Bacterial lipopolysaccharide activates suppressor B lymphocytes
(regulation of humoral immunity)

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ABSTRACT Lipopolysaccharide (LPS) extracted from the outer cell wall of Gram-negative bacteria modulates the immune response in vivo and in vitro. Depending on the experimental conditions, it may enhance or inhibit the production of humoral antibody. The pathway by which LPS suppresses antibody production is examined in this study. C57BL/6 spleen cells incubated with LPS (>10 μg/ml) not only fail to produce antibody to sheep erythrocytes in vitro but also, when transferred after stimulation with LPS, inhibit antibody production in spleen cells that were not treated with LPS. This observation suggested that LPS activates suppressor cells. We have identified a suppressor B cell as mediator of LPS-induced immune suppression and determined its cell surface antigen phenotype as IgG, IgM, CR, Ly-B-2+, FC1-. LPS does not induce suppressor macrophages or suppressor T cells, nor are macrophages or T cells required for the generation of suppressor B cells by LPS.

Lipopolysaccharide (LPS), primarily recognized as an immune adjuvant, also induces immune suppression. Both immune enhancement and immune suppression may occur simultaneously and, depending on the experimental conditions, one or the other may predominate. We have demonstrated that LPS enhances the antigen-dependent production of antibody in vitro by causing macrophages to release a B-cell differentiation factor (BDF). BDF stimulates antibody formation in the absence of helper T cells and of macrophages. BDF also selectively induces the phenotypic differentiation of B cells (1–3). LPS-induced immune suppression, by contrast, does not involve macrophages. We report here that LPS directly activates suppressor cells which carry the characteristic surface markers of B lymphocytes.

MATERIALS AND METHODS

Mice. Eight- to 12-week-old C57BL/6, (C57BL/6 × DBA/2)F1, and C3H/HeB/FJ female mice were purchased from Jackson Laboratory. C57BL/6-H-2b, C3H/He-B-2.1 Strb, and C57BL/6-Pc-1A were obtained from our own breeding colony.

Antisera. A.TH anti-A.TL (anti-laA), rabbit anti-MOPC 104 E (anti-IgM), rabbit anti-F(ab)2 fragment of MOPC 21 (anti-IgG), and (A/Th 1.1 × ACR F1 anti-ASL/1 (anti-Thy 1.2), raised as described (4), were supplied by U. Hammerling (Sloan-Kettering Institute, New York). (C3H.I × C57BL/6)F1 anti-I-29 (anti-Ly-b-2.1) and (DBA/2 × C57BL/6)F1 anti-MOPC 70 A (anti-Pc-1) were prepared (5, 6) by F. W. Shen (Sloan-Kettering Institute, New York). The 195 fraction of rabbit anti-sheep erythrocytes (SRBC) was purchased from Cordis Laboratories (Miami, FL). Rabbit anti-concanavalin A was prepared as described (7).

T-Cell Replacing Factor. Tumor necrosis serum (TNS) prepared in CD-1 mice was obtained from E. Carswell (Sloan-Kettering Institute, New York). T-cell replacing factors in this serum have been described (8).

LPS. LPS prepared from Salmonella abortus-equi, its sodium and triethylamine salt forms, and lipid A triethylamine, were provided by Chris Galanos (Freiburg, Germany). LPS from Salmonella minnesota S445 was supplied by Markus Simon (Heidelberg, Germany).

Antigens. SRBC were obtained from the Colorado Serum Company (Denver, CO).

Cell Culture. Spleen cells were cultured according to the method of Mishell and Dutton (9) except for the addition of 50 μM 2-mercaptoethanol to the culture medium (10). Two-phase cultures were set up as follows. One set of spleen cell cultures (recipient cultures) containing 0.6 or 0.7 ml of cells (10^7/ml) in complete medium (9) was set up in 35-mm dishes and immunized with SRBC. Another set of spleen cell cultures was initiated at the same time in the presence and absence of 10 μg of LPS. These cells were usually passed over Sephadex G-10 columns first to remove macrophages. After 20–24 hr, the cells were harvested by titration and washed twice in medium. Treatment of cells with antiserum and complement was performed at this time. Graded numbers (as indicated) were transferred in 0.4- or 0.3-ml volumes to the recipient cultures. Control cultures, to which no cells were transferred, received an equal volume of medium. Assays for plaque-forming cells (PFC) were performed 3 days later.

Cell Preparations. Macrophages were removed from spleen cell suspensions by passing them through Sephadex G-10 columns as described by Ly and Mishell (11). Cells bearing a receptor for the third component of complement (CR+) cells were removed by passage over Sephadex G-10 columns coated with antigen–antibody–complement complexes (7). Fractions of spleen cells rich in CR+ or CR- cells were prepared by rosetting CR+ lymphocytes with antibody and complement-coated erythrocytes and separating them from nonrosetted lymphocytes on a 1 x g velocity sedimentation gradient (12).

Cytotoxicity Test. A two-step test was performed by incubating cells (1–5 x 10^6 cells) with antiserum on ice for 30 min, pelleting the cells, and then resuspending them in 1:10 dilution of rabbit serum (complement) followed by incubation at 37°C for 35 min. The cells were then washed twice and resuspended in complete medium. Aliquots of cells treated with antiserum (and complement) were resuspended before use to the volume of the control cells which were treated with complement alone.

Assay for PFC. Cells producing antibody to SRBC were enumerated by the Jerne–Nordin hemolytic plaque assay (13) as modified by Mishell and Dutton (9).

Abbreviations: LPS, lipopolysaccharide; BDF, B-cell differentiation factor; SRBC, sheep erythrocytes; CR, receptor for third component of complement; PFC, plaque-forming cells; TNS, tumor necrosis serum.
concentrations and preparations of triethylamine. Dashed reagents; regard (1, g/ml) concentrations immune the production whereas higher doses (>PFC LPS of lipid C3HeB/FeJ, C3H/St, BALB/c, density from to spleen C57BL/6 spleen response production by Table Conditions It has been shown (14) that the addition of LPS at low concentrations (<1 µg/ml) to BDF₁ spleen cell cultures enhanced PFC production whereas higher doses (>1 µg/ml) suppressed the immune response. Suppression of BDF₁ spleen cells was achieved in a similar fashion with different preparations of LPS: LPS of S. abortus-equi, its sodium and triethylamine salts, and the lipid A portion (Fig. 1).

Suppression was not consistently achieved in cultures of C3HeB/FeJ, C3H/St, BALB/c, AKR, RF, and C58 spleen cells (in contrast to C57BL/6 or BDF₁ spleen cells) cultured at a density of 10⁵ cells per ml in the presence of 10 µg of LPS. Cells from these strains characteristically generated fewer PFCs in response to SRBC as compared with cells from C57BL/6 mice or their F₁ hybrids. By doubling the concentration of C3HeB/FeJ spleen cells, a substantial increase in the normal PFC response resulted, and this increase in responsiveness was found to be associated with an increased susceptibility to the immune suppressive activity of LPS (Table 1). These data and the observation that decreasing the cell concentration of C57BL/6 spleen cells resulted in enhancement of antibody production by LPS rather than suppression indicates that LPS is inhibitory only in cultures undergoing a vigorous immune response but is stimulatory in cultures undergoing a weaker response.

Table 1. Dependence of LPS immune suppression on cell density

<table>
<thead>
<tr>
<th>Spleen cell density, no. x 10⁶/ml</th>
<th>Anti-SRBC PFC/culture on day 4†</th>
<th>C57BL/6</th>
<th>C3HeB/FeJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>-</td>
<td>4225</td>
<td>4125</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>900</td>
<td>820</td>
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<tr>
<td>10</td>
<td>-</td>
<td>2250</td>
<td>725</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>550</td>
<td>1150</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>520</td>
<td>270</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>850</td>
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<td>-</td>
<td>275</td>
<td>10</td>
</tr>
<tr>
<td>2.5</td>
<td>+</td>
<td>1900</td>
<td>1770</td>
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</table>

* LPS from S. minnesota S445 (10 µg/ml).
† SRBC were added to culture on day 0.

The time of LPS administration with respect to antigen is critical in determining a state of enhancement or suppression. The immune response was suppressed only when LPS was added before or together with antigen, even when the addition of both LPS and antigen was delayed for a day (Table 2). These data confirm earlier in vivo and in vitro observations (14, 15). We document these experiments here because they provide the rationale for experiments described below.

It has been found (7) that spleen cell populations that were devoid of CR+ cells could not mount a PFC response when immunized with SRBC; however, responsiveness was restored by the addition of LPS. This was taken to indicate that cells carrying the CR are implicated in LPS-induced immune suppression. By manipulating the composition of cultured spleen cells, we attempted to identify further the cellular components involved in LPS-induced immune suppression.

Suppression by LPS Is Not Mediated by T Cells or Macrophages. To determine if a suppressor T cell could be involved, normal spleen cells were treated with anti-Thy 1 serum and complement and subsequently cultured in the presence and absence of LPS. The cultures were supplied with a T-cell replacing factor contained in TNS. T-cell depleted cultures containing LPS and TNS were suppressed compared to cultures without LPS (Table 3). Effectiveness of T-cell elimination is demonstrated by the lack of responsiveness in cultures to which TNS had not been added and was verified by the absence of a mitotic response to the T-cell mitogen concanavalin A (data not shown). Besides showing that suppression by LPS can occur in the absence of T cells, this experiment further implies that suppression is not due to limited T-cell helper function because the addition of a T-cell replacing factor did not restore responsiveness. Experiments using other helper factors [allogeneic effect factor (16), T-cell replacing factor (17), and B-cell differen-

Table 2. Effect of LPS on response of BDF₁ spleen cells when added simultaneously with or after antigen

<table>
<thead>
<tr>
<th>Time of addition, day</th>
<th>LPS*</th>
<th>Anti-SRBC PFC/culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-SRBC PFC/culture</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Day 4</td>
<td>Day 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>9,350</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>650</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>10,450</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
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<tr>
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<td>175</td>
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<tr>
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<td>1</td>
<td>1,243</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>5,125</td>
</tr>
</tbody>
</table>

* LPS from S. minnesota S445 (10 µg/ml) was added at times indicated to 10⁷ (C57BL/6 × DBA/2)F₁ spleen cells.

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Table 3. LPS immune suppression is not mediated by T cells

<table>
<thead>
<tr>
<th>Spleen cells</th>
<th>Addition*</th>
<th>Anti-SRBC PFC/culture on day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>—</td>
<td>2125</td>
</tr>
<tr>
<td>LPS</td>
<td>2125</td>
<td>2945</td>
</tr>
<tr>
<td>TNS</td>
<td>150</td>
<td>80</td>
</tr>
<tr>
<td>TNS + TNS</td>
<td>2575</td>
<td>6620</td>
</tr>
</tbody>
</table>

* LPS from S. minnesota S445 (10 µg/ml) was added to 10⁷ (C57BL/6 × DBA/2)F₁ spleen cells on day 0. TNS, 1%, was added on day 2. SRBC were added to all cultures on day 0.
† Cells treated with anti-Thy-1.2 antiserum plus complement.
ferentiation factor (2)) yielded similar results. Removal of macrophages by passage of spleen cells over Sephadex G-10 columns also did not affect the ability of LPS to inhibit antibody production. Our data are not shown here because this observation has been documented (3).

Characterization of the Suppressor Cell. Taken together, our experiments pointed to the possibility that LPS inhibits antibody formation by activating suppressor B cells. We attempted, therefore, to design an assay for suppressor B cells. A procedure that had previously succeeded in the identification of concanavalin A-induced suppressor T cells was used as a guide for devising this system. Concanavalin A, a mitogen for T cells, was found to inhibit antibody production in vitro when added to cultures at the start. Cells obtained from cultures stimulated with concanavalin A for 1 or 2 days inhibit antibody formation of fresh spleen cell cultures to which they are added together with antigen. The activity of the suppressor T cells could be distinguished from the inhibitory activity of concanavalin A itself through the use of α-methylnanoside, which competes with concanavalin A for its receptor on lymphocytes (18, 19). No such competitor has been described for LPS. Thus, using a similar procedure for the detection of LPS-induced suppressor B cells, we had to circumvent the problem of transfer of LPS. We tried to take advantage of the finding that addition of LPS to spleen cells after 24 hr of culture no longer induced suppression of the immune response (Table 2). Spleen cells were preincubated for 20–24 hr in the presence or absence of LPS and then transferred to cultures that had been set up and immunized 24 hr earlier. Generation of anti-SRBC PFCs was determined 3 days later. Spleen cells preincubated for 20 hr with LPS (but not spleen cells incubated without LPS) were found to be inhibitory for recipient cultures (Fig. 2). In most subsequent experiments, we first passed spleen cells over Sephadex G-10 columns to eliminate potentially complicating effects mediated by macrophages.

An experiment showing the effect of increasing numbers of pretreated spleen cells to recipient cultures is illustrated in Fig. 3. Spleen cells were preincubated for 24 hr or for 2 hr with and without LPS. Suppression was noted only in cultures of spleen cells that had at least 24 hr in which to develop suppressor cells in response to LPS.

Earlier experiments had indicated that CR+ cells are involved in the induction of LPS-induced immune suppression.

FIG. 3. Titration of precultured spleen cells for recipient cultures. (C57BL/6 × DBA/2)F1 spleen cells were passaged over Sephadex G-10 and cultured for 24 hr (A) or 2 hr (B) at 10^7 cells per ml in the absence (open symbols) or presence (solid symbols) of LPS (S. minnesota S445, 10 μg/ml). These cells were then harvested, washed, and added in increasing numbers to unseparated (C57BL/6 × DBA/2)F1 spleen cell (recipient) cultures that had been initiated 24 hr previously in the presence of SRBC. Recipient cultures contained the appropriate cell number and volume so that 10^3 cells in 1 ml was achieved after additions were made. The dashed line represents the response of control cultures to which no cells were added.

(3). We tested this possibility directly by preparing populations of CR+ and CR− cells that were cultured for 24 hr with and without LPS and subsequently were added to antigen-stimulated recipient cultures. The results showed that CR+ cells activated by LPS can suppress the immune response. Cells from the CR− fraction of spleen cells did not generate suppressor activity in response to treatment with LPS (Fig. 4).

Because this experiment strengthened the view that the cell mediating LPS-induced suppression was a B cell, further efforts were made to characterize the LPS-induced suppressor cell serologically. Portions of Sephadex-fractionated cells cultured for 20 hr in the presence or absence of LPS were treated in a two-step cytolytic procedure with cell surface antigen-specific antisera and complement. Antiserum directed against the following cell surface markers were tested for their ability to abrogate suppression of LPS-activated spleen cells.

La*. LPS-activated C57BL/6 H-2^d spleen cells treated with anti-La* antiserum and complement no longer displayed inhibitory activity. Anti-La* antiserum reacted with approximately 40% of splenic B cells, with macrophages, and with a small fraction of T cells (Fig. 5A).
treatment with surface C57BL/6; anti-Thy, (A/Thy-Pc-i and anti-I/st untreated). The following antisera were used for the detection of the cell surface antigens: Anti-Id, A.TH anti-A.TL, anti-slg, rabbit anti-IgM of myeloma MOPC 104E plus rabbit anti-mouse (Pab) of IgG; anti-Thy, (A/Thy 1.1 x AKR-i-2F2); anti-Ly-b-2, (C3H.1 x C57BL/6)F1, anti-Ist tumor 29. Spleen cells passed over Sephadex G-10 columns were cultured at 10^6 cells per ml for 20 hr in the presence (1) or absence (2) of LPS (S. minnesota S445, 10 μg/ml). Cells were harvested and divided into fractions that were either treated with antisera and complement (Ab + c), complement alone (c), or left untreated (none). These fractions were then added to recipient cultures (7 x 10^6 spleen cells cultured with SRBC for 24 hr) as indicated.

Ig and Thy-1.2. Spleen cells cultured with LPS lost their ability to suppress after treatment with purified anti-IgM antisera and anti-F(ab)2 of IgG; antisera but not after treatment with anti-Thy-1 antisera. These data indicate that the cell type responsible for LPS-induced suppression is a B cell rather than a T cell (Fig. 5 B and C).

Ly-b-2.1. Ly-b-2.1 is an alloantigen expressed exclusively on B cells (5). C57BL/6-Ly-b-2.1-StrB is a mouse strain congenic with C57BL/6, whose B cells express this surface marker. Treatment of LPS-activated cells with anti-Ly-b-2.1 antisera and complement eliminated suppressor cells in cultures of the congenic strain but not in the control cultures of (Ly-b-2.1 negative) C57BL/6 mice (Fig. 5 D and E).

Pc-1. This surface antigen is found on a small subpopulation of B cells and is associated with their differentiation into plasma cells. Treatment of LPS-stimulated cells from C57BL/6-Pc-1+ mice (a strain congenic in C57BL/6 whose cells express the Pc-1 surface antigen) with anti-Pc-1 antiserum and complement did not alleviate suppression (Fig. 5F).

Thus, on the assumption that LPS-induced immune suppression is mediated by one distinct cell type, its cell surface phenotype can be described as Ig+, Ia+, CR+, Ly-b-2+, Pc-1+.

Normal Mouse Serum Interferes with Induction of Suppression by LPS. Having identified a suppressor B cell as mediator of LPS-induced immune suppression, we turned to experiments aimed at defining the mechanism of suppression. We had previously noted that LPS fails to inhibit antibody production when normal mouse serum is added to the spleen cell cultures simultaneously. With the two-stage assay procedure described above, it became possible to distinguish whether the mouse serum interferes with the activation or the action of LPS-induced suppressor B cells.

When normal mouse serum was added to cultures that received LPS-stimulated spleen cells (effector phase) as well as to cultures treated with LPS prior to the cell transfer (induction phase), it did not interfere with the induction of suppressor cells but instead inhibited their function (Fig. 6).

**DISCUSSION**

We have described experiments that indicate that LPS-induced suppression of antibody formation in vitro is mediated by suppressor B cells. Indirect evidence for the involvement of B-cells stems from the finding that suppression occurs only in cultures containing cells that carry the CR and from the fact that removal of T cells and of macrophages does not affect the ability of LPS to inhibit the production of antibody. The sensitivity of LPS-activated splenic suppressor cells to cytolytic treatment with antisera directed against B-cell surface antigens provided direct evidence. Unless B-cell-mediated immune suppression represents a cooperative action, the LPS-induced suppressor B cell can be described by the phenotype Ig+, Ia+, CR+, Ly-b-2+, Pc-1+.

Evidence for the existence of suppressor B cells has been reported also in systems that do not involve LPS. Zembala et al. (20) found that a CR+ theta-negative cell can suppress the cell-mediated hypersensitivity response to picryl chloride. Zan-Bar et al. (21) reported that cells bearing predominantly IgG can suppress the IgM response of IgM- and IgD-bearing cells. Similar to our findings, Perrson (22), in experiments to study LPS-mediated suppression of the humoral immune response, found that removal of macrophages and T cells did not interfere with LPS-induced depression of PFC responses. Impairment of delayed hypersensitivity responses in mice by LPS has been reported by La Grange et al. (23). They noted a correlation between an increase in non-antigen-specific (polyclonal) antibody titers and the degree of suppression. They concluded that B cells can regulate T-cell activity. In light of the studies by LaGrange et al. and her own findings, Perrson suggested that LPS suppression was the result of B cells impairing T-cell helper function. If this were the case, one should be able to overcome suppression by the addition of a helper factor. This was not the case in our experiments. It is possible that T-cell helper function is also impaired by LPS. Yet, it alone could not account for the induction of LPS suppression.

Although the mechanisms by which B cells regulate the function of other B cells is not recognizable at present, some possibilities are raised by the finding that normal mouse serum interferes with the effecter function of LPS-activated suppressor cells. First, mouse serum inhibits the mitotic response of B cells to LPS (unpublished observations). It is conceivable that a large
number of proliferating cells might generate inhibitory conditions in tissue culture. Curtailment of their mitotic activity might simply prevent this effect. The CR—fraction of spleen cells, which displays a delayed mitotic response to LPS as compared to the CR+ fraction (24), is not suppressive but CR+ cells are.

Another possibility to consider takes into account the ability of LPS to activate Ig production by B cells polyclonally. Normal mouse serum has been shown to inhibit polyclonal B-cell activation. It was recently reported by Dresser (25) that approximately 50% of immunoglobulin-producing cells activated polyclonally by LPS secrete Ig-specific autoantibodies. Such LPS-induced anti-Ig B cells might be the LPS-induced suppressor cells. Our finding that normal mouse serum inhibits the effector phase rather than the induction phase of LPS-induced suppressor B cells must be considered in this context. If, indeed, suppressor B cells would inhibit antigen-reactive B cells by interaction with their surface Ig, interference by solubilized Ig copies in the serum seems plausible.

Induction of suppressor B cells must not be limited to activation of B cells with LPS. Evidence for suppressor B cells has been obtained in systems that do not involve LPS. Dresser’s experimental observation of LPS-induced anti-Ig B cells provides a possible explanation for polyclonally induced suppressor B cells, and Jerne’s theoretical postulate (26) of specific antibody-antiidiotype antibody interactions, as expressed in his network theory, might provide a possible explanation for antigen (or antibody) specific suppressor B cells. Jerne proposed that the immune system is maintained in a steady state by the “dualistic” nature of its elements. The Jerne model might provide for the possibility that an antigenic determinant induces Ig-producing B cells and corresponding suppressor B cells, just as LPS might induce Ig-producing B cells and corresponding suppressor B cells in a polyclonal manner.

On a more practical note, LPS has been used as a tool to investigate regulatory mechanisms of the immune response. As a result of these studies, clinical trials have been initiated to determine whether stimulation of the immune system with LPS has beneficial effects in cancer patients. Experiments reported here show that, in addition to its properties of enhancement of antibody production, LPS can stimulate B cells to inhibit other B cells from developing into antibody-forming cells. This dual effect of LPS might be characteristic of many immune modulators. In the mouse system, it was observed that, under identical conditions, some strains favor immune enhancement and others favor suppression in response to LPS. Similar patterns might occur among individual cancer patients treated with LPS. It would appear that immunotherapy would be most effective if the balance of inhibitory and stimulatory effects of a given immunopotentiator could be manipulated to its desired advantage. Prerequisite for the means to achieve this is a clear understanding of the biological pathways involved. With regard to LPS, the work presented here might be a step in this direction.

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