DISSOCIATION BETWEEN MITOGENICITY AND IMMUNOGENICITY OF TNP-LIPOPOLYSACCHARIDE, A T-INDEPENDENT ANTIGEN

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Two models have been proposed to explain triggering of B cells by so-called "T-independent antigen." Feldmann and Basten (1) proposed that the interaction of multiple repeating determinants on polymeric antigens with specific Ig receptors on the B-cell surface is sufficient to provide the signals for division of these cells and differentiation to antibody-forming cells. In contrast, Coutinho et al. (2, and see review, 3) have claimed that there is only one signal, a mitogenic signal, receptors acting merely as passive focussing devices to localize the antigen on specific cells where it delivers a mitogenic signal resulting in differentiation to an antibody-producing cell. This model rests primarily on the demonstration that at high concentration all T-independent antigens they have tested are mitogenic for B cells (4-6). Compatible with this hypothesis are the observations that hydrolysis of lipopolysaccharide (LPS) to remove the ester-linked fatty acids of the mitogenic lipid A component abrogates its mitogenic (7, 8) activity as well as its ability, when substituted with the TNP hapten, to induce a T-independent anti-TNP response (9). However, alkali treatment of LPS, although not changing its antigenic component (8), may also modify the molecule physically or chemically which could account for loss of immunogenic properties (10). We therefore investigated other reagents which interact with LPS in a more chemically defined manner in an effort to clarify the relationship between the mitogenic and immunogenic properties of this molecule.

Polymyxin B (PB) is one of a family of cyclic peptide antibiotics which are bactericidal for most gram-negative bacteria. It prevents the lethal endotoxic activity of LPS (11, 12) and changes the physical structure of LPS (13). We report here that low doses of PB added to cultures of mouse spleen cells inhibit the mitogenic activity of TNP-LPS, a T-independent antigen, and native LPS, but do not suppress the immune response to TNP-LPS. PB interacts with TNP-LPS and LPS causing a physical change in the molecule. In addition, polymyxin-treated LPS is no longer mitogenic. These results suggest a dissociation between the mitogenic and immunogenic properties of TNP-LPS.

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Materials and Methods

Mice. CBA/H Wehi were obtained from breeding colonies of The Salk Institute, and BDF, (C57BL/6 x DBA/2) mice were purchased from the Jackson Laboratories, Bar Harbor, Maine. Spleen cells from nu/nu mice (The Salk Institute) and adult thymectomized-antithymocyte serum-treated (ATx-ATS) BDF, (14) mice were used as a source of B cells.

Additions to Culture. Lipopolysaccharide W from Escherichia coli 055:B5 (LPS) was purchased from Difco Laboratories, Detroit, Mich. Trinitrophenylated-LPS (TNP-LPS) was prepared as previously described (9). Polymyxin-treated LPS (PB-LPS) was prepared by mixing 10 mg LPS and 30 mg PB at room temperature for 1 h followed by exhaustive dialysis against water. Sheep erythrocytes (SRBC) and horse erythrocytes (HRBC) were obtained from the Colorado Serum Co., Boulder, Colo. Other mitogens used were phytohemagglutinin (PHA) (Wellcome Research Laboratories, Beckenham, Kent, England), Concanavalin A (Con A) (Pharmacia Fine Chemicals AB, Uppealn, Sweden), and purified protein derivative (PPD) (Statens Seruminstitutet, Copenhagen, Denmark). Polymyxin B (8260 U/mg) was purchased from Sigma Chemical Co., St. Louis, Mo. DNP-dextran was generously contributed by Ms. Bonnie Blomberg, The Salk Institute. This antigen is immunogenic in cells from nude mice and other T-depleted spleen cells (Blomberg, unpublished observations).

Mitogen Assay. Half a million viable spleen cells, with or without mitogen, were cultured in 0.2 ml RPMI-1640 supplemented with 5% fetal bovine serum (FBS) (Grand Island Biological Co., Grand Island, N.Y.) in microtest plates (Cooke Engineering Co., Alexandria, Va.). Cultures were incubated for 48 h and then pulsed with 0.05 μCi [3H]UdR (spec act >200 Ci/mmol; New England Nuclear, Boston, Mass.) for a further 16 h. DNA was extracted using a modified multiple cell harvester (Otto Hiller Corp., Madison, Wis.), and radioactivity determined in an automated gamma counter.

Cell Cultures and Assays for Plaque-Forming Cells (PFC). Spleen cells were cultured in microtest plates and assayed as described previously (15). SRBC, TNP-LPS, and PB were diluted in BSS so that all additions could be made in a total vol of 10 μl. Cultures with no addition received 10 μl BSS.

Isopycnic Density Gradient Ultracentrifugation. TNP-LPS and TNP-LPS mixed with three parts by weight of PB were centrifuged in CsCl for 60 h and the fractions analyzed for TNP and abequose content as described previously (9).

Results

Polymyxin B at a concentration of 5-10 μg/ml (1-2 μg/0.2 ml culture) inhibits the mitogenic response of TNP-LPS and LPS without affecting the mitogenic response to either PHA, Con A, or PPD (Table I). At this concentration PB is thus inhibitory only for LPS and is not toxic for T or B lymphocytes. Only at levels of 50 μg/ml (10 μg/0.2 ml culture) is the response to PHA and Con A decreased to 50% of the control (data not shown). The effect is not T-cell-dependent, as it can be obtained in cultures of T-depleted spleen cells derived from nu/nu (Table I, Exp. 6-8) mice or ATx-ATS mice (Table I, Exp. 5).

In contrast, PB does not inhibit the immune response to TNP-LPS, and enhances the response considerably in cultures of normal spleen cells (Table II). It has no effect on the PFC response to SRBC. PB has no significant effect on the response to TNP-LPS, or to another T-independent antigen, DNP-Dextran, in the absence of T cells. The average level of TNP-PFC in unstimulated cultures of normal cells is fairly high here, and varies considerably from experiment to experiment. In individual experiments background TNP-PFC are inhibited by PB only when the background is high.

Several experiments were carried out to confirm the suggestion that the effect of PB on the mitogenic response was due to interaction of PB with LPS and TNP-LPS rather than to any effect on cells in culture. Polymyxin-treated LPS
### Table I

**Effect of Polymyxin B on Responses to T- and B-Cell Mitogens. Responses in Cultures with Polymyxin as % Control**

|                | Mitogen                  | TNP-LPS | LPS | PPD | Con A | PHA |
|----------------|--------------------------|---------|-----|-----|-------|-----|
| **Exp.**       | **Cell source**          | **Background** |     |     |       |     |
| 1              | Normal CBA               | 5.1     | 9.3 |     | 100   | 102 |
| 2              | Normal CBA               | 3.7     |     | 115 | 102   |     |
| 3              | Normal CBA               | 4.9     | 72  |     | 103   |     |
| 4              | Normal CBA               | 7.4     | 75  | 109 | 115   |     |
| 5              | Normal BDF,              | 2.3     | 3.8 | 101 | 85    |     |
|                | atx-als BDF,             | 3.6     | 6.4 |     |       |     |
| 6              | nu/nu                    | 15.3    | 15.3|     |       |     |
| 7              | nu/nu                    | 4.3     |     |     |       |     |
| 8              | nu/nu                    | 5.2     | 7.0 |     |       |     |

Additions were made in the following amounts: TNP-LPS: Exp. 1, 10 μg; Exp. 6, 5 μg; others, 1 μg. LPS: Exp. 1, 10 μg; others, 1 μg. PPD: 10 μg, Con A: 0.5 μg, PHA: 0.5 μg. PB: Exp. 1, 2, 6, 2 μg; others, 1 μg.

*Controls are cultures with added BSS instead of Polymyxin B.

### Table II

**Effect of Polymyxin B on Immune Responses of Normal and T-Depleted Spleen Cells**

| Cells cultured | Antigen*             | TNP-PFC/culture | SRBC-PFC/culture |
|----------------|----------------------|-----------------|------------------|
|                | -PB                  | +PB             | -PB              | +PB              |
| Normal         | No antigen           | 25 ± 7 (32)     | 8 ± 2 (37)       | No antigen       | 10 ± 4 (14)     | 18 ± 5 (17)     |
|                | 0.01 μg TNP-LPS      | 57 ± 5 (6)      | 367 ± 109 (6)    | 2 × 10³ SRBC     | 386 ± 37 (17)   | 378 ± 59 (17)   |
|                | 0.1 μg TNP-LPS       | 93 ± 17 (40)    | 753 ± 96 (56)    |                  |                  |                 |
| Nu/Nu          | No antigen           | 4 ± 7 (8)       | 5 ± 7 (8)        |                  |                  |                 |
|                | 0.1 μg TNP-LPS       | 67 ± 42 (8)     | 151 ± 56 (8)     |                  |                  |                 |
|                | 0.01 μg DNP- Dextran | 151 ± 138 (8)   | 136 ± 50 (8)     |                  |                  |                 |

Cells were cultured at 10°/well for the TNP-response, and 2 × 10°/well for the SRBC-response, and assayed on day 5 of culture. 1 μg PB per culture was added where indicated. PFC of individual cultures of normal BDF cells were averaged, and the means ± SEM given here. The numbers in parentheses indicate the total number of cultures included in each mean. Data given for TNP responses from six experiments (two for the lower antigen dose) and for SRBC responses from four experiments. Data from cultured nu/nu cells come from one experiment (Mean ± SD).

*Amount added per culture.

(PB-LPS) was prepared, and the mitogenic activity of this material compared with untreated LPS. A mitogenic assay yielded the following results: 1 μg LPS, 3,524 cpm; 1 μg PB-LPS, 422 cpm; unstimulated cultures, 90 cpm. Chemical determination of the free amino groups in LPS and PB-LPS indicated the amount of PB bound per μg LPS was 0.055 μg. Evidence for a physical change in

1Morrison, D. C., and D. M. Jacobs. 1975. Characterization of the interaction of lipopoly-saccharide with Polymyxin B. The effect on the activation of serum complement. Manuscript in preparation.
TNP-LPS was obtained by observing the CsCl density profile of TNP-LPS in the presence and absence of PB. As seen in Fig. 1, the density of TNP-LPS decreases on exposure to PB. Thus, treatment of LPS followed by removal of free PB yields a product with a small amount of bound PB which is reduced in mitogenic activity to less than 10% that of the original material. Further characterization of PB-LPS and PB-TNP-LPS and a determination of the quantitative and qualitative nature of the interaction will be described elsewhere.

Discussion and Summary

Our results indicate a dissociation between the immunogenic and mitogenic properties of TNP-LPS, a T-independent antigen. In the presence of PB the mitogenic response to this antigen and to unconjugated LPS is abrogated, while under the same conditions TNP-LPS stimulates the same or a higher TNP-PFC response. It is significant that PB does not affect the proliferative response to other T- and B-cell mitogens such as Con A, PHA, and PPD; nor does it affect the PFC response to another T-independent antigen, DNP-Dextran, or to a T-dependent antigen, SRBC. Furthermore, treatment of LPS with PB and subsequent removal of the unbound antibiotic reduces considerably the mitogenic capacity of LPS. Thus, PB does not exert its activity on the cells, and reacts here only with LPS. The enhancement of the response to TNP-LPS in the presence of PB may be due to a reversal of toxic effects of the LPS portion of the conjugate as the effect is more pronounced in cultures of normal spleen cells than in cultures of T-deprived spleen cells. This possibility rests on the observations that LPS added to normal spleen cells can inhibit the anti-SRBC response (16) and high levels of TNP-LPS are less stimulatory in vitro than low doses (9).
The precise nature of the interaction of PB with LPS and its haptenated derivative is unknown, although work on model membrane systems and the selective toxicity of PB for gram-negative bacteria suggest that the presence of phosphatidylethanolamine in the LPS of bacterial cell walls is important (11-17, Footnote 1). An interaction might also be expected between the highly charged PB, which contains five $\gamma$-amino groups, and the phosphate groups of LPS. Alternatively, the hydrophobic portion of the PB molecule could interact with the ester- and amide-linked fatty acids of the Lipid A portion of the molecule. Thus, we cannot yet make any correlation between the changes in structure caused by PB and the differences in biological activity achieved.

Nevertheless, it is of interest that TNP-LPS can be modified so that its general B-cell mitogenicity is abrogated yet immunogenicity is retained. These results strongly suggest that the property of antigens which allows the triggering of B cells in the absence of T cells is not the ability to be a mitogen for all B cells. It could be argued that TNP-LPS in the presence of PB binds only to specific cells via Ig receptors and acts only as a local mitogen. If this were a case, one would not be able to distinguish between T-dependent and T-independent antigens by their ability to induce cell division in antigen-specific B cells. For example, SRBC stimulates B cells to divide in the absence of T cells, but no antibody is produced unless T-cell replacing factors (TRF) are added 24 h later (18-21). Since TRF is the differentiation signal, all antigens may possess the signal which stimulates the specific precursors to divide. The additional "signal" possessed by T-independent antigens may thus be a differentiation signal rather than a mitogenic signal. The "signal" may not be delivered by a structure on the antigen, but could be delivered by the changes in conformation of the receptors after interaction with a polymeric antigen, as originally suggested by Feldmann and Basten (1). We expect TNP-LPS to be a good antigen with which to explore these possibilities further.

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