choice for these experiments, and therefore, the best demonstration of specificity in the polyclonal reaction we are dealing with.* Thus, while one of the anti-NP[b] monoclonal antibodies recognizes an epitope present in the *majority* of all primary anti-NP antibodies produced in C57BL/6 mice and is negative in our assays, another, which recognizes an epitope expressed on a *minority*

**Table 7.** Polyclonal reactivity of monoclonal anti-idiotypic antibodies: detection of cross-reactive determinants on B lymphocytes

| | | % IgM positive cells stained in | | | | |
|---|---|---|---|---|---|---|
| Donor of antibody | Antibody | BALB/c | A/J | CBA/J | SJL | DBA/2 |
| SJL | anti (anti-NP) As 79[a] | 21.1 | 17.5 | 15.3 | 12.4 | 9.4 |
| CBA | anti (anti-NP) Ac 38[a] | <0.01 | <0.01 | <0.01 | <0.01 | <0.01 |
| SJL | anti (Id H/k) As 87[ab] | 0.2 | 0.4 | N. D. | 0.3 | 0.7 |
| BALB/c | anti-MOPC460 F6 (51)[c] | 25.1 | 26.5 | N. D. | 22.2 | 31.0 |

[a] A gift from Dr. M. Reth (Ref. 69)
[b] The idiotypic immunoglobulin, without antibody activity, is made up of specific anti-NP heavy chains, and MOPC21 myeloma $\kappa$ chains (Ref. 69)
[c] A gift from Dr. P.-A. Cazenave (Ref. 9 and 14)

**Table 8.** Polyclonal stimulation of spleen B cells by monoclonal anti-idiotypic antibodies

| Specificity of anti-Ig antibody | Concentration of antibody or mitogen | Mouse strain of spleen cells | TdR: CPM $\times 10^{-3}$ culture | IgM PFC/culture |
|---|---|---|---|---|
| *Expt. 1* | | | | |
| no | – | BALB/c | 4.2 | 176 |
| LPS | 50 μg/ml | BALB/c | 53.1 | 20,466 |
| anti-J558[a] | 5 ng to 50 μg/ml | BALB/c | 2.2–4.8 | 279–650 |
| anti-TEPC 15[b] | 2.5 ng to 25 μg/ml | BALB/c | 1.8–4.1 | 73–291 |
| *Expt. 2* | | | | |
| no | – | C57BL/6 | 2.9 | 26 |
| LPS | 50 μg/ml | C57BL/6 | 46.0 | 8,741 |
| anti-(anti-NP) As79[c] | 50 ng/ml | C57BL/6 | 22.8 | 2,040 |
| anti-(H/kId) As87[c] | 5 ng to 50 μg/ml | C57BL/6 | 2.6–3.1 | 28–66 |
| anti-(anti-NP) Ac38[c] | 5 ng to 50 μg/ml | C57BL/6 | 2.6–3.5 | 25–45 |
| no | – | C3H/Tif | 5.9 | 840 |
| LPS | 50 μg/ml | C3H/Tif | 52.8 | 25,840 |
| As79 | 50 ng/ml | C3H/Tif | 45.3 | 16,800 |
| As87 | 5 ng to 50 μg/ml | C3H/Tif | 3.7–6.0 | 623–1, 196 |
| Ac38 | 5 ng tp 50 μg/ml | C3H/Tif | 3.2–6.2 | 590–956 |
| *Expt. 3* | | | | |
| no | – | BALB/c | N. D. | 145 |
| LP | 50 μg/ml | BALB/c | N. D. | 15,760 |
| As79[c] | 50 ng/ml | BALB/c | N. D. | 39,240 |
| *Expt. 4* | | | | |
| no | – | BALB/c | 1.5 | 30 |
| LPS | 50 μg/ml | BALB/c | 17.2 | 12,800 |
| anti-MOPC 460 F6(51)[d] | 2.5 μg/ml | BALB/c | 14.2 | 13,733 |
| no | – | C57BL/6 | 1.7 | 89 |
| LPS | 50 μg/ml | C57BL/6 | 37.3 | 19,400 |
| anti-MOPC 460 F6(51) | 2.5 μg/ml | C57BL/6 | 18.9 | 10,033 |
| no | – | C3H/Tif | 5.6 | 1,090 |
| LPS | 50 μg/ml | C3H/Tif | 60.0 | 20,266 |
| anti-MOPC 460 F6(51) | 2.5 μg/ml | C3H/Tif | 67.1 | 16,466 |

See footnote to Table 2 for conditions of culture. TdR uptake was measured on day 3 in all experiments, while the IgM PFC response was measured on day 4 in experiments 1 and 2, on day 6 in experiment 3, and on day 5 in experiment 4

[a] a gift from Dr. J. F. Kearney, University of Alabama, Birmingham
[b] a gift from Dr. C. Heusser, Ciba-Geigy
[c] gifts from Dr. M. Reth (Ref 69)
[d] a gift from Dr. P.-A. Cazenave (Ref. 9 and 14)

of antibodies, is a polyclonal reagent. This finding provides a strong argument against the possibility that we are detecting very low affinity reactions between anti-V region antibodies of broad specificity and large sets of Ig molecules.

2) Some bona fide idiotopes do cross-react with non-Ig structures on B lymphocytes surfaces. Both monoclonal anti-idiotypic antibodies found to react with at least 20% of all splenic B cells are combining-site related [14, 69]. This would

indicate their specificity for the determinants made up of contact residues or, at least, amino acid sequences on hypervariable regions, which should be considered idiotypic determinants par excellance. The stringent specificity of these antibodies in their reactions with immunoglobulins has been established indeed [14, 69].

3) The binding of anti-idiotypic antibodies to large sets of B cells does not correlate with the subclass of the antibodies and, therefore, it is likely to depend exclusively on their combining-site specificity. The monoclonal anti-MOPC460 antibody – which is "positive" – is an $IgG_1$ molecule and one of the anti-$NP^b$ monoclonal antibodies (Ac38), which is negative – is also $IgG_1$. On the other hand, the other positive antibody (As79) – anti-$NP^b$ Id – is an $IgG_{2b}$ molecule, but a third monoclonal antibody directed against idiotope(s) on molecules composed of anti-NP heavy chains and MOPC21 light chains (As87) – which is negative – is also an $IgG_{2b}$. This provisional conclusion is now being directly tested by using $F(ab)_2$ reagents prepared from the positive antibodies.

4) Although the number of different monoclonal antibodies tested is far too limited to give us solid indications as to the characteristics of Ig idiotopes that cross-react with non-Ig polyclonal B cell receptors, it might not be fortuitous that both of the "positive" antibodies are "hapten-modifiable" and therefore classified as combining-site related.

### *The Antibody Repertoire of Mature B Cells Expressing "Idiotype-Cross-reactive" Mitogen Receptors*

As will be discussed below, the finding of Ig-idiotopes on B cell mitogen receptors introduced the possibility of considering functional, triggering receptors included in a larger network of idiotype-anti-idiotype interactions. This idea would offer the functional basis missing in the network theory and it would simultaneously provide the tools to deal with selection of available repertoires in dynamic "steady states" characteristics of the immune system. It is clear that the mode of participation of such mitogen receptors in those processes has to be considered in relation to the idiotypic specificities of the antibody receptors simultaneously expressed by individual B lymphocytes. On the other hand, the large polyclonal representation of the various "idiotype-cross-reactive" mitogen receptors would necessarily indicate that individual B cells express more than one of these specificities. Since we know that clonal selection theories are correct as to the monospecificity of all the antibody receptor combining-sites [36, 63, 66] and idiotypes [34, 70], the *problem is framed into the study of the antigenic relationships between a single antibody idiotype and multiple mitogen receptor idiotypes expressed by single cells.*

We have first directly confirmed the supposition that single B cells express more than one polyclonal "idiotypic" specificity. As shown in Table 9, by staining the same B cell populations with two different anti-idiotypic antisera labeled with distinct fluorochromes, we could directly show the multiple expression of these determinants by single cells. The "overlaps" in cell populations stained by two different anti-idiotypes varies for each pair of reagents tested, but it consistently falls within 10 and 70% of all cells positive for each of the reagents. This observation constitutes a very strong argument in favor of the "idiotypic" specificity of these reactions and against the possibility that the antisera react with surface Ig receptors on these large

**Table 9.** Membrane expression of determinants cross-reactive with more than one idiotype by the same cells

| Staining reagent | Strain | Positive cells % $IgM^+$ cells | % cells stained[a] "Pure" | "Double" |
|---|---|---|---|---|
| BALB/c anti-MOPC 460 | | 20.8 | 42 | |
| + | BALB/c | | | 30 |
| A/He anti-W3129 | | 19.9 | 28 | |
| A/He anti-J558 | | 21.1 | 17 | |
| + | BALB/c | | | 68 |
| A/He anti-W3129 | | 19.5 | 15 | |
| A/He anti-J558 | | 20.2 | 50 | |
| + | BALB/c | | | 10 |
| Rabbit anti-MOPC 104E | | 14.6 | 40 | |
| BALB/c anti-MOPC 460 | | 20.1 | 16 | |
| + | DBA/2 | | | 40 |
| A/He anti-W3129 | | 37.9 | 44 | |
| A/He anti-J558 | | 13.5 | 15 | |
| + | DBA/2 | | | 30 |
| A/He anti-W3219 | | 38.3 | 55 | |

The percentage of "idiotype" positive Be cells was determined as described in the legend to Table 1

[a] For double stainings the A/He anti-W3129 was labeled with arsonate and detected by rhodamine-labeled rabbit anti-arsonate antibodies (courtesy of Dr. S. Cammisuli); the rabbit anti-MOPC 104E was used unlabeled and detected by rhodamine-labeled sheep anti-rabbit antibodies. The other antibodies were labeled with trinitrophenyl and detected by fluorescein-labeled goat anti-DNP antibodies. The staining was performed by exposing the cells to a mixture of two anti-idiotypic antibodies and then to a mixture of the detecting fluorescent reagents. Samples are screened field by field under specific illumination for fluorescein and rhodamine, and "pure green", "pure red", and "yellow" cells recorded

populations of B cells. Thus, each reagent binds only to a fraction of all B cells and, in addition, for each pair of reagents we have always found that a sizable fraction of all positive cells could react with one, but not with the other antiserum. In view of the monospecificity of Ig-receptors on single B cells, these results exclude reactivity of the antisera with surface antibody receptors. The extent of "overlaps" found with each pair of reagents is also variable with the strain of the spleen cell donor. This could suggest interesting possibilities on the genetic control of the selection of available repertoires, if the hypothesis on the interdependence of idiotope expression on antibodies and mitogen receptors were to be confirmed.

These results give us no indication about the expression of the various "idiotypic" determinants by the same cells on the same or on distinct molecules. Although the relative distributions of positive cells would suggest the likelihood of each determinant being associated with a distinct molecule, we cannot exclude, on the basis of these results, that all "idiotypic" determinants are present on the same complex surface structure which is not identical on all B cells.

Unfortunately, fluorescence techniques limit to two the number of reagents that can be tested simultaneously and, therefore, we have no indications as to "how multiple" the expression on single cells of these "idiotypic" determinants might be. In any case, again by inferring from the relative distribution of "overlaps" with several different pairs of reagents, we can safely conclude that each B lymphocyte expresses several, but not all, such determinants. The final conclusions on these questions will have to be reached using biochemical techniques, but the notions that already emerge from our results are in good agreement with the multiple expression of mitogen receptors by single B cells, as previously determined by functional responsiveness [4, 39].

These conclusions raise the central question of the degree of diversity of this set of non-Ig B lymphocyte receptors. We have at present no answer to this question nor a clear experimental approach to it, but we shall indicate below how large a "diversity" we would like to theoretically ascribe to this collection of receptors.

The general problem of the distribution of antibody V-gene specificities amongst B cell subsets, defined by functional reactivities to mitogens or by the expression of "idiotypic" mitogen receptors, will certainly require a long and systematic effort that has only recently been initiated. It suffices here to say that the current postulates of the random distribution of antibody specificities among mitogen-reactive B cell subsets [3, 21] may not be correct, as recently suggested by the results obtained in the polyclonal stimulation of B cells with T cell help [5]. While waiting for this complete, taxonomic description of the B cell system, we can ask a question that considers the central point in our considerations. Can a single, immunocompetent B lymphocyte express the same idiotypic determinant(s) both on antibody and mitogen receptors? The answer to this question would be of relevance for the elaboration of models explaining the regulation of available repertoires. The simplest experiments would consist of stimulating normal mature B cells with a particular anti-idiotype and measuring the occurrence of this idiotype in the polyclonal antibody response. As already discussed, an experiment of this type is shown in Table 6, but the results are not easily interpretable due to the very small numbers of cells secreting idiotype-positive antibodies. This difficulty may be overcome by measuring antibody secretion in a plaque assay where large numbers of cells can be screened for a single specificity. This was done by stimulating splenic B cells from BALB/c or C57BL/6 mice with anti-J558 anti-idiotypic antisera and measuring both the total numbers of IgM plaque-forming cells (PFC) and the numbers of cells secreting specific anti-$\alpha$, 1–3 linked dextran antibodies included in the response. As shown by other workers [8, 16], the majority of BALB/c anti-dextran PFC secrete antibodies bearing idiotypic determinants in common with that myeloma protein, while very few, if any, of the C57BL/6 anti-dextran PFC display this characteristic.

If the polyclonal stimulation with anti-idiotypic antibodies results in the production of anti-dextran specific PFC in BALB/c spleen cells, it is likely that cells expressing J558-like mitogen receptors – that would be stimulated – would also produce antibody molecules bearing the same determinants. As shown in Table 10, however, no anti-dextran PFC are included in the polyclonal response of BALB/c spleen stimulated by anti-J558. In contrast, the lipoprotein (LP)-induced polyclonal response of BALB/c cells does include anti-dextran PFC, as well as the anti-J558

**Table 10.** Failure of anti-J558 antibodies to induce anti-dextran α-1, 3 PFC in BALB/c mice and their ability to inhibit mitogen-induced development of specific PFC

| Donor of spleen cells | Additions to cultures | PFC/culture | | Ratio of specific/total IgM PFC |
|---|---|---|---|---|
| | | anti-dextran α-1, 3 PFC | IgM PFC | |
| BALB/c | no | <5 | 804 | – |
| | LP 50 μg/ml | 65 | 31,350 | 1/482 |
| | anti-J558 1:200 | <5 | 10,680 | <1/5,000 |
| | LP+anti-J558 | 32 | 99,400 | 1/3,101 |
| C57BL/6 | no | <5 | 983 | – |
| | LP 50 μg/ml | 73 | 33,060 | 1/452 |
| | anti-J558 1:200 | 53 | 41,600 | 1/785 |
| | LP+anti-J558 | 76 | 71,400 | 1/939 |

Normal spleen cells from the indicated strains were activated by either lipoprotein or the indicated dilution of an IgG fraction purified from A/J anti-J558 antiserum made specific by convenient absorptions. Specific PFC were detected by red cells coupled with dextran B1355, in the presence of soluble dextran B512 to inhibit anti-dextran α-1, 6 specificities. All the responses were measured after four days of culture

induced polyclonal response of C57BL/6 (Coutinho, Blomberg, and Forni, unpublished work). Since these results cannot be explained by passive transfer of anti-idiotypic antibodies to the plaque assay – because of the very different concentrations of antibodies required to inhibit PFC and to stimulate spleen cells in culture, in addition to the extensive washing of the cultured cells prior to the plaque assays, we conclude that the polyclonal induction with anti-J558 idiotype antibodies does not lead to the activation of B cells to secrete antibodies bearing J558 determinants.

This observation could be explained in at least two quite different ways. One possibility is that cross-reactive idiotypic determinants are mutually exclusive on mitogen and antibody receptors in the same cell. This would not necessarily be determined by constraints in the control of expression of the genes encoding these structures, but it could well be result of somatic selection of precursor cells. The above results, however, could also be explained by postulating that even if such "double idiotypic" B cell exist, the anti-idiotypic antibodies which, on the one hand, interact with mitogen receptors and activate the cell to clonal expansion, on the other hand also react with antibody receptors on that cell and, therefore, inhibit the terminal differentiation to antibody secretion in the activated clone. This is a well documented functional property of anti-μ or anti-κ antibodies reacting with surface IgM molecules on B cells stimulated by a polyclonal mitogen, namely inhibition of maturation to Ig synthesis without impairment of the proliferative response [2, 46]. The mechanism for this inhibition is undetermined and several alternative explanations have been proposed by various authors [2, 5, 25, 46, 73]. Furthermore, the biologic significance of the phenomenon is totally unclear. It is very surprising that the binding of antigen or antibody to surface IgM receptors on B cells has such distinct functional effects. Thus, while the former is claimed to be either of no functional significance or stimulatory when in the presence of "second signals", the latter is clearly inhibitory. One explanation for this discrepancy could be invoked by

purists of the specificity of signalling to B lymphocytes, by arguing that while anti-μ antibodies bind to "irrelevant" parts of the receptor molecules, the antigen is recognized by specific combining sites and this reaction has different consequences in the generation of signals [12, 19].

It appeared, therefore, important to test the ability of anti-idiotypic antibodies to suppress the LP-induced PFC responses of B cells secreting the relevant idiotypes, since we could obtain further insight into the mechanism of anti-Ig inhibition of maturation in activated B cells and simultaneously assess the validity of the alternatives posed by the results shown in Table 10. As also presented in Table 10, we have found that anti-idiotypic antibodies to J558, if added to cultures of BALB/c spleen cells undergoing stimulation by LP, suppress the appearance of anti-dextran PFC in these cultures. The inhibition is idiotype-specific, as shown by the fact that the total IgM polyclonal PFC response remains unaltered or is even expanded, and because it is not observed in C57BL/6 mice, as expected, since this strain does not express J558 idiotypes in a sizable fraction of anti-dextran antibodies.

This experiment demonstrated the ability of antibodies to the variable regions of IgM receptors to mediate "anti-Ig inhibition" of B cell maturation to synthesis and secretion of antibodies. It also provided an additional demonstration that anti-idiotypic antibodies react with a large fraction of B cells by recognizing surface molecules which are not IgM receptors. Furthermore, these results indicate that the mature B cell compartment represented in the spleen of BALB/c mice does not express detectable numbers of anti-dextran antibody specificities which do not bear J558 idiotypic determinants. In contrast, these experiments dismiss our possibilities of choosing between the two alternatives on the double expression of cross-reactive idiotypic determinants on mitogen and antibody receptors by the same B lymphocyte. These cells, therefore, may well exist but they cannot be induced to secrete specific antibodies by stimulation with anti-idiotypes. As discussed below, however, we prefer the alternative that excludes them from the immunocompetent cell pool.

Experiments of this type could be advantageously carried out with monoclonal reagents, since by using the mixtures of specificities present in conventional antisera it is impossible to ascertain whether the antibodies that stimulate by interaction with mitogen receptors are the same which suppress via IgM receptors. By looking at single idiotopes with monoclonal antibodies, this question can be asked with precision. Unfortunately, the monoclonal anti-idiotypic antibodies with mitogenic characteristics that we have found react with such a minority of all Ig molecules that their detection among polyclonal populations of antibodies faces serious difficulties. In addition, only a few percent of all the polyclonally induced PFC in the relevant strains with specificity to the corresponding hapten (NP/NIP or TNP) bear these idiotopes and, therefore, no major changes in the composition of the antibody responses induced by LPS or by these anti-idiotypes would be expected. We have found in preliminary experiments that the frequency of anti-hapten PFC among all IgM-secreting PFC induced by LPS is approximately the same as that observed after induction by the monoclonal antibodies (unpublished observations). These studies have to wait for the development of more monoclonal reagents with these characteristics.

## The Reaction of Anti-Idiotypic Antisera with Precursor Cells

### *Polyclonal Reactivity of Conventional and Monoclonal Anti-Idiotypic Antibodies with Ig-Negative Cells in Bone Marrow and Fetal Liver*

Two quite different indications led us to investigate the expression of idiotypic determinants on precursor cells. On the one hand, the finding that the majority of splenic cells stained by anti-idiotypic reagents were μ-positive but δ-negative suggested that these cells were at a rather early stage along the differentiative pathway of B cells. The possibility that early B cells and B cell precursors expressed mitogen receptors was in good agreement with previous findings on the ability of dextran sulfate to stimulate bone marrow and fetal liver cells into proliferation and enhanced plaque responses upon rechallenge with LPS [38]. Other workers have also found that adult bone marrow cell populations and neonatal spleen cells develop large memory responses upon challenge with thymus-independent mitogens, such as hapten-ficoll conjugates [58] and pneumococcal phosphorylcholine (Augustin, personal communication), indicating the ability of precursors for Ig-secreting cells to be directly triggered into mitosis by such ligands.

These observations, on the other hand, contrast with the well established finding that responsiveness to other B cell mitogens, such as LPS, is only acquired after the precursors have differentiated into B lymphocytes expressing surface Ig molecules [57]. This acquisition of responsiveness appears to correspond to the expression of serologically detectable triggering receptors for LPS which are exclusively present on Ig-positive cells in the spleen and bone marrow [31, 33]. In addition, most of the cells expressing serologically defined LPS receptors, and that are therefore LPS-reactive, are $\mu^+$ und $\delta^+$, suggesting their more advanced differentiation stage [32, 72]. Other mitogens, such as PPD or lipoprotein, appear to activate B cells at an even later stage of differentiation, as previously pointed out by us and others [37, 50]. The independent indication for the expression of idiotypic determinants on precursor cells was provided by purely theoretical speculations on the role of such determinants in the process of precursor cell expansion and selection into the immunocompetent cell pools, as will be discussed below.

We have therefore investigated the reactivity of a number of anti-idiotypic antisera and purified antibodies with cell populations containing B cell precursors, namely fetal liver and adult bone marrow. Mature B lymphocytes are first detectable in murine fetal liver by days 16–17 of gestation, as demonstrated by the expression of surface IgM in a very small number of cells with morphologic characteristics of lymphocytes [67]. Before this point in embryonic development, there is also general agreement that no antigen or mitogen-reactive cells are present in the liver, but that these populations contain large numbers of precursor cells already committed to differentiate along the B cell line [56, 64]. The failure to stain day 15 fetal liver cells with anti-μ antibodies confirmed these previous reports. Most importantly however, a sizeable fraction of these cells could be specifically stained by anti-idiotype reagents. Already from day 13 of gestation positive cells are detected, and from then to day 19 their frequency increases steadily, both in vivo and if fetal liver cells are allowed to differentiate in culture (Tables 11 and 12). The evidence for the specificity of these staining reactions has been previously described

**Table 11.** In vivo development of "idiotype"-positive, Ig-negative fetal liver precursors[a]

| | Cells bearing "idiotype-cross-reactive" membrane structures as % of total cells | | | |
|---|---|---|---|---|
| Idiotypic marker | Days of gestation 13 | 14 | 15 | 16 |
| J558 | 0.09 | 0.45 | 0.78 | 1.46 |
| TEPC15 | | 0.07 | 0.77 | 1.2 |
| MOPC460 | 0.1 | | 0.82 | 0.90 |
| W3219 | 0.09 | | 0.79 | 1.07 |

[a] $(C57BL/6 \times DBA/2)F_1$

on the basis of the absence of stainings with other antibodies of the same classes but without anti-idiotypic activity, and of the specific inhibition of the stainings with the corresponding ligands [31].

An interesting observation to stress here is the fact that none of the cells stained on the membrane with the anti-idiotypic reagents could be double-stained in the cytoplasm with anti-μ antibodies. Thus, μ-containing surface Ig-negative cells have been described in these populations already from day 13 and 14 of gestation and postulated to be immediate progenitors of mature B cells [13, 67]. Our findings demonstrating that the cyt $\mu^+$, $m\mu^-$ "pre-B cells' are not included in the $m\mu^-$, $Id^+$ cell population that we are describing, would suggest pathways of B cell development different from those currently accepted. It should be pointed out that the evidence suggesting that such "pre B-cells" are precursors is only circumstantial and other possibilities for their biologic significance must be considered.

**Table 12.** In vitro development of J558 Id-positive fetal liver precursors and its stimulation by the relevant anti-idiotypic antibodies[a]

| | Cells bearing "J558-cross-reactive" membrane structures as % of total cells | | | | | |
|---|---|---|---|---|---|---|
| Period of in vitro culture corresponding to gestation time | Culture started on day 13 | | 14 | | 15 | |
| | 0.09 | | 0.45 | | 0.78 | |
| | no | anti-J558 | no | anti-J558 | no | anti-J558 |
| Day 16 | | | 0.61 | 1.93 | | |
| Day 17 | | | | | 0.90 | 2.0 |
| Day 19 | 1.83 | 3.89 | 1.71 | 4.68 | | |
| Day 20 | | | | | 2.3 | 4.8 |

[a] $(C57BL/6 \times DBA/2)F_1$

Furthermore, different types of results that are often contradictory have accumulated around this issue, namely in relation to the questions on the surface expression of IgM molecules – that have been reported in biochemical and rosetting experiments, but not in fluorescence – and the production of whole IgM molecules or of isolated μ chains. Whole IgM secretion was first reported in very early fetal liver cell populations and attributed an embryonic origin by biosynthetic labeling, immunoprecipitation, and gel analysis of the secreted products. Thereafter, a number of reports described the existence of "pre-B cells" defined by immunofluorescence as producing whole IgM molecules [67]. Lately, however, evidence has been presented for the inability of early fetal liver cells to produce IgM and for the intracellular expression of single μ chains, both by fluorescence and by biochemical techniques [13]. It appears, therefore, that the definition of pre-B cells on such a basis must await further clarification. In particular their progenitor-descendent relationships with B cells must be established before they can be considered precursors.

On the other hand, our failure to detect fetal liver and bone marrow cells simultaneously expressing intracellular μ chains and surface cross-reactive idiotypic determinants could indicate that either the two cell types are independent and only one of them is, in fact, a precursor cell for B lymphocytes, or else that they are sequential stages in the process of B cell differentiation.

Very similar findings were obtained by the staining of adult bone marrow cell populations with these reagents. With some reagents, including the monoclonal ones, the Id-positive cells constituted an impressively large fraction of all bone marrow cells (Table 13). These numbers would certainly account for most of the B lymphocyte precursors if all positive cells were members of the B cell lineage. As described before for fetal liver cells, most of the positive cells can be doubly stained by another anti-Id antiserum and consequently it is likely that each of these reagents can mark the majority of all the cells in this category. This finding, on the other hand, solves the problem with numbers of positive cells upon testing a few anti-Id specificities only. As discussed above for the splenic positive cells, we have at present

**Table 13.** Expression of "idiotype-cross-reactive" determinants on $\mu^+$ and $\mu^-$ bone marrow cells

| Anti-idiotypic antibody | "Idiotype-positive" cells % total cells[a] | | | | |
|---|---|---|---|---|---|
| | BALB/c | C57BL/6 | A/J | SJL | DBA/2 |
| BALB/c anti-J558 | 0.8/2.8 | 1.0/4.9 | 1.9/4.6 | 4.3/1.3 | 2.7/3.8 |
| A/He anti-EPC109 | 2.0/22.9 | 4.9/6.1 | 0.5/10.1 | 1.0/3.1 | 0.9/0.4 |
| A/J anti-TEPC15 | 2.0/18.1 | 4.5/9.6 | 1.3/46.2 | 1.6/7.0 | 3.7/10.1 |
| anti-(anti-NP) As79[b] | 1.1/13.5 | 1.2/7.0 | 0.5/14.2 | 0.5/6.1 | 2.0/4.5 |
| anti-(anti-NP) Ac38[b] | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| anti-MOPC460 F6(51)[c] | 1.6/25.4 | 2.6/7.0 | 2.0/27.4 | 1.5/4.4 | 2.4/10.9 |

[a] The values reported refer to $Id^+\mu^+/Id^+\mu^-$ cells, respectively. The total value of $Id^+$ cells as percent of total bone marrow cells is the sum of the reported figures.
Staining was performed on pooled bone marrow from 5–10 mice. See legend to Table 1 for the staining technique

[b] Monoclonal anti-idiotypes; see Ref. 69

[c] Monoclonal anti-idiotype; see Ref. 14

no information as to whether all determinants are expressed in the same or in different molecules on the cell membrane or whether a few antigenic determinants on such structures could be seen by various antibody specificities present in the different antisera. In the latter case, not only the molecular structures would be different from Ig, but also their antigenic determinants would differ from those on Ig molecules and a few of these could "cross-react" with a number of distinct idiotypes.

While all $Id^+$ cells in fetal liver were μ-negative both on the membrane and cytoplasm, a fraction of those in adult bone marrow were $\mu^+$. This finding is in agreement with the idea that the $Id^+, \mu^-$ cells belong to the common pathway of B cell differentiation, since the large majority of $\mu^+$ small lymphocytes present in the marrow are newly generated B cells on the way out to the periphery [60]. The large differences in numbers of positive cells detected with each particular anti-Id reagent, as well as the variation in the fraction of all Id cells co-expressing μ – that is maintaining Id expression after acquisition of immunocompetence – could be due to a number of reasons. Perhaps the most attractive possibility is related to the expression of each particular Id on antibody molecules in the mature immune system, its ontogenic development and clonal dominance. On the other hand, it is interesting that among all the B cells ($\mu^+$ cells) in the marrow roughly the same proportion as in the spleen are labeled by each anti-Id. This observation indicated that the splenic content in B cells is largely constituted by short-lived lymphocytes recently produced in the marrow, as independent experiments have suggested (Phillips, personal communication; Freitas, Forni, and Coutinho, unpublished observations). Furthermore, those findings could suggest two independent lines of B cell differentiation, only one of which expresses this type of Id-cross-reactive mitogen receptor structures.

### *Evidence that Ig-Negative, Id-Positive Cells in the Fetal Liver and Bone Marrow Include B Lymphocyte Precursors*

The above reactions, although their specificity was demonstrated, do not indicate the nature of the target cells. Fetal liver and bone marrow cell populations are extremely heterogeneous and contain precursors for all hemopoietic cell lines. We searched, therefore, for direct indications of whether or not the target cells for anti-idiotypic antibodies were in fact B lymphocyte precursors.

This question is difficult to answer with precision, as we are interested in studying a cell for which we have no markers. That is, whenever a cell already expresses surface Ig or becomes mitogen- or antigen-reactive, it is no longer relevant for this question as it is no longer a B cell precursor. Since the definition of a precursor can only be done by the direct demonstration of progenitor-descendant relationships, definitive experiments of this type will have to consist in cloning precursor cells and in demonstrating the correlation between their functional ability to develop into B lymphocytes with any other characteristic under investigation. While attempting to reach this degree of certainty, we have performed experiments that, although not clonal, provide direct functional information on the nature of the target cells for anti-idiotypic reagents in fetal livers and adult bone marrow.

The addition of anti-idiotypic antisera to cultures of fetal liver cells from day 13 of gestation onward has marked effects on the development of B lymphocytes in

these cultures. First, there is an increased proliferation in the cultures that results in higher numbers of recovered cells and can be detected by TdR incorporation. As shown in Fig. 2, four different reagents specific for BALB/c idiotypes markedly increase TdR uptake in (C57BL × DBA/2)$F_1$ fetal liver cell cultures from day 13 of gestation. Most important, the number of B lymphocytes generated in the presence of the anti-idiotypic reagents, as compared to those developed in untreated cultures, is increased ten-fold in most of the experiments (Table 14, Fig. 2) and, in some cases, one- or two hundred-fold. B cells arising in these cultures are defined by their morphologic appearance as small lymphocytes and by surface staining with anti-μ antibodies. This excludes the possibility that we are detecting passively absorbed anti-idiotypic antibodies to non-B cells, since we add to the cultures purified IgG fractions of the corresponding anti-Id antisera and, in addition, anti-idiotypic antibodies in immune sera have never been observed in the IgM class. Anti-μ antibodies or purified IgG fractions from antisera with other specificities do not have these enhancing effects in cell proliferation and B cell generation. LPS also has no influence in these cultures, as expected from the late embryonic development of LPS-reactivity [56]. These findings demonstrate that *anti-idiotypic antibodies have a profound influence on the polyclonal development of B lymphocytes from Ig-negative precursors* suggesting, therefore, that they recognize precursor B cells. The simplest interpretation of the results would be that anti-idiotypic antibodies, specifically recognizing a surface structure on precursor B cells, stimulate these to divide. Probably as a result of this increased precursor cell proliferation, but by completely obscure mechanism, a higher number of mature B cells is generated. These appear to be resting, as we have counted $\mu^+$ small lymphocytes in these experiments,

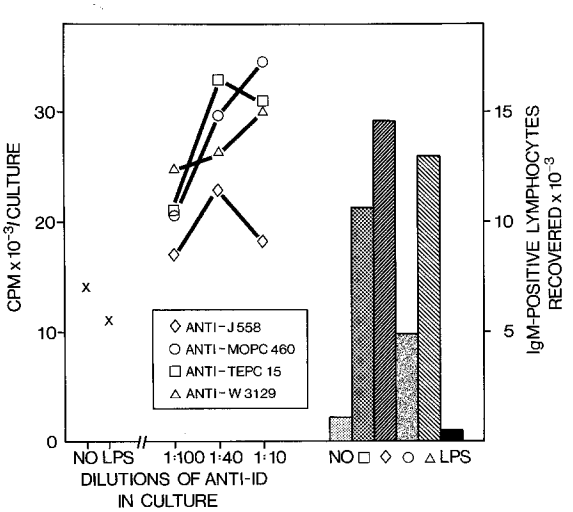


**Fig. 2.** On day 13 of gestation C57BL/6 females mated to DBA/2 males were sacrificed and liver cells from the fetus were placed in 0.2 ml cultures containing $4 \times 10^5$ cells, in the presence of the indicated concentrations of the various reagents. TdR uptake, and the total number of cells and of small lymphocytes expressing surface μ were determined from days 2 to 6 of culture. Figure 1 shows the results observed on day 3 for TdR uptake and on day 6 for numbers of B cells (in cultures stimulated by 1 : 100 dilutions of the antisera). Similar results were obtained on other days of assay.

**Table 14.** Expansion of $Id^+$ cells and enhanced B lymphocyte differentiation in fetal liver cultures stimulated by anti-idiotypic antisera

| | | Cells/culture, input at time 0 | | | |
|---|---|---|---|---|---|
| Expt. 1 day 14 | Addition to culture | Total | $IgM^+$ | $J558^+$ | $TEPC15^+$ |
| | | $3 \times 10^6$ | <300 | 13,500 | 1,980 |
| | | | cells/culture recovered at day 5 | | |
| | no | $3{,}85 \times 10^5$ | 3,773 | 6,583 | 2,196 |
| | anti-J558 1:2,500 | $8{,}14 \times 10^5$ | 46,235 | 38,095 | N. D. |
| | anti-TEPC15 1:2,500 | $3{,}78 \times 10^5$ | 11,831 | 5,216 | 16,594 |
| Expt.2 day 16 | | | cells/culture input at time 0 | | |
| | no | $3 \times 10^6$ | N. D. | N. D. | N. D. |
| | | | cells/culture recovered at day 2 | | |
| | no | $3{,}35 \times 10^6$ | <3,000 | 30,150 | N. D. |
| | anti-J558 1:2,500 | $7{,}31 \times 10^6$ | *54,825* | *146,200* | N. D. |

Fetal liver cells from the indicated days of gestation were cultured as indicated, and harvested five or two days later in Expt. 1 and 2, respectively. The numbers of cells stained with anti-μ, anti-J558 Id, or anti-TEPC15 Id were determined using fluorescence

suggesting that if they are direct descendants from the Id-positive proliferating precursors, they have become refractory to the stimulatory activity of the available concentrations of anti-idiotypic antibodies. Transposed into physiologic situations, this would indicate that *acquisition of immunocompetence by a B cell corresponds to the loss of its ability to be stimulated by whatever driving forces were active at the precursor level.*

B lymphocytes generated in vitro by differentiation of fetal liver cells include LPS-reactive cells [56], even though these should be expected to constitute only a minority of the total population of newly differentiated cells. We can, therefore, assess the influence of anti-Id reagents not only on the generation of small, resting B cells, but also on the development of LPS-reactive cells. This possibility provides the additional advantage of measuring immunocompetence of the differentiated cells, namely their ability to be stimulated by LPS and to give rise to IgM-secreting plaque forming cells.

The stimulation of fetal liver cell cultures by concentrations of anti-Id antisera displaying the effects described above, does not result in the appearance of IgM-secreting PFC, in contrast to the observations with spleen cells. Since large numbers of B cells are generated in these cultures which could be expected to respond to the anti-Id, this finding also suggests that the differentiation of B cells in the presence of an anti-Id antiserum leads to the generation of mature B cells that are unable to respond to the *available concentrations* of that ligand. If the anti-Id stimulated cultures, however, are further activated by addition of LPS, a large increase in the number of IgM-secreting PFC is observed, in comparison with the responses

observed in cultures stimulated by LPS only. The responsiveness to LPS develops in fetal liver cultures only at the time equivalent to day 19 or 20 of gestation [56]. We had shown reactivity to the anti-Id reagents already from day 13 and, therefore, the synergy between anti-Id and LPS appeared to be the result of sequential stimulation of the same cells. We have performed most of these experiments by pre-treating the fetal liver cell cultures with the anti-Id reagents for several days and by sequentially stimulating with LPS in the absence of further addition of anti-Id. The enhanced IgM PFC responses observed in these experiments, an example of which is shown in Table 15, directly demonstrates the influence of anti-Id upon the generation of immunocompetent, LPS-reactive B lymphocytes.

The reverse type of experiments provided more direct evidence for the expression of "idiotypes" by precursor B cells. Fetal liver cell populations, containing no detectable B cells, were exposed to high concentrations of various anti-Id reagents and complement and thereafter cultured under conditions permissive for B cell differentiation. The LPS-induced IgM-PFC responses in these cultures are shown in Table 16. The drastic reduction of the PFC response in pretreated cells directly demonstrates the expression by B cell precursors of determinants recognized by the antisera. Very similar results have been obtained with adult bone marrow cell populations depleted of B lymphocytes by velocity sedimentation or anti-Ig columns. The most definitive evidence obtained so far was provided by experiments in which the killing of Ig-negative, Id-positive bone marrow cells with a monoclonal anti-idiotypic antibody and complement resulted in the corresponding decrease in the numbers of B cell precursors in the population, as measured by limiting dilution analysis of LPS-reactive IgM-secreting clones (unpublished observations).

Taken together these findings strongly support the conclusion that precursor B cells, before they express IgM on the surface, do express other structures which cross- react with Ig-idiotypes and are readily detected by anti-idiotypic antisera. Keeping to the parallel with our previous results with spleen cells, these Id-cross-reactive surface structures are probably growth receptors, as also indicated by the fact that anti-Id antibodies are mitogenic for fetal liver and bone marrow Ig-

**Table 15.** Enhanced differentiation of LPS-reactive, IgM-secreting cells in fetal liver cell cultures stimulated by anti-J558 Id or dextran B1355

| Additions to precultures | Secondary cultures | IgM PFC/$10^6$ cells precultured |
|---|---|---|
| no | no | 700 |
| no | LPS | 11,733 |
| anti-J558 (1 : 2, 500) | no | 150 |
| anti-J558 (1 : 2, 500) | LPS | 31,600 |
| Dx B1355 100 ng | no | 825 |
| Dx B1355 100 ng | LPS | 85,066 |

Day 16 fetal liver cells were precultured ($3 \times 10^6$/ml) in the presence of the indicated reagents for five days. The cultures were then harvested, the cells washed extensively, and recultured ($2 \times 10^5$/ml) in the presence of 50 μg/ml of LPS or without further additions

**Table 16.** Reduction in the numbers of LPS-induced IgM PFC upon treatment of fetal liver cells with anti-idiotypic reagents and complement

| Pretreatment | Additions to culture | IgM PFC/cultures |
|---|---|---|
| *Expt. 1* | | |
| C | no | 8 |
| C + anti-J558 | no | 9 |
| C | LPS | 1,800 |
| C + anti-J558 | LPS | 10 |
| *Expt. 2* | | |
| C | LPS | 3,020 |
| C + anti-LPS receptor | LPS | 3,400 |
| C + anti-J558 | LPS | 255 |
| C + anti-W3129 | LPS | 1,080 |
| C + anti-MOPC460 | LPS | 145 |
| C + anti-MOPC104E | LPS | 1,865 |

All fetal livers were from (C57BL/6 × DBA/2)$F_1$, and rabbit complement was used. After the indicated treatments and washings, $2.5 \times 10^6$ viable cells were set up in cultures containing LPS (50 μg/ml) as indicated, and assayed for IgM PFC seven or eight days later. Expt. 1: Fetal liver cells from day 16 of gestation; the specific cytotoxicity in the presence of anti-J558 was 23%; assay for PFC on day 7. Expt. 2: Fetal liver cells from day 15 of gestation, the viable cell recoveries in the various groups varied from 57 to 76%; assay for PFC on day 8

negative cells. Results shown in Tables 12 and 14 strengthen this conclusion by showing that stimulation of fetal liver cell cultures with anti-Id antisera, in addition to enhancing B cell development, also results in a clearcut selective increase in the numbers of cells recovered from the cultures that express the corresponding Id-cross-reactive membrane structure. This finding can only be explained by the proliferation of the $Id^+$ cells directly stimulated by the specific antibodies, some of which will subsequently complete their differentiative pathway by reverting to a stage of small lymphocyte and acquiring immunocompetence. As also shown in Table 15, the mitogenic ligand corresponding to the anti-Id antibodies, e. g., dextran α-1,3 linked as compared to anti-J558 antisera, is also competent to induce a much higher number of fetal liver cells in parallel cultures to become immunocompetent and develop into IgM secreting PFC upon stimulation with LPS. Since we have previously shown that dextran competes with anti-J558-Id antibodies for the binding to fetal liver cells, we conclude that the surface structures expressed on precursor B cells present in fetal liver which cross-react with Ig idiotypes are growth receptors.

These conclusions prompted us to search for the expression of such mitogen receptors in cell lines of presumed or established B cell precursor characteristics, in order to facilitate further studies in the molecular description of these structures. We have now found several Ig-negative Abelson's lymphomas which selectively express surface determinants regognized by conventional or monoclonal anti-idiotypic antibodies. The absence of detectable staining with several anti-Id reagents in cell lines in which all the cells are positive with one or two similar reagents demonstrates once again the specificity of these reactions and the non-Ig nature of these receptors.

*Consequence of the Interaction Between Anti-Idiotypic Antibodies and Precursor Cells on the Antibody Repertoire of the Differentiated B Lymphocyte Population*

Being aware of the fundamental importance that the above findings might have on the somatic generation of antibody diversity, we have investigated in detail the antibody repertoire of the mature B cell populations expanded and developed as a consequence of the interaction between anti-Id antibodies and $\mu^-$, $Id^+$ precursor cells. Significant experiments must be performed with monoclonal reagents since we are addressing the question of the idiotypic similarity between growth receptors on precursor and antibody receptors on competent cells. These experiments have recently been initiated and we will summarize here the main results obtained so far.

In brief, Ig-negative precursors were purified from the adult bone marrow and stimulated in culture for one week periods by a particular monoclonal anti-Id. The cells are then washed free of antibodies and stimulated, either in mass cultures or under limiting dilution conditions, by LPS. Another week later, we assay for the frequency of LPS-reactive, IgM-secreting clones in those populations, as well as for the frequency of clones secreting antibodies with a given combining site specificity and/or a given idiotype. Control cultures are not stimulated by anti-Id in the first one-week culture, or else two distinct monoclonal anti-Id antibodies are used in criss-cross experiments.

These experiments gave us results that can be summarized as follows. (1) The presence of small amounts of anti-Id monoclonal antibodies in cultures of Ig-negative precursors results in a large increase in the number of cells that can be stimulated by LPS into clonal expansion and IgM secretion. (2) The frequency of IgM-secreting clones with a combining site specificity similar to that of the target idiotype for the monoclonal antibodies used to stimulate the precursor cell cultures is 5- to 10-fold increased, compared to that found in untreated cultures or in cultures stimulated by anti-idiotypic antibodies of different specificity. (3) The frequency of clones secreting IgM molecules bearing the idiotype, on the other hand, is not proportionally increased. Although the different sensitivity in the test system does not yet allow for definite conclusions, it appears that the stimulation of precursor cell cultures with an anti-idiotypic antibody selects *against* the differentiation of mature B cells expressing that idiotope on antibody molecules. As an example, culturing bone marrow precursors with 50 ng/ml of monoclonal anti-MOPC460 antibodies results in a large increase in the numbers of LPS-reactive competent B cells recovered from the cultures. These cells contain 5- to 10-fold more anti-TNP clones than controls but they do not contain more (possibly less) competent cells expressing MOPC460 Id on antibody molecules.

These results contrast with the findings obtained with the stimulation of mature splenic B cells where we observed no detectable influence of the anti-idiotypic antibodies in the repertoire of antibody specificities among stimulated cells other than the suppression of the corresponding idiotype.

## Summing up the Results and Inserting Them in the Immunologic Theory

Considering the results described here, in particular those obtained under the very stringent conditions provided by monoclonal antibodies, we have to conclude that

mature B lymphocytes and their precursors express on their membrane polyclonally distributed structures which cross-react with a number of idiotypic determinants characteristic of various antibodies. *As we do not in any way question the validity of the clonal selection theory, we consider that those surface structures bearing idiotypic determinants are not immunoglobulin in nature.* This conclusion is supported by a large number of observations, in particular those demonstrating that antibodies produced by single cells do not bear the idiotypes expressed by those cells on the membrane, by the demonstration that precursor cells express such idiotypic surface structures *before* Ig chains are expressed, either inside or on the cell surface, and by the genetic control of these determinants which does not agree with that of IgV-genes. On the other hand, the functional data summarized here demonstrate, in our opinion beyond doubt, that the idiotypic determinants are, at least in part, associated with growth or mitogen receptors, both in precursor and in mature cells that can be triggered via appropriate interactions mediated by these receptors.

We do not find it very fruitful at the present stage to speculate on the structural and genetic basis for these cross-reactivities. "Real" hypervariable region idiotypes, or subgroup specificities, or even carbohydrate-associated determinants, each or all of these categories may in fact turn out to be responsible for our observations, without in any way dismissing their interest. Thus, the only fundamental conclusion is that antibodies share clonal markers with polyclonally distributed non-Ig receptors which are multiply expressed by B lymphocytes and their precursors. Since these polyclonal receptors are functionally competent to trigger the cells into proliferation, our observations suggest a number of very attractive possibilities for mechanisms involved in the development and regulation of the immune system. On the basis of the network theory that postulates the availability in the normal immune system of the appropriate anti-idiotypic specificities, our findings could in fact be used to explain:

1) The very high turnover rate of B lymphocytes, by providing the driving force necessary for precursor cell expansion.

2) The selection of precursor cell pools, *before* acquisition of immunocompetence and Ig-expression, in various immune steady states.

3) The mechanisms involved in somatic generation of antibody diversity, including the selection *for* somatic variants.

4) The controlled, sequential expression of germ-line antibody genes in the developing immune system.

5) The influence of maternal antibodies on the antibody repertoire of the progenies.

6) The anti-idiotype mediated suppression and stimulation in the developing or mature immune system.

7) The selection and regulation of antibody repertoires in the absence of (extraneous) antigen.

8) The internal activity of mature cells in the system, namely background plasma cells and natural antibodies in germ- and antigen-free "nude" mice.

In essence, all these phenomena can be satisfactorily explained within a framework in which available *anti-idiotypic antibodies* are the *internal mitogens* able to "screen" individual cells at two structurally and functionally distinct receptor

sites: one that triggers the cell into proliferation, the other that inhibits maturation and antibody synthesis. Postulating various threshold levels of anti-idiotypes necessary for fruitful interactions at each of those two types of receptors, as well as differences in the numbers of receptors of each type expressed by individual cells, a large number of models can be built to deal with the above questions. In addition, while clonal selection rules impose the single expression of antibody idiotypes in each committed cell or precursor, the observed multiple expression of non-Ig idiotypes by single cells allows for the postulate of models in which defined "sets" of such idiotypes are expressed in different subpopulations of precursors, making it possible that anti-idiotypic antibodies may positively or negatively influence the production of a given antibody idiotype depending on the properties of the target cells other than expression of that idiotype on Ig receptors.

Above all, our findings provide the functional basis so far missing in the network theory, in particular in thymus-deprived networks. Thus, apart from a few honorable exceptions, it has been found impossible to stimulate B lymphocytes by the binding of thymus-dependent protein antigens or antibodies to surface immunoglobulin receptors. The conclusion that surface antibodies on B cells are not triggering receptors necessarily deprives the network of idiotypes and anti-idiotypes of all dynamic functionality and it makes it a sterile exercise of static elements. The inclusion of triggering receptors in such a network ensures its functionality by providing the internal mitogens to the system, in addition to allowing for the *completeness of the antibody repertoire without endangering functional, self-nonself discrimination.* In the light of these observations, the immune system contains in itself all the necessary elements to ensure its own activity, development, and regulation. Selection of the antibody repertoire is based on what the system "sees" on the cells (idiotypes) and not on what the cells "see" in the environment (combining sites), which is the only way of explaining the establishment of available antibody repertoires in the absence of antigens. In addition, our findings bring into the idiotypic network a large pool of cells that so far had been considered outside of the system, namely committed B cell precursors before they express Ig. Here again, selection can operate already at the level of the precursor pool, although these cells cannot yet "see," because the system can "see" whether or not their surface idiotypes fit into the particular steady state in which they differentiate. The possibility of dealing with this very large pool of cells and to select the output of precursors into immunocompetence appears particularly important in the context of the postulated Eigen-behavior [44] or recursive properties of the immune system [77].

Some of these questions have been discussed in detail elsewhere [22]. Let us consider here an example of these possibilities by addressing the problem of the selection *for* somatic variants among precursor cells that must necessarily be solved before we can accept theories on the somatic origin of antibody diversity. We follow the network postulates on the availability of anti-idiotypic antibodies in the internal environment where precursor cell generation and differentiation takes place. These could be either maternally derived, contained in the egg, transferred at the placenta or absorbed from the colostrum, or produced locally by background plasma cells or, in ontogeny, by a cell type differentiated for this particular purpose. Let us consider now two different precursor cells in which the same antibody V-region sequence is

translocated and expressed. The difference between these two cells arises from the differential expression of idiotypic growth receptors. Each cell expresses a given set of these, but while in the first cell none of the growth receptors cross-reacts with the antibody idiotype, in the second cell this cross-reactivity exists. In the presence of sub-threshold concentrations of anti-idiotypic antibodies that are insufficient to stimulate precursors, the expression of surface Ig-idiotype will provide the conditions for exposing the cell to higher concentrations of competent antibodies as the whole cell membrane will constitute an immunoabsorbent. In the first cell of our example, this accumulation of anti-idiotypic antibodies will have no functional consequences, as the cell does not express triggering receptors bearing that idiotype. We assume then that the cell proceeds along the differentiative pathway without being expanded and it will become a mature cell expressing a germ-line antibody. In contrast, the second cell in our example is confronted with triggering concentrations of anti-idiotypic antibodies which, by interacting with idiotypic growth receptors, will activate clonal expansion of the target cell. Simultaneously, however, the stimulatory antibodies are likewise interacting with antibody receptor idiotypes and inhibiting further differentiation of the clonal progeny. It follows that such an exhaustive proliferation will be maintained until either the cells die or variants arise which escaped the anti-idiotypic pressure by mutating away from the germ-line idiotype on antibody receptor or on triggering structures. Since the former are those responsible for the "concentration" of stimulatory antibodies, stepwise variation might be envisaged leading to a largely expanded progeny of mature cells expressing variant, somatically derived antibody receptors.

This particular model provides convenient mechanisms for expanding somatic variants without selecting against germ-line gene expression. These are necessary requirements for the process of somatic selection. Thus, as argued above, in order to conciliate the rarity of mutations with the idea that a large part of the antibody repertoire is somatically derived, we must consider mechanisms for expanding variants. On the other hand, since germ-line specificities appear to be frequently expressed in mature cells, the mechanisms of selection for mutants cannot involve an absolute selection against germ-line products. The above model would satisfy both of these requirements and it agrees with the results obtained by exposing precursor cell cultures to anti-idiotypic antibodies. In these experiments we obtained expansion of precursor cells differentiating to immunocompetence by stimulation with anti-Id antibodies, concomitantly with selection against expression of that idiotype on antibody molecules. In addition, we observed a large increase in the number of clones with an antibody combining site specificity similar to that of the original idiotype, suggesting that these represent variants that have lost the idiotype but maintained similar combining sites. These, however, do not account for the increase in the total numbers of IgM PFC clones and, therefore, we consider the latter as variants that mutated away both combining site and idiotypic specificities.

Obviously, the fraction of all competent cells expressing either germ-line products or "filtered" through that mechanism of selection for variants depends solely on how large is the set of idiotypic growth receptors expressed by each individual precursor cell. At the limit, if every cell expresses all the collection of such receptors there would be no germ-line antibody expression, provided that the

idiotypic universe of mitogen receptors equals the idiotypic repertoire of germ-line antibody genes. The last premise is likely to be correct, since this model requires cross-reactivity between the two types of receptors in order to select against an overwhelming predominance of germ-line antibody expression. In fact, we postulate that the distinctive characteristic of germ-line genes is their idiotypic cross-reactivity with mitogen receptors.

These models are clearly compatible with the wealth of observations on suppression of idiotypes by anti-idiotypic antibodies, particularly when these are administered to a developing system. Our views only provide the mechanistic details on how this suppression operates, and in addition they would include three predictions: (1) the suppression of a particular idiotype at the antibody level is accompanied by an increase of the expression of the same idiotype at the growth receptor level; (2) the suppression of a particular antibody idiotype is concomitant with the expansion of variants of that idiotype in the mature cell pool (the affinity and the concentration of the suppressive anti-idiotypes injected will determine how different the variants will be from the gemline idiotype); (3) Ig-negative precursor cell pools can be selected – in terms of their expression of idiotypic growth receptors – before acquisition of immunocompetence.

Clearly, we can only now start to understand the experimental and theoretical possibilities of this approach. Alternative models could postulate higher *available* concentrations of stimulatory anti-idiotypic antibodies under physiologic conditions, or low thresholds for activation of precursor cells, and therefore expect precursor cell expansion before expression of Ig chains. The selective distribution of idiotypic growth receptor "sets" in cell subpopulations and the nonrandom expression of Ig V-genes among these subpopulations would necessarily lead to antibody repertoire selection operating before acquisition of immunocompetence. Yet another alternative model could postulate the inactivation of precursor cells expressing Ig idiotypes recognized by available anti-idiotypic antibodies in the absence of any other non-Ig mediated interactions. This model, however, cannot be accepted in absolute terms, since germ-line antibodies are abundantly expressed in the mature antibody repertoire, but it could be made dependent on higher levels of some but not all anti-idiotypes. Although we assume that *selection operates on the basis of idiotypic and not of combining site specificities*, it is also clear that each antibody can both recognize and be recognized, and consequently these considerations are to be taken in pure network terms, that is, within the "plus-minus" equilibria characteristic of the system [40].

## A Hypothesis on Antibody Repertoires: Specificity of Germ-line Antibody Genes and Their Evolution; Somatic Generation of Further Diversity; Selection of Available Repertoires

We would like here to give the main principles of a hypothesis developed to account for those basic problems in the understanding of the immune system and which uses some of the results and ideas presented in this review. The major evolutionary pressure determining the present day strategies of the immune system is considered to be the defense against intra- and extracellular infectious microorganisms.

Extracellular bacteria appear to be particularly important in the context of B cells and antibodies. The primary postulate in these ideas is that of the *completeness* of the antibody repertoire. Due to the very short life cycles of the invasive microorganism and the consequent possibility of rapid variation in the molecular patterns displayed by the parasite, a successful immune system could only be that endowed with the ability to recognize all the possible alternatives. Such a completeness is primarily based on the degeneracy of antibody-antigen reactions which provide the possibility of recognizing an infinite number of antigens using a relatively small collection of antibodies. Furthermore, the requirements for degeneracy posed by the necessity of completeness, which resulted in the particular evolution of the structure of antibodies and of the effector mechanisms operating in antibody responses, must have also determined the ways by which the responses are activated, since both triggering and effector functions must be suited to degenerated recognition. On the other hand, the completeness of the antibody repertoire encompasses all self antigens and, therefore, the activation of responses must be determined on a basis other than antibody recognition per se, as the system cannot be self-aggressive.

The universality of recognition by a small collection of antibodies not only does not include, but it necessarily excludes the possibilities for detailed recognition. The collection of antibodies ensuring completeness must be relatively small and germ-line-encoded since it needs to be expressed at all times in the development (and maturity) of the individual and it is therefore limited by the numbers of available cells. On the one hand, the small size of the germ-line pool is required from the evolutionary point of view, as it would appear difficult to maintain survival pressures in large numbers of very similar genes and avoid losses of duplicates. On the other hand, the survival value of the *completeness* of recognition cannot be left to the hazards of somatic variation. Individual (somatic) diversification is considered to represent the possibilities of the system to develop detailed recognition through further diversification from the germ-line substrate, and therefore it must be inbuilt in the very system by the nature of its elements and the ways they interact and it must occur as a necessary consequence of these interactions.

In order to provide the necessary evolutionary pressures maintaining germ-line genes in the absence of external ligands (antigens), the germ-line collection of antibodies is viewed as a closed network which is complete both in the repertoire of combining sites and in the idiotypic universe that provides the targets for combining site recognition.

Many such collections of antibodies could be conceived. The origin of the one adopted by the succesful immune system must be reached, we assume, at the functional level. Since a *resting state* of "immunocytes" must have been evolved before diversification – otherwise diversification would provide no advantage – it follows that before the appearance of antibodies, the cells in the primitive immune system could already exist in two distinct functional states, resting and activated, and therefore these ancestors of lymphocytes displayed non-antibody triggering receptors. These are considered equivalent to the contemporary mitogen receptors, polyclonally expressed by B lymphocytes of vertebrates, displaying exquisite specificity for bacterial products. Their specificity for ligands, which are very different from self-components, ensures lack of self-aggression and allows their

inclusion in the germ-line genome and expression in every individual without any requirements for the ontogenic "learning" of self. Probably, when effector molecules (antibodies) evolved and diversified, their original binding specificity was primarily directed to the same "pathogenic" determinants also recognized by the "triggering" receptors (regardless of whether or not antibody genes evolved from those primitive receptor genes). On the other hand, the "nonself" specificity of both antibodies and triggering receptors limited the possibility of expansion within the system to the availability of external stimulation. It follows that further somatic variation of antibodies, being dependent upon extensive proliferation, could not operate in the absence of external stimulation; that is all diversification, if existing at all, would be antigen-directed and consequently epimetheic [59]. We propose that the chance events accompanying the appearance of diversified antibodies solved this problem and introduced the possibilities for the completeness and the prometheic characteristic of the highly evolved immune system of vertebrates [59]. It would appear that antibodies with binding site specificities to determinants expressed on triggering receptors would be advantageous, since they could function as internal mitogens, providing the conditions for expanding the cellular components of the system with the consequent possibilities of diversification prior to introduction of antigens. A network reached by this process of accumulating convenient antibody specificities generated at random would appear as a necessary consequence of the original cross-reactivity between mitogen and antibody receptors.

We propose, therefore, that the accumulation of germ-line genes was determined by either their similarity or their complementarity with mitogen receptors. The survival value of these cross-reactivities is obvious, as we find them in the mammalian immune system. The completeness of the antibody repertoire, on the other hand, appears as the other selective pressure operating throughout evolution to ensure responses against unexperienced molecular patterns, and it must have determined the accumulation of the particular constellation of germ-line antibodies with this and the former properties. A germ-line collection of antibodies which is a complete network, cross-reactive with functionally distinct receptors present on the same cells that express antibodies, provides phylogenetic and ontogenetic autonomy for the immune system, and it ensures self-nonself discrimination without limiting the completeness of antibody recognition.

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