THE ROLE OF SURFACE IgD IN THE RESPONSE TO THYMIC-INDEPENDENT ANTIGENS

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Although most immunoglobulin classes are represented on the surface of thymus-independent (B) lymphocytes and as secreted products, immunoglobulin D (IgD) is expressed principally as a cell-bound molecule (1, 2) and is found on the vast majority of splenic B cells in the adult mouse (3–5). This unique status among immunoglobulin classes and the widespread distribution strongly suggest that surface IgD (sIgD) has a critical role in B-cell activation or regulation. Such a role has yet to be demonstrated, however. In this paper we show that sIgD is important in the in vitro primary antibody response to the thymic-independent (T-I) antigen, trinitrophenyl-aminoethylcarbamylmethyl-Ficoll (TNP-AECM-Ficoll). Thus, an alloanti-δ reagent inhibits the primary anti-TNP response to TNP-AECM-Ficoll. By contrast, this reagent has no detectable effect on the anti-TNP response to a second T-I antigen, TNP-Brucella abortus (TNP-BA). This result is particularly interesting as we have previously defined two classes of T-I antigens, differing in their ability to stimulate antibody formation in the immune-defective CBA/N strain (6). TNP-BA is a representative of T-I antigens which stimulate anti-TNP antibody formation by early neonatal and CBA/N spleen cells. TNP-AECM-Ficoll fails to induce a response in CBA/N cells and cannot stimulate neonatal cells until 7–10 days after birth (6). The selective inhibitory activity of the anti-δ reagent strongly suggests that sIgD plays a crucial role in the triggering of the class of B cells addressed by TNP-AECM-Ficoll, but is not essential to the response of B cells to TNP-BA.

Materials and Methods

Animals. Mice of strains C57Bl/Ka and BC8 were kindly provided by Dr. M. Potter (National Cancer Institute, National Institutes of Health). The BC8 strain is congenic with C57Bl/Ka in

Abbreviations used in this paper: B lymphocytes, thymus-independent lymphocytes; IgD, immunoglobulin D; pfc, plaque-forming cells; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; sIgD, surface immunoglobulin D; T-I, thymic-independent; TNP-AECM-Ficoll, trinitrophenyl-aminoethylcarbamylmethyl-Ficoll; TNP-BA, trinitrophenyl-Brucella abortus.

The murine immunoglobulin isotype referred to as IgD has not yet been formally demonstrated to be the analogue of human IgD. Consequently, it should be designated putative murine IgD. However, for simplicity, we will refer to it as IgD and to its heavy (H) chain as "δ."

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that it bears the same "background" genes but possesses the Ig heavy chain markers of BALB/c, rather than those of C57Bl/Ka.

Culture System. The method employed is a modification of that originally devised by Mishell and Dutton (7) and has been described (8). Briefly, single cell suspensions of mouse spleen were cultured in flat-bottom microtiter plates (IS-FB-96-TC; Linbro Chemical Co., New Haven, Conn.), with 200-μl cultures containing the indicated number of cells (10^6 unless otherwise stated). Cultures were incubated at 37°C, in a humidified atmosphere of 5% CO_2 in air. Experimental groups were assayed in triplicate. Determination of numbers of plaque-forming cells (pfc) was carried out on the day of peak response (day 3 or 4). Anti-δ or control reagents were present throughout the entire culture period. In some experiments, irradiated cells were used as "fillers." They were irradiated (1,500 rads) in a ^{137}Cs source (Isomedix Inc., Parsippany, N.J.) at a dose-rate of 1,875 rads/min.

Antigens. TNP-AECM-Ficoll was prepared as described by Inman (9). The Ficoll used as starting material had an average molecular weight of 400,000 daltons; the molar ratio of TNP to Ficoll in the product was 56.

TNP-BA was prepared as described by Mond (see footnote 3). The stock suspension used contained approximately 10^8 microorganisms per milliliter. Dilutions of antigen expressed in this paper are dilutions of the stock; thus a 200-μl microculture challenged with a 10^-2 dilution would contain about 2 × 10^5 microorganisms.

Plaque-Forming Cell Assay. The method employed was a modification for microscope slides of the haemolytic plaque assay of Jerne and Nordin (10). TNP-sheep erythrocytes were prepared by the method of Rittenberg and Pratt (11). Only data for direct (IgM) pfc are given.

Numbers of pfc in individual culture wells were determined, and geometric mean and relative standard error for results from triplicate wells were calculated. Data are expressed as pfc per culture or pfc per known number of cells. The antigen-specific pfc value (antigen-induced minus background) is the difference between the geometric means. The standard error of the geometric mean was transformed to an arithmetic scale by multiplying the geometric mean by the antilog of the standard error of the mean of the logarithmic values. The standard error of the differences of the geometric means was then calculated by a conventional method (12).

Cell Surface Iodination and Solubilization. This procedure was performed as described by Kessler (13). 50 × 10^6 nucleated spleen cells, depleted of erythrocytes by hypotonic lysis, were radiolabeled with 1 mCi ^{125}I; the reaction was catalyzed by 1.5 IU lactoperoxidase (grade B, 42 IU/mg; Calbiochem, San Diego, Calif.). The washed cells were solubilized with 0.5% NP-40. This was followed by ultracentrifugation (110,000 g for 30 min) and preabsorption with Staphylococcus aureus Cowan I strain (ATCC 12598). 50 μl of a 10% suspension (vol/vol) was used for the NP-40 extract obtained from the equivalent of 25 × 10^6 cells. The extract was incubated with S. aureus for 30 min.

Immunoprecipitation. This procedure was performed as described by Cullen and Schwartz (14). The rabbit anti-mouse-κ reagent used was kindly provided by Dr. Rose Mage (Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health). Sequential precipitations were performed as described by Cullen et al. (15).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE Analysis). The method used was a modification of that originally described by Laemmli (16). All gels used for analysis contained 10% (wt/vol) acrylamide and all samples were reduced with 2-mercaptoethanol before analysis.

Anti-δ and Control Reagents. The reagents used were ascitic fluids generated in C57Bl/Ka mice by the method of Tung et al. (17). To elicit the anti-δ reagent, C57Bl/Ka mice received 10^7 spleen cells from adult BALB/c donors intraperitoneally twice at weekly intervals. They were then rested for a month and weekly injections of spleen cells resumed for 4 wk, during which time the ascites were induced. To produce the control reagent, Hanks' balanced salt solution was substituted for the cell suspension in the immunization protocol; otherwise, timing of injections and ascites induction were identical. Before use, either for immunoprecipitation or culture, reagents were deggregated by ultracentrifugation (130,000 g for 30 min in a Beckman airfuge; Beckman Instruments, Inc., Cedar Grove, N.J.).

The strain combination used here to produce alloanti-δ (C57Bl/Ka immunized with BALB/c), although different from that employed by Goding et al. (4; C57Bl/6 anti-CBA), should nonetheless recognize the same specificity, because IgD from both BALB/c and CBA bear the Ig 5.1
specificity, which is lacking from C57Bl IgD (N. L. Warner, personal communication). Our reagent will hereafter be referred to as anti-Ig 5.1.

Ig Preparation from Ascitic Fluids. Ig was precipitated with 40% saturated ammonium sulphate. Precipitated material was redissolved in phosphate-buffered saline, dialyzed extensively, and finally deaggregated by ultracentrifugation before use.

Results

Specificity of the Anti-Ig 5.1. We initially wished to confirm that the reagent generated by alloimmunization was, in fact, specific for the δ-heavy chain when tested on BC8 spleen cells. Inasmuch as no murine IgD plasmacytomas have been reported, we have analyzed reagents by their ability to precipitate iodinated spleen cell membrane components. Spleen cells from BC8 and C57Bl/Ka mice were iodinated, and extracts prepared for immunoprecipitation studies as described above. Gel profiles showing radioactivity precipitated by rabbit antimouse-κ and putative anti-Ig 5.1 from such membrane preparations are shown in Fig. 1. Rabbit antimouse-κ precipitates proteins with the mobility of μ- and δ-heavy chains using extracts from either strain (Fig. 1 a and b). The light chain peak is indistinguishable from the running front using 10% gels. The mobility of authentic μ-chain is indicated in the figures and was determined by the migration of fluoresceinated μ-chain used as an internal marker. In contrast to the rabbit antimouse light chain antiserum, the alloantibodies precipitated radioactive material from the BC8 membrane preparation (Fig. 1 c), but not from the C57Bl/Ka (Fig. 1 d), demonstrating specificity for determinants encoded in the BALB/c allotype linkage group. The radioactive material precipitated from the solubilized BC8 membrane preparation did not comigrate with the μ-chain marker, but had the mobility of the non-μ peak precipitated by anti-κ. This indicates that there is no detectable anti-μ activity in the anti-Ig 5.1. To confirm the identity of the non-μ material precipitated by anti-κ and anti-Ig 5.1, sequential precipitations were performed as described in Materials and Methods. Iodinated spleen cell extracts from BC8 mice were incubated in the first step with either rabbit antimouse-κ or normal rabbit serum, followed by precipitation of immune complexes with S. aureus. The rabbit anti-κ precipitated the material usually seen with this reagent (data not shown, but identical to that in Fig. 1 a); no discrete peaks of radioactivity were precipitated by normal rabbit serum. The supernate from the first step was divided into four aliquots for the second precipitation; the reagents used were rabbit anti-κ, normal rabbit serum, anti-Ig 5.1 and nonimmune ascites. Neither control reagent precipitated any radioactivity, nor did anti-κ after anti-κ. The gel profiles shown in Fig. 2 represent results with anti-Ig 5.1 after either normal rabbit serum (Fig. 2 a) or rabbit antimouse-κ preprecipitation (Fig. 2 b). The anti-κ removed the bulk of the material potentially precipitable by anti-Ig 5.1, whereas normal rabbit serum left this unaffected.

Effect of Anti-Ig 5.1 on PFC Responses In Vitro. We next wished to determine whether anti-δ could inhibit in vitro antibody responses. We initially evaluated the effect of the continuous presence of anti-Ig 5.1 on the in vitro response of BC8 cells, which possess the 5.1 allotypic determinant, and of congenic C57Bl/Ka cells, which lack this determinant. BC8 or C57Bl/Ka spleen cells (10⁶) were cultured with a 1:50 dilution of anti-Ig 5.1, with a 1:50 dilution
of normal ascitic fluid or with no ascitic fluid in the presence or absence of TNP-AECM-Ficoll. No source of exogenous complement was added. Direct anti-TNP pfc were measured on day 4. As shown in Table I, anti-Ig 5.1 suppressed the response of BC8 spleen cells to TNP-AECM-Ficoll at all antigen concentrations tested, when compared either with cultures containing no ascitic fluid or with nonimmune ascitic fluid. Indeed, nonimmune ascitic fluid appeared to somewhat enhance the antibody response to TNP-AECM-Ficoll and thus the effect of anti-Ig 5.1 is even more dramatic when compared with nonimmune ascitic fluid. On the other hand, the anti-Ig 5.1 reagent had no
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Fig. 2. Analysis by SDS-PAGE of radioactivity precipitated by anti-Ig 5.1 plus S. aureus from radiiodinated BC8 spleen cell extracts which had first been exposed to (a) normal rabbit serum plus S. aureus and (b) rabbit antimouse-κ antiserum plus S. aureus.

Table I
The Effect of Anti-Ig 5.1 Antibodies on the In Vitro PFC Responses of Spleen Cells from Congenic Mice Bearing Different Allotypic Determinants

| Additions to Culture | No ascites | Nonimmune ascites | Anti-Ig 5.1 |
|---------------------|-----------|-------------------|------------|
|                     | (1:50)    | (1:50)            | (1:50)     |
| **BC8 (Ig 5.1*)**   |           |                   |            |
| TNP-AECM-Ficol      |           |                   |            |
| 10⁻² μg/ml          | 156.0 ± 1.3 | 323.8 ± 4.1 | 79.4 ± 20.4 |
| 10⁻³ μg/ml          | 185.1 ± 5.4 | 202.2 ± 14.7 | 50.7 ± 4.9  |
| 10⁻⁴ μg/ml          | 66.2 ± 2.5  | 129.8 ± 12.4 | 16.8 ± 1.3  |
| **C57BL/12** (Ig 5.1⁻) |           |                   |            |
| TNP-AECM-Ficol      |           |                   |            |
| 10⁻² μg/ml          | 143.4 ± 5.4 | 196.1 ± 18.9 | 273.7 ± 8.5 |
| 10⁻³ μg/ml          | 199.6 ± 23.7 | 292.7 ± 17.4 |            |
| 10⁻⁴ μg/ml          | 100.3 ± 20.5 | 169.4 ± 13.9 |            |

*Calculation of antigen-specific pfc/10⁶ cells and standard error as described in Materials and Methods.
Effect on Nonimmune and Anti-Ig 5.1 Ascitic Fluids on the In Vitro PFC Responses of BC8 Spleen Cells to T-I Antigens

| Antigen       | Additions to culture | Direct anti-TNP pfc/10^6 input cells* |
|---------------|----------------------|--------------------------------------|
| TNP-AECM-Ficoll | None                 | 147.6 ± 11.6 (1:50) 165.8 ± 3.0 (1:100) 9.5 ± 5.3 (1:100) |
|               | Nonimmune ascites    | 216.3 ± 17.7 (1:50) 111.7 ± 9.8 (1:100) 24.5 ± 6.1 (1:100) |
|               | Anti-Ig 5.1 ascites  | 146.2 ± 14.1 (1:50) 156.4 ± 3.4 (1:100) 24.3 ± 3.3 (1:100) |
| TNP-BA        | None                 | 148.1 ± 4.6 (1:50) 191.8 ± 15.7 (1:100) |
|               | Nonimmune ascites    | 173.3 ± 3.1 (1:50)   |
|               | Anti-Ig 5.1 ascites  | 175.3 ± 3.1 (1:100)  |

* Calculation of antigen-specific pfc/10^6 cells and standard error as described in Materials and Methods.

inhibitory effect on the response of C57Bl/Ka cells to TNP-AECM-Ficoll. This establishes that the blocking effect is a function of the interaction of the immune ascitic fluid with a product of the IgH chain region, presumably IgD.

Because TNP-BA can stimulate responses by cell populations that cannot respond to TNP-AECM-Ficoll, we then tested the effect of anti-Ig 5.1 upon the response of BC8 spleen cells to this antigen and obtained a strikingly different result. Anti-Ig 5.1 at 1:50 or 1:100 dilutions failed to inhibit the response of 10^6 BC8 spleen cells to a 5 × 10^-5 dilution of TNP-BA, although the same dilutions of anti-Ig 5.1 inhibited responses to TNP-AECM-Ficoll at all antigen doses tested (Table II).

Although the results above clearly show differential effects of anti-δ reagents on the in vitro antibody responses to TNP-BA and TNP-AECM-Ficoll, a relatively trivial explanation was available which was derived from a study of precursor frequency for both antigens. In normal adult spleen cells, TNP-BA activates roughly fivefold more precursors than does TNP-AECM-Ficoll (D. E. Mosier, unpublished observation). Consequently, the usual culture conditions might provide only a slight excess of precursors for TNP-AECM-Ficoll but a very substantial excess of precursors for TNP-BA (i.e., the response is already at a level at which increasing the cell number fails to increase the pfc response). If the anti-Ig 5.1 antibodies were blocking only a fraction of B cells in culture, their effect would be more readily manifest on the TNP-AECM-Ficoll response. To rule out this possibility, the cell dilution experiments described below were performed, data from which are shown in Figs. 3 and 4. In these experiments, only the number of responding cells were varied, whereas the antigen concentration, dilution of suppressing or control reagent, and total cell number in the
culture were held constant. Constant total cell density was achieved by using BC8 spleen cells exposed to 1,500 rads as nonresponding "filler" cells. Instead of using the ascitic fluids in these cultures, Ig prepared from ascitic fluid, as described in Materials and Methods, was used. The final concentration of Ig in culture was 50 μg/ml.

The cell dilution curve for the response to 10⁻² μg/ml TNP-AECM-Ficoll is shown in Fig. 3. Data are plotted as antigen-specific pfc per microwell against the number of responding cells. The anti-Ig 5.1 caused greater than 90% suppression at responding cell numbers of 10⁶ and 1.5 × 10⁶. With 2 × 10⁶ responding cells, the suppression was only of the order of 40%. These data indicate that even in a response highly susceptible to suppression, the suppression may be minimized by increasing the number of responding cells exposed to the reagent. The above data made it important to perform a similar experiment with TNP-BA as the antigen, and these results are shown in Fig. 4. At no cell concentration was the TNP-BA response susceptible to anti-Ig 5.1 suppression.

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**Fig. 3.** Cell dilution curve for BC8 spleen cells responding to TNP-AECM-Ficoll (10⁻² μg/ml) in the presence of nonimmune (●) or anti-Ig 5.1 (▲) immunoglobulin.
A final series of experiments were performed to examine the effect of challenge antigen concentration on the suppression by anti-Ig 5.1. This was tested by assaying the day 4 responses of $10^6$ BC8 spleen cells in the presence of either anti-Ig 5.1 or control ascites (at a 1:50 dilution) over a wide range of concentration of each antigen. The data are shown in Figs. 5a (response to TNP-AECM-Ficoll) and 5b (response to TNP-BA). The suppression of the TNP-AECM-Ficoll response is consistent over the antigen dose range $10^{-10} - 10^{-4} \mu g/ml$. The dose-response curves for TNP-BA in the presence of anti-Ig 5.1 and nonimmune ascites are identical over the range of antigen dilutions $10^{-4} - 10^{-5}$; at the $10^{-6}$ dilution there is a small but significant response in the presence of nonimmune ascites, which was eliminated by anti-Ig 5.1.

**Discussion**

The results presented here show that the in vitro anti-TNP response to one T-1 antigen, TNP-AECM-Ficoll, is inhibited by anti-Ig 5.1 antibodies, whereas
that to a second T-I antigen, TNP-BA is resistant to this inhibition. In fact, our data demonstrate that the response to TNP-AECM-Ficoll is suppressed at all antigen doses tested and all cell numbers employed, although the inhibition when $2 \times 10^6$ cells are used is less striking. The relative insensitivity to inhibition of cultures with high cell numbers presumably reflects a relatively low titer of anti-Ig 5.1 in the inhibiting reagent. The response to TNP-BA is not inhibited at any cell concentration tested nor at any antigen dose except the lowest ($10^{-6}$ dilution). Indeed, when TNP-BA is used at $10^{-6}$ dilution, the response observed is quite small and neither neonatal mice nor mice with the X-linked CBA/N defect respond, although both respond quite well to higher concentrations of TNP-BA (see footnote 3).

Two alternative explanations may be proposed to explain the differential sensitivities of responses to TNP-AECM-Ficoll and to optimal concentrations of TNP-BA to anti-Ig 5.1. The first is that the two antigens address distinct subpopulations of B cells which differ in their expression of sIgD. The second explanation is that a single population of cells is responsive to both antigens, but that TNP-AECM-Ficoll and TNP-BA employ different mechanisms to trigger cells to antibody production.

The former explanation has support from several sets of observations. It has been shown that TNP-BA can elicit good antibody responses from splenic B cells of neonatal mice, whereas the response to TNP-AECM-Ficoll is marginal until the mice are 1 to 2 wk of age (6). Thus, the acquisition of responsiveness to TNP-AECM-Ficoll is similar in timing to the appearance of sIgD because this isotype is poorly expressed by neonatal B cells but rises in relative
amounts over the first 4 wk of life (18). Similarly, mice with the CBA/N X-linked B-lymphocyte defect respond to high concentrations of TNP-BA, but do not respond to TNP-AECM-Ficoll at any concentration. The B cells of adult defective mice are similar, in terms of sIg phenotype, to those of young, nondefective animals, in that the ratio of sIgD to sIgM is only about one-third that found in nondefective adult spleen cell populations (19). These findings support the existence of at least two subpopulations of B cells that differ in sIg phenotype. To further support the notion of a distinctive cell type responsive to each of the antigens, preliminary results indicate the complete susceptibility of the TNP-AECM-Ficoll response (in normal strains) to anti-Lyb 5 and complement; the TNP-BA response is only partially affected by this treatment. We would suggest further that each of these two antigens may be seen as a prototype of a class of T-I antigens, distinguished operationally by their ability to elicit antibody responses from neonatal, nondefective spleen cells and from CBA/N mice. In the group capable of eliciting such responses would be TNP-BA and, with it, TNP-derivatives of lipopolysaccharide and polyacrylamide beads. We refer to these as T-I 1 antigens (6). The group of antigens capable of stimulating only more mature cells includes TNP-AECM-Ficoll and levan, dextran, pneumococcal polysaccharide, and polyinosinic:polycytidilic acid. These are designated T-I 2 antigens.

As noted above, an alternative explanation for our data is that at least some of the response to TNP-BA in the nondefective adult is mediated by cells that can also respond to TNP-AECM-Ficoll. Inasmuch as only the response to TNP-AECM-Ficoll is anti-Ig 5.1 inhibitable, this implies different mechanisms of triggering the same cell by two T-I antigens bearing the same haptenic determinant.

Our experiments do not address the question of the mechanism by which anti-Ig 5.1 inhibits the response to TNP-AECM-Ficoll. Essentially, three basic mechanisms may be envisaged. The first, and simplest, is that for the class of T-I antigens represented by TNP-AECM-Ficoll, effective induction involves a transmembrane "signal" which requires the participation of sIgD, sIgM alone being inadequate. Thus, blocking the sIgD receptors with antibody prevents antigen binding and signal generation. The second possibility is that induction requires occupancy, by antigen, of a certain threshold fraction of the total sIg molecules. Blocking of sIgD might prevent the binding of antigen to this critical number of receptors. Both of these proposals imply that occupancy of sIgD receptors by antigen and by anti-Ig causes very different outcomes, the former leading to signal generation, the latter not. Finally, it is also possible that the suppression by anti-Ig 5.1 is active, rather than passive, and that the binding of anti-Ig 5.1 to sIgD generates a "negative signal" leading to an inhibited response.

Our success in showing a role for sIgD was based upon the choice of TNP-BA and TNP-AECM-Ficoll as test antigens. The choice of these antigens was not fortuitous, but based upon our knowledge of the ontogeny of responsiveness to a range of T-I antigens and the development of splenic B cells with respect to sIg phenotype in normal mice (6).

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Summary

An alloanti-δ antibody was prepared by immunizing C57BL/Ka mice with BALB/c spleen cells. Its specificity for δ-chain was demonstrated by immunoprecipitation and SDS-PAGE of 125I-labeled membrane proteins from BC8 spleen cells. BC8 mice possess C57BL/Ka "background" genes and BALB/c IgH genes.

The anti-δ reagent without complement inhibited the primary in vitro anti-TNP antibody response to TNP-AECM-Ficoll by BC8 spleen cells, although it had no effect on the anti-TNP response of congenic C57BL/Ka spleen cells, which lack the δ-allotype identified by this antibody. On the other hand, the anti-δ antibody had no effect on the anti-TNP response of BC8 spleen cells to TNP-BA, except at limiting antigen concentrations. Both TNP-AECM-Ficoll and TNP-BA are T-I antigens, but they differ in that TNP-AECM-Ficoll fails to stimulate in vitro responses by immunologically defective CBA/N and neonatal spleen cells whereas TNP-BA can cause responses from both these animals. These results suggest that the IgD receptor is critical to T-I antibody responses initiated by TNP-AECM-Ficoll but that it is not required for T-I responses stimulated by TNP-BA.

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