SPECIFIC ACTIVATION OF THE BONE MARROW-DERIVED LYMPHOCYTE BY ANTIGEN PRESENTED IN A NON-MULTIVALENT FORM

EVIDENCE FOR A TWO-SIGNAL MECHANISM OF TRIGGERING

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Antigen-induced activation of bone marrow-derived (B) lymphocytes involves interactions between specific antigenic determinants and immunoglobulin receptors on the lymphocyte surface (1). Soluble antigens such as serum proteins can trigger B lymphocytes only if thymus-derived (T) cells are provided (2), thus implying a requirement for some element besides a simple antigen-receptor interaction. Triggering may result from the presentation to the B cell of a multivalent configuration of identical antigenic determinants produced on, or through the action of, the T cell (3, 4). Alternatively, it has been postulated that, as well as the binding of antigen by receptors, the B cell must react with a second substance, this second signal being some product of collaborating T cells (5).

The first hypothesis has been supported by the observation that antigens not requiring T cell help to activate B cells share a common polymeric structure (3, 6), enabling them to directly form multiple receptor-antigen bonds. The experiments to be reported offer an alternative explanation for the role of one such thymus-independent antigen, polymerized flagellin (POL) (6) in B cell triggering. They suggest that multivalent presentation of antigen is not an absolute requirement for B cell activation by demonstrating that antigen presented in a non-multivalent form can trigger B cells in the absence of T cells, under at least one circumstance.

Materials and Methods

Animals.—Congenitally athymic “nude” (nu/nu) mice, from a closed but not inbred colony (7), were used at 6-8 wk of age.

Fowl Gamma Globulin (F3′G).—F3′G was rendered aggregate-free by centrifugation at 100,000 g for 180 min (8), at a protein concentration of 10 mg/ml, the top one-third of the supernatant being designated “deaggregated-F3′G” (De-F3′G).

Flagellin.—Polymerized flagellin (POL) was prepared by Mr. J. Pye by an established method (9) from Salmonella adelaidae. A preparation of bacterial flagella (FLA) was prepared from Salmonella typhimurium as described previously (9).

Tissue Culture.—A modification of the Diener-Marbrook culture system was used (6). Briefly, 1.5 × 10⁷ viable nucleated spleen cells were cultured with appropriate amounts of the reagents under test in 1 ml of tissue culture medium (Eagle's minimal essential medium [Grand Island Biological Co., Grand Island, N. Y.] with 5% fetal calf serum [Commonwealth Serum Laboratories, Parkville, Australia]) within a chamber separated by a dialysis membrane from a reservoir of 50 ml of medium. Cultures were incubated for 3.7 days in 10% CO₂ and humidified air.

Antibody-Forming Cell (AFC) Assay.—A modified hemolytic plaque method employing sheep erythrocytes (SRC) coated with F3′G in the form of a fowl-anti-SRC antibody (10)
was used to detect cells forming anti-FyG antibodies. The antibodies detected are presumably those directed against the Fc end of the FyG molecule that is exposed on the SRC surface. The specificity of plaque formation was established by specific inhibition on addition of free FyG to the assay. Cells forming antibodies against sheep erythrocytes, so-called "background plaques," were assayed in aliquots of each culture harvested using uncoated SRC for the plaque method.

**Absorption of De-FyG with FLA.**—De-FyG (200 μg) was incubated with 1 mg of FLA in Dulbecco's solution for 60 min at 37°C and then 120 min at 4°C. The mixture was then centrifuged at 75,000 g for 40 min so as to sediment the FLA and the supernatant was used as "FLA-absorbed De-FyG."

**Absorption of De-FyG with Spleen Cells.**—De-FyG (400 μg) was incubated in tissue culture medium with 100 × 10⁶ spleen cells of either CBA/H/Wehi mice or nu/nu mice for 15 min at 37°C and 180 min at 4°C and then centrifuged to sediment the cells.

**Binding Studies with Labeled De-FyG.**—De-FyG was trace labeled with ¹²⁵I by a chloramine-T method (11). Varying amounts of De-FyG were mixed with labeled De-FyG and mixed in Dulbecco's solution with POL or FLA. After incubation for 60 min at 37°C and overnight at 4°C, the mixtures were centrifuged at 75,000 g for 40 min to sediment POL and FLA and the counts were determined in 1 ml of supernatant and in the sedimented button.

**RESULTS AND DISCUSSION**

De-FyG, given alone, did not produce an in vivo AFC response in the spleens of intact nude mice, nor in cultures of spleen cells from nude mice that lack detectable T cells (7). However, when De-FyG was added to cultures of spleen cells in the presence of polymerized flagellin (POL), a definite anti-FyG response was observed (Fig. 1), which showed a clear relationship between the concentration of De-FyG in the culture and the anti-FyG AFC response. As little as 0.1 μg/ml of POL added per culture was sufficient to allow a detectable response to FyG. There was no specific anti-FyG response in the absence of De-FyG with any dose of POL tried (0.01–100 μg/ml). The maximal response of 400–500 AFC/culture is equal to that observed when cultures of nu/nu spleen are immunized with FyG in a multivalent form, complexed to POL (J. Schrader and M. Feldmann, manuscript in preparation). These results were confirmed using flagella (FLA) from a different non-cross-reacting *Salmonella* strain (*Salmonella typhimurium*) and also in in vivo experiments (data to be published).

To investigate the possibility that multivalent complexes of antigen were being formed by aggregation of De-FyG with POL or by the reaction of possible natural anti-POL antibody in the De-FyG, trace-labeled De-FyG was incubated with POL and the counts sedimentable with POL were determined. No binding was detected by this method. To exclude the possible existence of minute amounts of fowl anti-POL antibody that were not detected thus, De-FyG was preabsorbed with FLA and an amount of the supernatant equal to the volume of the original solution of De-FyG containing 10 μg was added to cultures with 10 μg of FLA. No significant diminution in the anti-FyG response was seen (Table I), even with this suboptimal quantity of 10 μg/ml of De-FyG (Fig. 1).

As it was possible that natural antimouse antibody in the De-FyG preparation was producing a multivalent configuration of De-FyG by binding to mouse cells, De-FyG was absorbed with mouse spleen cells. The ability to
immunize cultures containing POL remained intact (Table I). Furthermore, in additional studies (to be published) a good AFC response to De-FyG has been observed when nu/nu spleen cells are incubated for a short time with De-FyG (in the absence of POL) to allow binding to B cell receptors and washed to remove unbound De-FyG, before culturing in the presence of POL. Thus it seems established that FyG is not encountering the B cell in an aggregated state.

The possibility of contamination of POL by endotoxin being responsible for the effects reported here is peripheral to the main theme of interest, namely that B cells in the presence of some second substance respond to antigen even though it is not multivalently presented. Nevertheless it should be noted that the carbohydrate content of the POL preparation is only 0.2% (9) and the possible amount of endotoxin in 10 µg of POL therefore is of the order of 0.02 µg. This compares with the 10-100 µg of endotoxin used in pertinent studies (12, 13). The enhancing effect on the anti-SRC background response seen at the highest dose of POL (100 µg/ml) (Table II) suggests that POL might increase the rate of B cell division. However this effect is not apparent at the doses of 10 µg and less shown to be effective in allowing a response to De-FyG. For this reason it is unlikely that, at these smaller doses of POL, the observed anti-FyG response is due to an amplification by such an effect.
TABLE I
Persistence of Immunogenicity of De-FγG after Absorption with Spleen Cells or FLA

| De-FγG added to cultures | Anti-FγG AFC culture¶ |
|--------------------------|------------------------|
| 100 μg of spleen-absorbed De-FγG* | 487 ± 73               |
| 100 μg of spleen-absorbed De-FγG§ | 453 ± 150              |
| 10 μg of FLA-absorbed De-FγG | 243 ± 51**             |
| 10 μg of De-FγG | 169 ± 41**              |

* Absorbed with CBA spleen.
† Absorbed with nu/nu spleen.
§ Either POL 10 μg (§) or FLA 10 μg (§) was added to each culture.
¶ Results represent the arithmetic mean of four cultures ± SEM.
** Not significantly different by Student's t test.

TABLE II
Dose Dependence of the Effect of POL on Anti-SRC Background

| Dose of POL* | Anti-SRC AFC/culture‡ |
|--------------|------------------------|
| μg/ml        |                        |
| 0            | 33 ± 12                |
| 10           | 21 ± 5                 |
| 100          | 318 ± 66               |

* Spleen cells were cultured alone or with two doses of POL in the absence of any other antigen (apart from the fetal calf serum constituents in the medium). The figures displayed represent the pooled results of cultures set up as controls in three experiments.
‡ Results represent the arithmetic mean of 4-12 cultures ± SEM.

of an amount, normally undetected with FγG, of thymus-independent B cell triggering, such as occurs with erythrocyte antigens in nude mice (7). In the latter case, natural multivalency of antigen is a possible source of such thymus-independent triggering, but as outlined above this is unlikely to be a factor with De-FγG.

The data presented suggest that POL possesses an intrinsic ability to influence the triggering of B cells so that they can be stimulated by nonmultivalent antigen in the absence of T cells. Thus the thymus independence of POL itself as an antigen (6) would rest not necessarily on its ability, intrinsic to its physical polymeric form, to interact multivalently with antigen-specific receptors, but on some other property affecting B cells in general. There is evidence to suggest that at least one other thymus-independent antigen, lipopolysaccharide (13), has a similar ability to render all B cells thymus independent. It is established that a range of polymeric substances such as endotoxin (14), polynucleotides (15), dextran (16), and plant and mammalian tissue polysaccharides (17) share a common pyrogenic action, probably related to their ability to interact with the granulocyte or macrophage to produce "endogenous pyrogen" (14). Members of this group have also been shown to stimulate macrophages to produce interferon (18) and a "colony-stimulating factor" allowing in vitro proliferation and differentiation of bone marrow stem cells (19). It is of interest that polymers as a group also seem active in stimulating the immune response. Substances studied include lipopolysaccharide (20), dextran sulfate, polyacrylic acid, polynucleotides and pyran...
copolymer (discussed in reference 21), DEAE-dextran (my personal observation), and POL (this study and data in preparation). Although all these substances may not share a common mechanism of immune stimulation, two possibilities stand out. Firstly, the macrophage may be stimulated to release a B cell-activating signal in analogy to the production of endogenous pyrogen or interferon, or secondly, as the lymphocyte shares a bone marrow origin with granulocytes and macrophages, it too may react directly with these polymers. These alternatives are at present under investigation, but in the case of POL it seems possible that it reacts directly with the B cell, as it is established that POL and dinitrophenylated POL (22) activate specific B cells at least, in the absence of macrophages.

The action of POL might be explained as lowering the triggering threshold through the release of subthreshold amounts of an intracellular messenger, normally the result of receptor-antigen interactions, until monomeric antigen despite its thermodynamic disadvantage could trigger. Against this is the observation that at all the POL concentrations studied the presence of De-FyG was obligatory (Fig. 1 b), and the converse that at even the highest concentration of De-FyG examined so far (1,000 μg/ml), a threshold dose (0.1 μg/ml of POL) produced only a minimal response (42 ± 12 anti-FyG AFC/culture). If, on the other hand, antigen in a multivalent configuration induces a rearrangement of receptor structures that constitutes the actual signal (23), the action of POL in this study may be explained as a cross-linking of membrane-receptor-antigen structures. Alternatively, in the absence of evidence that POL induces a cross-linking of receptor-antigen structures on the B cell, the present demonstration that antigen need not be presented to the B cell in a multivalent form to produce antigen-specific triggering admits the possibility that cross-linking or aggregation of receptors is not the mechanism of B cell triggering. The experiments reported here are consistent with a two-signal mechanism of triggering, such as proposed by Cohn (5), one signal being a simple receptor-antigen interaction, the second being interaction with a non-antigen-specific factor, in this case provided directly or indirectly by POL.

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