It is now well established that B cells use two distinct receptors to respond to an antigen (1, 2). One of these, surface immunoglobulin receptors, recognize epitopes with high affinity and ensure clonal selection by the antigen. The other class of receptors controls triggering and growth, and recognizes mitogenic moieties on thymus-independent antigens or T-cell derived helper factors in the response to thymus-dependent antigens (1).

The genetic and structural characterization of mitogen receptors is very limited. So far, only the receptor for lipopolysaccharide has been studied in some detail, due to the availability of two mutant strains which appear to carry nonfunctional alleles of the gene coding for that receptor (3-7). Other ways of obtaining reagents specific for these types of receptors appear of great importance, for they are fundamental components of immune reactivity.

We envisaged the possibility that simple molecules, such as polysaccharides, which display thymus independence and, therefore, direct mitogenicity for B cells, could be a useful tool in this search. Our hypothesis was that the same structure in these bifunctional molecules would be specifically recognized both by antibody combining sites and by polyclonally distributed mitogen receptors. The possibility could exist that these two structures, recognizing the same simple determinant, would be similar enough to cross-react when analyzed with an antibody.

We chose to test this possibility in the dextran B1355 system, where good reagents were available and where the genetics of the antibody counterpart were well studied (8, 9). The prediction was that an anti-idiotypic antibody, specific for dextran binding sites on antibody molecules would also recognize the mitogen receptor for dextran B1355, responsible for the polyclonal activation of B cells by dextran.

In this paper we report experiments confirming this prediction, and we start some elaboration on the significance of this finding.

**Materials and Methods**

Anti-J558 idiotypic antisera to the purified myeloma protein J558 (αλ) with anti-dextran B1355 antibody activity was raised in A/He mice. Sera or ascites of BALB/c mice carrying the J558 tumor were immunoadsorbed onto a dextran B1355 polyacrylamide gel (10). A/He mice received weekly injections of J558 subcutaneously in the inguinal and axial node areas and in the hind foot pads. The antigen was in complete Freund's adjuvant, incomplete Freund's adjuvant, and phosphate-buffered saline for the first, second, and subsequent injections, respectively. After four injections mice were assayed for anti-J558 activity by inhibition of J558-dextran B1355 hemagglutination and strong sera were pooled. The antiserum was finally

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absorbed on Sepharose-coupled normal mouse IgG and 19S fraction from normal mouse sera.

**Dextran B1355.** Fraction S from NRRL B1355 was a kind gift from Dr. Allene Jeanes, retired from Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill.

**Immunofluorescence.** An Ig fraction of anti-J558 antiserum was prepared by repeated precipitation with 1.6-M ammonium sulfate and labeled with trinitrophenylsulfonic acid (11). Rabbit anti-mouse \( \mu \)-chains, and goat anti-DNP antibodies were labeled with tetramethylrhodamine and fluorescein isothiocyanate, respectively (12). For the staining, spleen cells were incubated for 30 min in ice with the TNP-labeled anti-idiotype antiserum in the presence of \( 5 \times 10^{-4} \) M TNP-aminocaproic acid, washed thoroughly with balanced salt solution containing 10% fetal calf serum and 10 mM sodium azide and exposed to FITC-labeled anti-DNP antibodies, again for 30 min in the cold. When double staining was performed, the cells were incubated in TRITC-labeled anti-mouse \( \mu \)-antibodies, either before or after the staining with the anti-idiotype antiserum. For redistribution studies, cells were exposed to the first reagent for 20 min in the cold and then for 10 min at 37°C, washed in cold medium and exposed to the other reagents strictly in the cold and in the presence of 20 mM sodium azide. The stained preparations were observed under fluorescence microscopes (Zeiss Photomikroskop II or Leitz Orthoplan) equipped with vertical illuminators and filter combinations specific for fluorescein and rhodamine. Total cells in the microscopic field were counted in phase contrast.

**Cell Cultures.** Cell cultures and reagents were as previously described (13). Briefly, \( 10^6 \) or 2 \( \times 10^6 \) cells were cultured in RPMI-1640 supplemented with glutamine antibiotics, Hepes, 2-mercaptoethanol and 10% fetal calf serum (Gibco U155701D, Gibco-Bio Cult, Glasgow, Scotland) in 0.2-ml aliquots in Microtiter tissue culture plates. Anti-idiotypic antibodies, normal mouse serum, or dextran B1355 were titrated in the cultures, and lipopolysaccharide (LPS), kindly provided by Doctors C. Galanos and O. Luderitz, Max-Planck Institute für Immunobiologie, Freiburg, West Germany, was used as a control. The cultures were assayed at various incubation times for proliferation by measuring tritiated thymidine incorporation, and for B-cell maturation to immunoglobulin-secreting cells, by counting numbers of plaque-forming cells (PFC) in the protein-A plaque assay (14). These tests were performed exactly as described before (5, 14).

**Results**

**B-Cell Mitogenicity of Dextran 1355.** The thymus-independence of the antibody response to dextran B1355 was likely to be due, as postulated before (15) to the B-cell mitogenicity of these molecules. We have purposely tested dextran B1355 for this property, because the low responsiveness of some mouse strains, such as C57BL/6 (8) could be due to lack of recognition of the \( \alpha-1,3 \) determinants either at the antibody level, or at the mitogen receptor level. The latter appeared unlikely, since high responsiveness was allotype linked and antibody low-responders to dextran could develop good anti-hapten responses when dextran was used as a carrier (10), in contrast to the situations where the defect is at the mitogen receptor level (2). It was not surprising therefore, to find that both BALB/c and C57BL/6 mice, high- and low-responders to dextran, respectively, both developed good polyclonal responses to surprisingly low concentrations of dextran (Table I). We conclude that both strains display a functional mitogen receptor for dextran B1355, in a large fraction of all spleen cells. Although frequency determinations have not been performed yet, the relative magnitude of the response, as compared to the response to LPS, would suggest that the dextran-sensitive B-cell subset is roughly three to five times smaller than the LPS-sensitive B-cell subset. The latter has recently been determined by two independent methods to be \( \approx 30\% \) of all splenic B cells (16, 17).

**Antibodies to J558 Idiotype Recognize Dextran-Binding Surface Structures on the Membrane of a Large Fraction of all B Cells.** Having established the presence of a functional mitogen
receptor, polyclonally distributed in a sizable fraction of splenic B cells of both high and low responder mice, we have directly tested the possibility outlined above, namely that the anti-J558 idiotype could in fact recognize the dextran-specific mitogen receptor. As shown in Table II, this appears to be the case. A sizable fraction of splenic B cells, in average from 10 to 20% of all splenic B cells from both BALB/c and C57BL/6 (as well as several other strains) are stained by the anti-idiotype.

To prove that the staining of the cells was actually due to the anti-idiotypic antibodies and not to other antibodies present in the antiserum, and was directed to a molecule involved in the binding of dextran, the staining was repeated after absorption of the antiserum on the J558 myeloma protein or with other myeloma proteins of the same class and type, or performed in the presence of B1355 dextran or of the α-1,6 linked dextran B512. The results are reported in Table III. The staining was significantly inhibited both by the J558 myeloma protein and by dextran B1355,
TABLE III
Inhibition of the Staining of BALB/c and C57BL/6 Spleen Cells by Anti-J558 Idiotypic Antibodies

| Strain   | Inhibitor | J-558 Positive cells | Inhibition |
|----------|-----------|----------------------|------------|
| BALB/c   | None      | 5.4                  | %          |
|          | Dx B1355* | 1.2                  | 78         |
|          | Dx B512   | 5.7                  | 0          |
|          | J558      | 1.4                  | 75         |
|          | MOPC 315+‡| 4.8                  | 12         |
|          | RPC 20    | 5.1                  | 6          |
| C57BL/6  | None      | 5.4                  | %          |
|          | Dx 1355*  | 1.4                  | 75         |
|          | Dx 512    | 5.7                  | 0          |
|          | J-558     | 1.01                 | 82         |
|          | MOPC 315+‡| 5.1                  | 6          |

* Dextran B1355 and dextran B512 were present in the staining mixture at a concentration of 1 mg/ml. Dextran B1355 carries α-1,3 linkages to which J558 antibodies bind, while dextran B512 has only α-1,6 linkages and does not bind J558.

‡ J558 (αλ), MOPC 315 (αλ) and RPC-20 (λ) proteins were reacted with the anti-idiotype antiserum for 3 h in the cold at a concentration of 2 mg/ml of undiluted serum.

whereas it was unaffected by other myeloma proteins and by dextran B512. This proves that the staining we observed is not due to antibodies to α- or λ-chains, and is in fact due to antibodies specific for the idiotypic determinants on the J558 molecule. Furthermore, the inhibition of the binding of anti-idiotypic antibodies in the presence of dextran B1355 demonstrates that the anti-J558 idiotype recognizes a dextran-specific receptor on the cell surface.

The Dextran-Binding Receptor, Cross-Reactive with J558 Idiotype, is not an Immunoglobulin Molecule. As shown in Table II, all cells positively stained by the anti-J558 antibodies were also positive for surface IgM, regardless of which receptor was stained first. There was, however, no positive correlation in the intensity of staining displayed by individual cells with the two reagents. In view of these results, it was important to exclude the possibility that our anti-idiotypic antiserum contained antibodies to immunoglobulin iso- or allotypes. From the way the antiserum has been prepared, no such antibodies should be expected except for the remote possibility of some antiallotypic determinants of the IgA molecules. Using immunodiffusion techniques, we have failed to detect in this antiserum antibodies to IgM, IgA, and all IgG subclasses, at a concentration of the antiserum over 100 times higher than that used for cell staining. In spite of that, we have additionally absorbed the antiserum on Sepharose-coupled IgG and a 19S fraction from mouse serum, as well as on a IgA (TEPC 15) and λ (RPC20) myeloma proteins, and we did not find any difference in the number or intensity of staining of cells detected by the unabsorbed versus the absorbed antiserum. Furthermore, as shown above, the activity of the antiserum was absorbed by the immunizing idiotype and not by other BALB/c α- or λ-chains.
There is no reason to assume, on the other hand, that an anti-allotype to BALB/c would react with C57BL/6 and all other strains tested, nor that an anti-α chain allotype would react with 10–20% of the IgM positive splenic B cells. Furthermore, it is not conceivable that Ig molecules on that large fraction of all B cells would carry the J558 idiotype and be dextran-binding.

We have performed inhibition and redistribution experiments to further analyze the nonimmunoglobulin nature of the J558 cross-reactive membrane structure. BALB/c spleen cells were stained with either anti-μ or anti-J558 in conditions to induce the redistribution ("capping") of the reacted molecules, and then counterstained with the alternative reagent. The results are reported in Table IV. It was found that redistribution of IgM does not affect the J558-cross-reacting structure, indicating that they are independent molecules on the B-cell surface. On the other hand, redistribution of the molecules detected by the anti-J558 idiotype antibodies co-caps the surface IgM on roughly two thirds of the cells, a situation that closely parallels the one observed with the mitogen receptor for lipopolysaccharide (18). The latter finding also excludes the possibility that the anti-J558 idiotype antiserum could react with surface IgD, since IgM and IgD "cap" independently in both directions (18, 19).

Finally, preliminary experiments have demonstrated the presence of cells positive for the J558-cross-reactive structure in 15 days old fetal liver in the complete absence of cells bearing surface immunoglobulins.

Anti-Idiotype Antibodies to J558 are Polyclonal B-Cell Mitogens. The final proof for the recognition of a dextran-specific mitogen receptor by anti-J558 idiotype antibodies would be of a functional nature. Thus, we have shown before that anti-mitogen receptor antibodies are mitogenic for the B cells expressing that receptor (6). Therefore, we have investigated the mitogenic properties of this anti-idiotype antiserum.

As shown in Fig. 1, anti-J558 induces a strong polyclonal response in spleen cells from both BALB/c and C57BL/6 strains. Activation results in proliferation and
maturation of B cells to high rate immunoglobulin secreting PFC of both the IgM and the IgG classes. The magnitude of the response is comparable to that observed with dextran B1355 itself and, in comparison with the LPS induced response, with the number of cells stained by the antiserum.

It is highly probable that the cells responding to the anti-J558 antibodies are those that we can label with the antiserum. Furthermore, for our prediction to be correct, these should be the dextran-reactive B cells. We performed an experiment attempting to test this possibility. Small cells from C57BL/6 spleen were purified on a 1-g gradient (20), and stimulated in culture with dextran B1355 or with LPS for 36 h. Thereafter, we scored the positivity for the J558-cross-reactive molecules of the blasts (mitogen-reactive cells) and of the cells which had remained small after that period of stimulation (mitogen-unreactive). The presence of the receptor for LPS (17) was checked as a control. Results shown in Table V indicate that in fact the cells reactive
to dextran were those bearing the J558-cross-reactive structure. Thus, dextran-unreactive cells (small cell fraction) are completely depleted of J558-positive cells, while cells with this marker are enriched over 10 times among the dextran reactive blasts. Parallel cultures stimulated with LPS still showed J558-positive cells in the small cell fraction (LPS unreactive), although in a lower number than in the starting preparation, indicating a partial overlap among the B-cell subsets responding to these two mitogens.

Discussion

We conclude from the present experiments that an anti-idiotypic antiserum to a dextran-binding myeloma protein recognizes the mitogen receptor for dextran on B cells. It should be made clear that such mitogenicity is not carried out through surface immunoglobulin receptors on B cells. Thus, although the biochemical characterization of this novel type of receptor is still in progress, several findings exclude the direct participation of surface immunoglobulins in the polyclonal triggering observed in response to this anti-idiotypic. The number of cells responding to the anti-idiotypic, comparable to the number directly stained, are orders of magnitude above those that should be expected from an immunoglobulin idiotypic-anti-idiotypic interaction. Furthermore, these numbers parallel those observed in the response to dextran B1355 itself, which is a B-cell mitogen as all other thymus-independent antigens (15), and which inhibits the interaction of the anti-J558 idiotype with this large subset of B cells. We have evidence (Table V) for the presence of the J558-idiotypic-cross-reactive structure exclusively on the subset of cells responding to dextran as a mitogen, and not on cells which are dextran-unreactive. In addition, the binding of the anti-idiotypic was inhibited by the idiotype itself, and not by other immunoglobulin molecules of the same class and type, excluding that our antiserum was recognizing other immunoglobulin determinants on the cell surface. The experiments on the redistribution of surface IgM and the J558-cross-reactive mitogen receptor, also demonstrated that these are two independent structures on the B-cell surface. Although we have not performed direct experiments to test this possibility, it is also unlikely that stimulation takes place through surface Fc receptors, since we and many others have failed to activate B cells by exposing them to immune complexes or nonaggregated IgG (21, 22). Furthermore, the selectivity of the anti-idiotypic for a subset of B cells also points against this possibility, since the vast majority of the splenic murine B cells display Fc receptors (23). Finally, there is no a priori reason why dextran should inhibit Fc-receptor binding as it does in the present experiments.

Before the confirmation that the phenomenon here described also applies to other idiotype-anti-idiotype systems, general conclusions should be taken with care. However, the same simple rule should, in principle, apply to all other anti-idiotypes to antibodies to other simple molecules like dextran, in which mitogenic moieties and antigenic determinants are structurally similar. Actually, recent results demonstrating interference of anti-idiotypic antibodies to anti-levan antibodies, with the anti-TNP response to TNP-levan support our suggestion (C. Bona, personal communication).

We are analyzing several other anti-idiotypes, namely anti-MOPC460, anti-3129, and anti-MOPC104E myeloma proteins, as well as anti-idiotypes to anti-PVP and anti-streptococcal (A5A) antibodies (kindly provided by Doctors P. A. Cazenave, M. Cohn, K. Forsbeck, B. Andersson, and K. Eichmann) and the results so far obtained closely parallel the situation described here for the anti-J558 idiotype system.
On the other hand, the cross-reactivity between such a mitogen receptor and an antibody idiootype of likely germ-line origin (24) suggests new ways of thinking. Thus, the possibility should be kept in mind that mitogen receptors are, in fact, encoded by primordial genes where from the germ-line V genes were derived. The maintenance of the structural similarities that allow for the cross-reaction here observed throughout long periods of evolution, poses further requirements for the internal regulation of the immune system.

Summary

The antibody response to dextran B1355 is thymus independent, and in high responder mice, over 90% of the antibodies carry the idiootype of an α-1,3 binding myeloma protein (J558). The present experiments demonstrate: (a) dextran B1355 is a B-cell mitogen both in a strain which carries the J558 idiootype on antibodies and in a low-responder strain which does not express that idiootype on antibodies to dextran; (b) anti-idiotypic antibodies to J558 recognize a dextran-specific surface receptor on 10-15% of all splenic B cells in those two strains as well as in all strains so far tested; (c) as shown by inhibition experiments such surface receptors cross-react with J558, and (d) anti-idiotypic antibodies are mitogenic for spleen cells of both strains resulting in B-cell proliferation and maturation to polyclonal antibody secretion.

Note added in proof. Identical results to those shown in this paper have been obtained by using immunoabsorbent purified anti-J558 idiotypic antibodies.

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