

CELLULAR REQUIREMENTS FOR
LIPOPOLYSACCHARIDE ADJUVANTICITY
A Role for Both T Lymphocytes and Macrophages for
In Vitro Responses to Particulate Antigens

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It has been known for over 30 yr that gram-negative bacteria enhance immune responses to vaccines in man (1) and that the active adjuvant component is cell wall lipopolysaccharide (LPS)¹ (2). However, the precise mechanism by which LPS mediates its adjuvant effect has not yet been resolved.

Because LPS is a potent B-cell mitogen, a polyclonal B-cell activator, and acts as a T-independent antigen, several investigators have suggested that the adjuvant properties of LPS are most easily ascribed to a direct effect of LPS on the B cells (3–6). Because LPS reverses tolerance to immunity, even in the presence of T-suppressor cells, this has been taken as further proof that LPS enhances B-cell responses (5, 7). LPS can also bypass the requirement for T-helper cells by facilitating immune responses in mice given haptened, homologous erythrocytes (8, 9). Finally, the lack of tolerance reversal to human gamma globulin by LPS in C3H/HeJ mice (whose B cells are unresponsive to LPS) has been taken as evidence that potentiation is occurring at the level of the B cell (3, 10, 11).

Alternatively, a number of studies have suggested that the adjuvant properties of LPS are best explained through activation of T cells (12–17). Allison and Davies (12), utilizing both thymectomized mice and mice treated with anti-lymphocyte serum, demonstrated that LPS adjuvant activity required normal T-cell populations. In a series of experiments, Katz and coworkers (13–15) also provided evidence for a T-cell requirement during LPS-mediated augmentation of antibody responses. In the first experiments, removal of T cells from antigen-primed spleen cells before adoptive transfer and subsequent challenge abrogated the capacity of LPS to mediate its adjuvant effect (13). Subsequent studies (14, 15) provided evidence that the adjuvant effect of LPS on hapten-specific IgM, IgG, and IgE plaque-forming cell (PFC) responses was exerted on carrier primed, T-helper cell activity. More recent evidence

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¹ *Abbreviations used in this paper:* Bu-LPS, LPS prepared by butanol-water extraction; FCS, fetal calf serum; LAF, lymphocyte-activating factor; LAP, lipid A-associated protein; LPS, lipopolysaccharide; MLR, mixed lymphocyte reaction; MØ, macrophage; nu/nu, athymic nude mice; nu/+, normal littermates of athymic nude mice; PFC, plaque-forming cells; Ph-LPS, LPS prepared by phenol-water extraction; SRC, sheep erythrocytes; TNF, tumor necrosis factor; TNP, trinitrophenyl.

suggests that a subpopulation of T cells regulates B-cell responses to LPS and eventual immunoglobulin production (16, 17).

Because the macrophage (MØ) is an important accessory cell in the immune response, enhancement of its activity by LPS could also account for adjuvanticity. Allison and coworkers (18, 19) found that administration of antigen with MØ and LPS greatly enhanced immune responses. More recently, it has been found that LPS causes the release from MØ of lymphocyte-activating factor (LAF) (20). Subsequent biochemical characterization studies have shown that highly purified LAF does, in fact, enhance the antibody response (21, 22), although LAF-containing supernates augment antibody production only in the presence of T cells (23). Finally, Hoffmann and coworkers (9) have shown that administration of LPS to mice induces the production of a serum factor (presumably MØ derived) that potentiates antibody responses of C3H/HeJ (LPS nonresponsive) B cells.

Given this continuing controversy over the precise lymphoid cell types affected by LPS, we have studied potentiation of immune responses to particulate antigens *in vitro* as a correlate of LPS adjuvanticity. Spleen cells from either LPS responsive or nonresponsive mice were used as the source of T and B lymphocytes and MØ. Subsequent mixing of these different cell populations in culture allowed us to assess the importance of each cell type in LPS potentiation of immune responses. Our results clearly indicate that both T lymphocytes and MØ must be derived from LPS-responsive mice to obtain an LPS-induced enhancement of the immune response to particulate antigens.

Materials and Methods

Animals. C3H/HeJ and C57BL/10Sn were obtained from The Jackson Laboratories (Bar Harbor, Maine). C3H/HeN, C3H/HeN nude (nu/nu) and normal littermates (nu/+) (the progeny of eight cycles of crossing into the C3H/HeN strain), and C57BL/10ScN nu/nu and nu/+ (the progeny of nine cycles of crossing into the C57BL/10ScN strain) mice were provided by the Division of Research Resources (National Institutes of Health, Bethesda, Maryland). 7- to 12-wk-old mice were used for all studies.

Endotoxin. *Escherichia coli* K235 LPS prepared by a phenol-water extraction (Ph) procedure (24) or by a butanol-water extraction (Bu) method (25) was employed in these studies.

Culture Methods. Mouse spleens were aseptically removed and single cell suspensions were prepared by passage through a sterile screen (60 mesh). Cells were washed and cultured by the method of Mishell and Dutton (26) with modification. Specifically, cultures containing 5×10^6 spleen cells in 0.5 ml complete medium (26) in 16-mm multiwell Linbro trays (Linbro Chemical Co., Hamden, Conn.) were immunized with either 2×10^6 sheep erythrocytes (SRC) or trinitrophenyl conjugated SRC² (McGhee et al.). Briefly, 20 mg of 2,4,6-trinitrobenzene sulfonic acid (Pierce Chemical Co., Rockford, Ill.) in 2 ml cacodylate buffer, pH 6.9 was added to 0.5 ml packed SRC and reacted for 30 min at room temperature. The reaction mixture was stopped with excess glycylglycine buffer and the cells were washed extensively with medium. After the addition of either Ph-LPS or Bu-LPS, cultures were incubated (37°C) with rocking in a humidified chamber containing 7% O₂, 10% CO₂, and 83% N₂. The nutritional mixture (0.05 ml) described by Mishell and Dutton (26) was added daily to each culture. Cultures were assayed for either anti-SRC or anti-TNP PFC on day 5.

Similar culture methods were employed in cell mixing experiments. In these studies, various combinations of spleen cell subpopulations (described below) of either nonadherent lymphocytes (3×10^6 /culture), T cells (1×10^6 /culture), B cells (1×10^6 /culture), and/or a monolayer of adherent cells were cultured in 0.5 ml complete medium (Results).

² McGhee, J. R., S. M. Michalek, R. N. Moore, S. E. Mergenhausen, and D. L. Rosenstreich. 1979. *J. Immunol.* In press.

Purification of Splenic Macrophages. MØ-rich populations were prepared from spleen cells by an adherence technique. The concentration of single spleen cell suspensions was adjusted to 10^7 spleen cells/ml. Subsequent to centrifugation, the pellet of cells was irradiated with 1,000 rads, the supernate removed, and the cells resuspended in medium. 0.5 ml aliquots (containing 5×10^6 cells) were added to individual wells and incubated at room temperature for 2 h. Nonadherent cells were removed by repeated addition and removal of medium (three to four times). The remaining adherent monolayer of cells served as the source of MØ in all of the experiments reported herein.

Purification of Splenic T and B Lymphocytes. MØ-depleted spleen cell preparations were prepared as previously described (27) by consecutive passage through two Sephadex G-10 columns. (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.). The effluent cells were used as the source of nonadherent lymphocytes. These preparations did not support immune responses to particulate antigen (Results).

Purified individual T and B populations were prepared from spleen cell suspensions. The spleen cells were first treated with ammonium chloride erythrocyte-lysing solution-lysing buffer (Media Unit, National Institutes of Health, Bethesda, Md.) for 10 min at 4°C and subsequently washed with medium supplemented with 2% fetal calf serum (FCS). The resultant cell preparations were passed through 7-ml glass wool columns to remove adherent cells. The nonadherent cells were eluted with medium supplemented with 2% FCS (at room temperature) and subsequently applied to a nylon wool column according to the method of Julius et al. (28). T cells were eluted from this column with warm medium. Splenic B cells were prepared by compression of the nylon wool using the method of Handwerger and Schwartz (29). In some experiments, primed T cells were prepared by using spleens of mice which had been intravenously injected 1 wk before with SRC (0.1 ml of 0.01% suspension, reference 30). The T-cell preparations were further treated to remove residual B cells by using anti-mouse immunoglobulin serum (Meloy Laboratories Inc., Springfield, Va.) and complement as previously described (31). In certain experiments B-cell preparations were incubated with anti-theta serum and complement to remove residual T cells (31).

PFC Assay. After a 5-d incubation, nonadherent cells were removed from culture wells, washed, and resuspended to an appropriate volume for assay. Cultures in triplicate were tested for anti-SRC or anti-TNP PFC using the slide modification (26) of the Jerne and Nordin hemolytic plaque assay. For the TNP assay, lightly conjugated, TNP-SRC were prepared by the method of Rittenberg and Pratt (32).

Mixed Lymphocyte Reaction (MLR) Assay. The MLR assay was performed as described previously (33). Single spleen cell suspensions were prepared as described above and resuspended (5×10^6 cells/ml) in RPMI-1640 (Grand Island Biological Co., Grand Island, N. Y.) supplemented with glutamine, penicillin, streptomycin, Hepes buffer, and 10% FCS. Triplicate cultures consisting of 0.1 ml of responder cells and 0.1 ml of X-irradiated stimulator cells (2,500 rads) in round bottom, plastic microtrays were incubated for 96 h at 37°C in a humidified atmosphere of 5% CO₂ in air. Cultures were harvested (Mash II, Microbiological Associates, Walkersville, Md.) after a 4-h pulse of tritiated thymidine (0.5 µCi ³H-TdR; 6.0 Ci/mM, Schwarz/Mann Div. Becton, Dickinson & Co., Orangeburg, N. Y.).

Statistics. All results are expressed as the mean PFC response per culture \pm standard error. The significance of differences between means was determined by Student's *t* test.

Results

Optimal Conditions for LPS-Induced Adjuvanticity In Vitro. For these studies, we have utilized the LPS responsive C3H/HeN mouse strain and the histocompatible, LPS nonresponsive C3H/HeJ mouse (33, 34). In preliminary experiments, it was found that maximum numbers of anti-SRC PFC were obtained with 5×10^6 spleen cells in 0.5 ml medium from either C3H/HeN or C3H/HeJ mice (Fig. 1). This concentration of cells was maintained throughout all of the experiments reported herein. In these studies we have employed a Ph-LPS preparation which selectively stimulates LPS responsive mice (33, 34). As can be seen in Fig. 1 A, this preparation of endotoxin

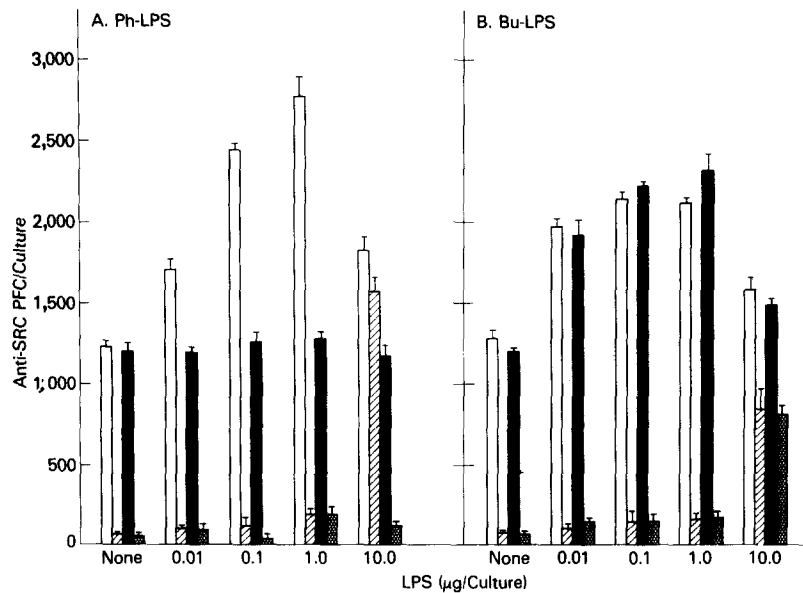


FIG. 1. Titration of LPS-induced adjuvanticity of specific (antigen dependent) and polyclonal (antigen independent) anti-SRC PFC responses. Primary spleen cell cultures (5×10^6 cells/0.5 ml) from either C3H/HeN (responder) or C3H/HeJ (nonresponder) mice were incubated with increasing concentrations of either: A. Ph-LPS or B. Bu-LPS for 5 d. Control cultures (no LPS) represent background anti-SRC PFC responses. □, C3H/HeN specific; ▨, C3H/HeN polyclonal; ■, C3H/HeJ specific; ▩, C3H/HeJ polyclonal.

enhanced the antibody response of C3H/HeN spleen cell cultures. Concentrations of 0.1 and 1.0 μg Ph-LPS were most effective, whereas a log higher concentration (10 μg) elicited a polyclonal response (antigen-independent generation of anti-SRC PFC). Ph-LPS did not enhance the anti-SRC PFC response or induce a polyclonal response in C3H/HeJ (nonresponsive) mouse spleen cell cultures. In contrast, a Bu-LPS preparation, which contains lipid A-associated protein (LAP), elicited both adjuvant and polyclonal responses in C3H/HeJ, as well as C3H/HeN, mouse spleen cell cultures (Fig. 1 B), thus demonstrating that the C3H/HeJ spleen cells were able to manifest an adjuvant response when appropriately stimulated. Our results concerning polyclonal activation by Bu-LPS and by high concentrations of Ph-LPS were in agreement with previous findings by others (9, 35).

Using this system as an *in vitro* model of LPS-induced adjuvanticity, we proceeded to analyze which cell or cells had to be LPS responsive for enhancement of the immune response to occur. Because 0.1 or 1.0 μg of either LPS preparations were maximally effective, these concentrations were utilized in all subsequent experiments.

Evidence for Macrophage Requirement in LPS-Induced Adjuvanticity. We first examined the contribution of adherent cells in potentiation of immune responses. Spleen cells from C3H/HeN (responder) mice were fractionated into nonadherent (G-10 passed) and adherent subpopulations. The two subpopulations of cells were cultured either by themselves or together in the presence and absence of Ph-LPS and the antibody responses to SRC assessed. Table I shows that in the absence of M ϕ , the antibody response of the lymphocytes was minimal. Moreover, Ph-LPS (1.0 μg /culture) failed to enhance the PFC response unless M ϕ were present.

TABLE I
Macrophage Requirement for LPS Adjuvanticity

C3H/HeN (responder) cell cultures*	LPS added (1.0 µg/culture)	
	None	Ph-LPS
Lymphocytes and MØ	1,085 ± 60‡	1,950 ± 100
Lymphocytes only	62 ± 19	81 ± 25
MØ only	25 ± 11	15 ± 6

* Splenic lymphocytes (T and B cells; 3×10^6 /culture) and splenic macrophages from spleen cell preparations given 1,000 rads of irradiation were cultured alone or in combination and incubated with SRC (2×10^6 /culture) in the presence or absence of Ph-LPS.

‡ Anti-SRC PFC determined on day 5 after incubation; mean PFC ± SE of cultures in triplicate/experiment; mean of three experiments.

To investigate the requirement for LPS-responsive MØ during LPS-induced enhancement of antibody responses, splenic MØ from either C3H/HeN (responder) or C3H/HeJ (nonresponder) mice was cocultivated with splenic lymphocytes (G-10 passed) from either responder or nonresponder mice. Although C3H/HeN MØ were fully functional (Fig. 2 A), the adjuvant response of C3H/HeN lymphocytes was not restored by C3H/HeJ MØ (Fig. 2 B). These results demonstrate that the MØ must be LPS responsive to produce an adjuvant response. However, it is clear that the MØ is not the only obligatory LPS-responsive cell in this system because neither C3H/HeN nor C3H/HeJ MØ could restore the adjuvant response of C3H/HeJ lymphocytes (Fig. 2). Bu-LPS, on the other hand, potentiated immune responses with both LPS responsive and nonresponsive cell populations (Fig. 2) indicating that all the cell populations were functional.

Evidence for a T-Cell Requirement in LPS-Induced Adjuvanticity. The above data strongly suggested that some LPS-responsive cell(s) other than the macrophage was also important for LPS potentiation of immune responses in vitro. The following experiments were carried out to determine if LPS-sensitive T cells were required. C3H/HeN nude (nu/nu) mouse spleen cells served as the source of T-cell deficient, LPS responsive population. Purified splenic T cells (1×10^6 /culture) from either responder (C3H/HeN) or nonresponder (C3H/HeJ) mice were added to C3H/HeN (nu/nu) spleen cells and the LPS-induced adjuvant response was measured. From the data presented in Fig. 3, it can be seen that T cells were required for in vitro immune responses to SRC and that potentiation with Ph-LPS occurred only in the presence of C3H/HeN T cells. Although C3H/HeJ splenic T cells could support immune responses similar to controls, no enhancement by Ph-LPS was observed, indicating that LPS responsive T cells, as well as macrophages, were required for adjuvanticity. Bu-LPS (containing LAP) enhanced the anti-SRC PFC responses with either T-cell preparation. No response was observed in LPS-treated cultures in the absence of T lymphocytes. In agreement with Parks et al. (36), a higher concentration of Ph-LPS ($>1.0 \mu\text{g}/\text{culture}$) was required to obtain an immune response in nude mouse spleen cell cultures in the absence of added T cells (data not shown).

In separate experiments, the requirement for T cells for LPS-mediated enhancement of immune responses was examined in cultures of responder nude mouse spleen cells immunized with another antigen, TNP-SRC. These studies employed carrier (SRC)-

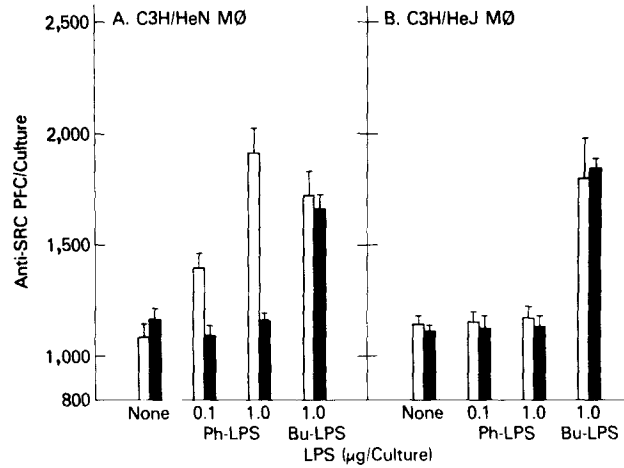


FIG. 2. Requirement for LPS responsive MØ for LPS-induced adjuvanticity. Adherent cell populations (MØ) prepared from irradiated (1,000 rads) spleen cell preparations (5×10^6 cells/0.5 ml) from responder (A. C3H/HeN) and nonresponder (B. C3H/HeJ) mice were cultured with nonadherent splenic lymphocytes (3×10^6 cells/culture) from either responder (C3H/HeN □) or nonresponder (C3H/HeJ ■) mice. Cultures were incubated in the presence or absence of either Ph-LPS (0.1 or 1.0 µg/culture) or Bu-LPS (1.0 µg/culture). LPS-induced enhancement of specific anti-SRC PFC responses was determined on day 5. Control cultures (no LPS) represent background anti-SRC PFC responses. □, C3H/HeN T + B cells; ■, C3H/HeJ T + B cells.

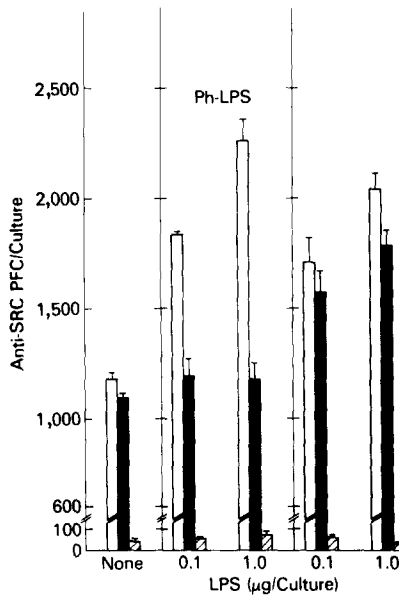


FIG. 3. Requirement for LPS responsive T cells for LPS-induced adjuvanticity. C3H/HeN (responder) nude mouse spleen cells (5×10^6 culture) were cultured alone (no T cells ▨) or together with splenic T cells (1×10^6 /culture) from either responder (C3H/HeN □) or nonresponder (C3H/HeJ ■) mice. Cultures were incubated in the presence or absence of either Ph-LPS (0.1 or 1.0 µg/culture) or Bu-LPS (0.1 or 1.0 µg/culture). LPS-induced enhancement of specific anti-SRC PFC responses was determined on day 5. Control cultures (no LPS) represent background anti-SRC PFC responses. □, C3H/HeN T cells; ■, C3H/HeJ T cells; ▨, no T cells.

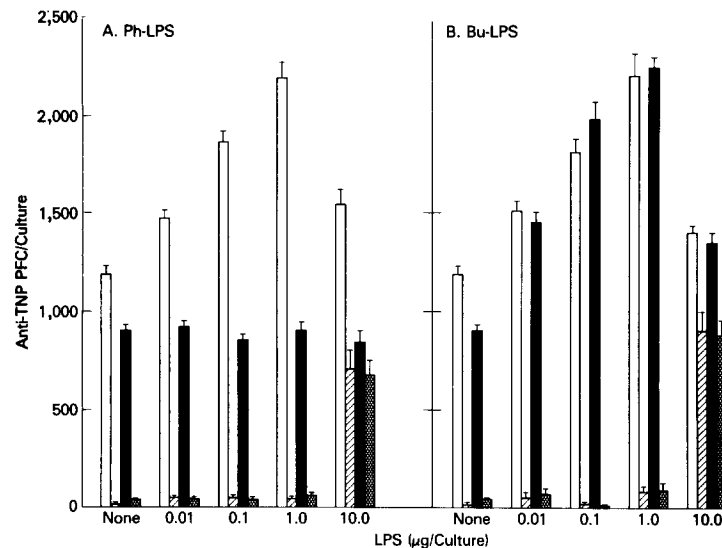


FIG. 4. Titration of LPS-induced augmentation of specific (antigen-dependent) and polyclonal (antigen-independent) anti-TNP PFC responses. Spleen cells ($5 \times 10^6/0.5$ ml) from C3H/HeN (responder) nude mice were cultured with carrier (SRC)-primed T cells (1×10^6 /culture) from either responder (C3H/HeN) or nonresponder (C3H/HeJ) mice. Cultures were incubated with increasing concentrations of either: A. Ph-LPS or B. Bu-LPS for 5 d. Control cultures (no LPS) represent background anti-TNP PFC responses. □, C3H/HeN T cell: specific; ▨, C3H/HeN T cell: polyclonal; ■, C3H/HeJ T cell: specific; ▩, C3H/HeJ T cell: polyclonal.

primed T cells and enhancement of anti-TNP PFC responses was determined. Nude mouse spleen cells cultured with purified splenic T cells from either C3H/HeN or C3H/HeJ mice elicited an immune response to TNP-SRC (Fig. 4). Optimal doses of Ph-LPS (0.1 and 1.0 μ g) induced significant enhancement of anti-TNP PFC, and at higher doses (10 μ g) nonspecific, polyclonal responses were observed (Fig. 4 A), similar to those obtained when SRC was used as antigen (Fig. 1). As was seen with the anti-SRC PFC response (Fig. 3), the specific anti-TNP PFC response of cultures containing nonresponsive (C3H/HeJ) T cells was not augmented with Ph-LPS. Potentiation of antibody responses occurred in all cases when Bu-LPS was used (Fig. 4 B). These results (Figs. 3 and 4) indicate that LPS sensitive T cells are necessary for the adjuvant effect of LPS. Taken together, these data would suggest that both LPS-responsive M ϕ and T cells are required for LPS-induced adjuvanticity.

Responder T Cells and Macrophages Control LPS-Induced Augmentation of B-Cell Responses. To determine if LPS responsive B cells were also required for adjuvanticity, we next investigated the ability of LPS-responsive T cells and M ϕ to support Ph-LPS enhanced anti-TNP PFC responses in cultures containing either purified LPS responsive or nonresponsive B cells. Purified populations of B or T lymphocytes were prepared by fractionation of either C3H/HeN or C3H/HeJ glass wool passed, nonadherent spleen cells on nylon wool columns. The B- and T-lymphocyte subpopulations were subsequently treated with either anti-theta or anti-immunoglobulin sera, respectively, and complement. Adherent cells were obtained from C3H/HeN nude spleen cells. Each cell population was added individually to culture and the adjuvant response of the cell mixture was determined. Responder T cells plus

TABLE II
Requirement for T Cells and Macrophages for LPS Adjuvanticity in Both Responder and Nonresponder B Cells*

Group	Cell source (C3H)			LPS added to culture (1.0 µg/culture)		
	B cell	T cell	MØ‡	None	Ph-LPS	Bu-LPS
1	HeN	HeN	HeN nude	920 ± 60§	1,505 ± 75	1,680 ± 92
2	HeJ	HeN	HeN nude	695 ± 35	1,035 ± 46	1,210 ± 85
3	HeN	HeJ	HeN nude	1,101 ± 60	1,185 ± 84	1,440 ± 60
4	HeJ	HeJ	HeN nude	806 ± 85	747 ± 62	1,368 ± 49
5	HeN	None	HeN nude	22 ± 10	40 ± 20	35 ± 18
6	HeJ	None	HeN nude	2 ± 2	4 ± 2	3 ± 3
7	None	None	HeN nude	22 ± 11	15 ± 5	20 ± 9

* Purified splenic T or B cells (1×10^6 /culture) from either C3H/HeN or C3H/HeJ mice previously injected (1 wk earlier) i.v. with SRC (0.1 ml of 0.01% suspension).

‡ Splenic macrophages from C3H/HeN nu/nu spleen cell preparations given 1,000 rads of irradiation and plated at equivalent (5×10^6 cells/0.5 ml) concentration and incubated with TNP-SRC (2×10^6 /culture) with or without Ph- or Bu-LPS.

§ Anti-TNP PFC determined on day 5 after incubation; mean PFC ± SE of cultures in triplicate/experiment; mean of three experiments.

responder MØ supported Ph-LPS adjuvant responses to TNP-SRC when B cells were derived from either responder or nonresponder mice (Table II; groups 1 and 2). When T cells were derived from C3H/HeJ mice and incubated with responder MØ and either responder or nonresponder B cells (groups 3 and 4), no enhancement in the immune response was observed, again emphasizing the need for LPS responsive T lymphocytes. Cultures of B cells and/or MØ alone did not respond to antigen either in the presence or absence of LPS.

Another series of experiments were carried out with B cells from LPS nonresponder nude mice (C57BL/10ScN)³ as an alternate source of B cells. Spleen cells from C57BL/10Sn mice were used as the histocompatible, LPS responsive cell populations. Again, each cell population was prepared and added individually to culture. Macrophage-depleted B cells were prepared by passage of nu/nu spleen cells over glass wool columns. T cells were prepared by fractionation of spleen cells on glass wool and nylon wool columns and adherent cells served as the source of MØ.

LPS nonresponsive (C57BL/10ScN) B cells exhibited an adjuvant response after incubation with LPS responsive (C57BL/10Sn) T cells and MØ (Fig. 5), again demonstrating that LPS responsive B cells were not essential for adjuvanticity in vitro. In contrast, no Ph-LPS augmentation of the immune response of nonresponder B cells occurred when either T cells or MØ were derived from nonresponder mice (C57BL/10ScN), confirming that T cells and MØ must be LPS responsive to produce an adjuvant response.

As a control, the same experiment was performed using splenic B cells from C3H/HeN (responder) nude mice. These LPS responsive B cells exhibited an adjuvant response in the presence of responder T cells and MØ, but not in the presence of nonresponder T cells and MØ (Fig. 6). A slight enhancement in the immune response occurred when C3H/HeJ T cells and responder MØ were tested. All cell cultures were capable of exhibiting LPS-induced potentiation of anti-TNP PFC responses,

³ Vogel, S. N., C. T. Hansen, and D. L. Rosenstreich. 1979. *J. Immunol.* In press.

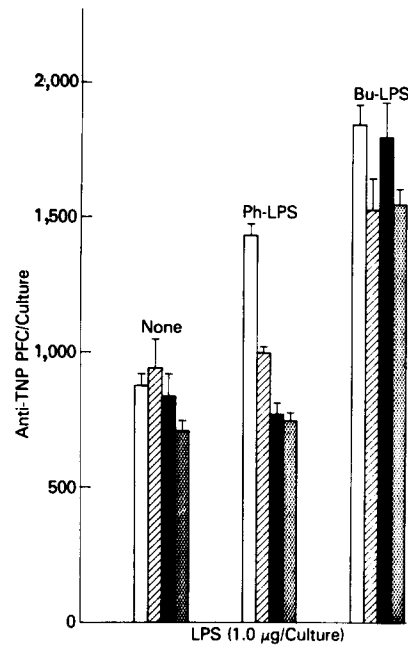


FIG. 5. Requirement for LPS responsive T cells and MØ for LPS-induced adjuvanticity. Splenic B cells (1×10^6 /culture) from LPS nonresponder (C57BL/10ScN) nude mice were cultured with splenic T cells (1×10^6 /culture) and adherent cells (prepared from 5×10^6 spleen cells/0.5 ml given 1,000 rads) derived from either responder (C57BL/10Sn) or nonresponder (C57BL/10ScN) mice. Cultures were incubated for 5 d in the presence or absence of either Ph-LPS or Bu-LPS (1.0 µg/culture). Control cultures (no LPS) represent background anti-TNP PFC responses. □, C57BL/10Sn T cells + MØ; ▨, C57BL/10ScN T cells + C57BL/10ScN MØ; ■, C57BL/10ScN T cells + C57BL/10ScN MØ; ▩, C57BL/10ScN T cells + MØ.

because Bu-LPS elicited enhanced responses with all cell combinations (Figs. 5 and 6). These studies demonstrate that responder T cells and MØ can support LPS potentiation of the immune response regardless of the LPS sensitivity of the B lymphocyte.

To insure that these observations were not due, in part, to an allogeneic effect, we have examined the MLR of each strain employed. As reported earlier (33), C3H/HeN and C3H/HeJ mice were completely histocompatible (Table III). We have extended these observations to C3H/HeN nu/nu and nu/+ mice, and as can be seen in Table III, no MLR was observed with any combination of C3H strains. Table IV lists the results of similar studies with C57BL/10Sn (responder) and C57BL/10ScN (nonresponder) mice. The C57BL substrains employed in these studies exhibited no significant MLR. Thus, it is unlikely that the adjuvant results obtained in this study were due to an allogeneic effect.

Discussion

The results of this study clearly indicate that both T lymphocytes and MØ contribute to LPS-induced potentiation of the antibody response *in vitro*. Therefore, it is not surprising that earlier studies which focused on either of these cells, observed that one or the other of these cell types was important in adjuvanticity (9, 12–15, 19, 20). Thus, only by careful separation of cells from either LPS responder or nonre-

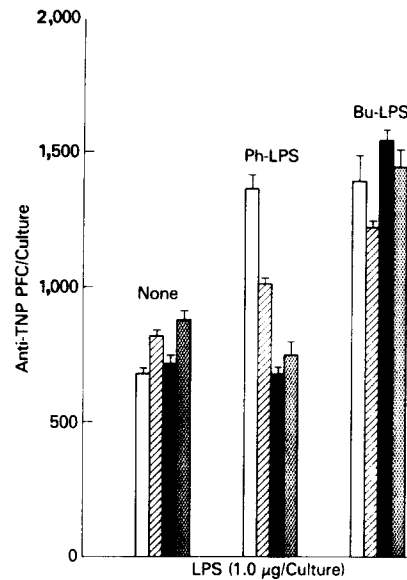


FIG. 6. Requirement for LPS responsive T cells and MØ for LPS-induced adjuvanticity. Splenic B cells (1×10^6 culture) from LPS responder (C3H/HeN) nude mice were cultured with splenic T cells (1×10^6 /culture) and adherent cells (prepared from 5×10^6 spleen cells/0.5 ml given 1,000 rads) derived from either responder (C3H/HeN) or nonresponder (C3H/HeJ) mice. Cultures were incubated for 5 d in the presence or absence of either Ph-LPS or Bu-LPS ($1.0 \mu\text{g}$ /culture). Control cultures (no LPS) represent background anti-TNP PFC responses. □, C3H/HeN T cells + MØ; ▨, C3H/HeJ T cells + C3H/HeN MØ; ■, C3H/HeN T cells + C3H/HeJ MØ; ▩, C3H/HeJ T cells + MØ.

TABLE III
Absence of Mixed Lymphocyte Reactions between C3H/HeN Nude and C3H/HeN and C3H/HeJ Mice

Responder strain	Stimulator strain*	MLR reaction
HeJ	HeN	1,104 ± 61‡
HeJ	HeN (nude)	874 ± 76
HeJ	HeJ	592 ± 29
HeJ	C57BL/6J	7,682 ± 603
HeJ	HeN (Nu/+)	609 ± 111
HeN	HeJ	705 ± 93
HeN	HeN (nude)	819 ± 72
HeN	HeN	1,182 ± 230
HeN	C57BL/6J	4,646 ± 661
HeN	HeN (Nu/+)	728 ± 48
C57BL/6J	HeN	10,109 ± 357
C57BL/6J	HeN (nude)	8,876 ± 206
C57BL/6J	HeJ	13,115 ± 1,287
C57BL/6J	C57BL/6J	1,380 ± 77
C57BL/6J	HeN (Nu/+)	681 ± 52

* Stimulator cells in MLR received 2,500 rads of irradiation.

‡ CPM ^3H -TdR incorporated by 5×10^5 cells (4-d culture).

sponder mice and examination of their ability to support adjuvant responses, could we evaluate their contribution to enhancement of immune responses in vitro.

From the data presented in Figs. 2 and 5, it is clear that the MØ is an important contributor to LPS-induced adjuvanticity, as had been suggested earlier by Hoffmann

TABLE IV
Absence of Mixed Lymphocyte Reactions between C57BL/10ScN Nude and C57BL/10ScN and C57BL/10Sn Mice

Responder strain	Stimulator strain*	MLR reaction
C57BL/10ScN	C57BL/10Sn	717 ± 68‡
C57BL/10ScN	C57BL/10ScN (Nu/Nu)	914 ± 76
C57BL/10ScN	C57BL/10ScN	1,102 ± 201
C57BL/10ScN	C3H/HeN	8,907 ± 406
C57BL/10Sn	C57BL/10Sn	1,029 ± 114
C57BL/10Sn	C57BL/10ScN (Nu/Nu)	776 ± 61
C57BL/10Sn	C57BL/10ScN	911 ± 116
C57BL/10Sn	C3H/HeN	11,209 ± 782
C3H/HeN	C57BL/10Sn	13,208 ± 942
C3H/HeN	C57BL/10ScN (Nu/Nu)	11,371 ± 806
C3H/HeN	C57BL/10ScN	13,862 ± 1,124
C3H/HeN	C3H/HeN	1,182 ± 230

* Stimulator cells in MLR received 2,500 rads of irradiation.

‡ CPM ³H-TdR incorporated by 5 × 10⁶ cells (4-d culture).

and colleagues (9). Nevertheless, the MØ alone was incapable of potentiating the immune response unless responder lymphocytes were present. Thus, the accessory cells (the MØ) cannot totally account for the enhancement of responses by endotoxin. The above mentioned studies which pointed to the MØ as the most important cell type for LPS potentiation of immune responses could have utilized MØ preparations with contaminating LPS responsive T cells (9). In this study, we have overcome this problem by obtaining MØ from T-cell-deficient nude mice.

In support of earlier work (13–15), our data clearly suggests that the T cell is important in LPS potentiation of immune responses. It is likely that endotoxin is acting on the T-helper cell population because T cells derived from carrier-primed, responder mouse spleen enhanced immune responsiveness to the hapten, TNP. Similarly treated splenic T cells from C3H/HeJ mice supported immune responses in the presence of Ph-LPS and adjuvant responses in the presence of Bu-LPS, further suggesting that LPS acts on the T-helper cell population. Although the T cell itself is not generally regarded as an LPS-responsive cell, there is evidence that this may not be true. Koenig et al. (37) showed that gradient-purified T cells responded to LPS as manifested by differentiation in vitro. Furthermore, Narayanan and Sundharadas (38) found that LPS would augment the development of cytotoxic T cells in the absence of macrophages. Therefore, it is certainly possible that LPS is acting directly on the T cell to induce its adjuvant effect. However, it must be emphasized that both LPS responsive MØ and T-helper cells are important in potentiation of immune responses.

The fact that an LPS responsive B cell was not required for adjuvanticity in vitro was somewhat surprising. Because of the failure of C3H/HeJ spleen cells to exhibit an adjuvant effect and because C3H/HeJ mice were thought to possess an isolated B-cell defect, previous workers (3–5) felt that adjuvanticity was mediated by a direct effect of LPS on B cells. However, subsequent studies showing the macrophage (39) and T-cell (37) defects in these mice make the former hypothesis untenable. The findings of Hoffmann et al. (9), which showed that C3H/HeJ spleen cells could exhibit an adjuvant response in vitro in the presence of tumor necrosis factor (TNF)-

rich serum are consistent with the idea that LPS responsive B cells are not essential for this effect. Our findings confirm and extend those of Hoffmann and coworkers.

The apparent separation of LPS adjuvanticity from mitogenicity in responder mice achieved by Poe and Michael (40) also lends support to the conclusion that enhancement of immune responses can occur by LPS action on lymphoid elements other than the B cell. In this regard, Frank and coworkers (41) utilized a detoxified, polysaccharide-rich fraction from *Serratia* LPS which could enhance immune responses in experimental cultures very similar to those reported here, although the fraction was not mitogenic for B cells. These studies taken together would suggest that LPS can be modified to allow adjuvant responses in the absence of a B-cell mitogenic effect. Our own results also suggest that with low concentrations of Ph-LPS (0.1 or 1.0 μg), good adjuvant responses can be obtained only in the presence of T cells. At these concentrations Ph-LPS, immunized spleen cell cultures from nude mice do not elicit normal immune responses to antigen. However, addition of T cells to these cultures results in normal and endotoxin-induced enhanced immune responses. These studies indirectly argue for other mechanisms for LPS adjuvanticity, and our own work reported here, clearly shows that potentiation of immune responses by LPS can be mediated by the T lymphocyte and the M ϕ .

The mechanisms whereby endotoxin potentiates immune responses via T cells and M ϕ remain unclear. It is well known that LPS can enhance the production of M ϕ -derived mediators such as lymphocyte-activating factor (LAF) which could then enhance T-helper cell production (42) with subsequent enhancement of antibody responses (21). The action of LPS on the T cell is not as clear. One possibility is that by enhancing differentiation of T cells, as shown by Koenig et al. (37), LPS may increase the pool of helper cells and thereby augment antibody production. Alternatively, LPS may induce the production of some T-cell-derived helper factor. Although such a mechanism has never been demonstrated for LPS, it is interesting that Hoffmann et al. (9) can substitute TNF-rich serum for both M ϕ and T cells. Because we have shown that T cells are also required for adjuvanticity, it is likely that TNF-rich serum contains both a T-cell product as well as a M ϕ product. This is likely because TNF is produced in response to a challenge that involves T cells (Bacillus Calmette-Guerin treatment). One would predict then, that nonresponder B cells could be induced to exhibit an adjuvant-like response in the presence of separately added, LPS-induced T cell and/or M ϕ -derived factors. Studies are currently underway in our laboratory to investigate this possibility.

Summary

By employing primary cultures of purified spleen cells from lipopolysaccharide (LPS) responder (C3H/HeN or C57BL/10Sn) or nonresponder (C3H/HeJ or C57BL/10ScN) mice incubated with particulate antigen and LPS prepared by phenol-water extraction (Ph), we have presented evidence that both T cells and macrophages (M ϕ) are required for LPS-induced adjuvanticity. First, M ϕ derived from C3H/HeN spleen cells, when mixed with responder, C3H/HeN lymphocytes and Ph-LPS, elicited enhanced antibody responses to sheep erythrocyte (SRC) antigen, whereas lymphocytes from the nonresponder, C3H/HeJ mouse strain did not evoke this response. Similarly, purified T cells from C3H/HeN spleens, when cultured with responder, nu/nu spleen cells, and Ph-LPS yielded enhanced anti-TNP PFC re-

sponses; whereas, C3H/HeJ T cells did not potentiate immune responses when mixed with optimal concentrations of Ph-LPS. LPS prepared by butanol-water extraction elicited significant adjuvant effects with all cell combinations. Finally, purified responder T cells and MØ enabled either responder or nonresponder B cells to elicit LPS potentiation. These data indicate that T cells and MØ are controlling LPS-induced augmentation of B-cell responses.

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References

1. Fleming, D. S., L. Greenberg, and E. M. Beith. 1948. Use of combined antigens in the immunization of infants. *Can. Med. Assoc. J.* **59**:101.
2. Johnson, A. G., S. Gaines, and M. Landy. 1956. Studies on the O antigen of *Salmonella typhosa*. V. Enhancement of antibody response to protein antigen by the purified lipopolysaccharide. *J. Exp. Med.* **103**:225.
3. Skidmore, B. J., J. M. Chiller, D. C. Morrison, and W. O. Weigle. 1975. Immunologic properties of bacterial lipopolysaccharide (LPS): correlation between the mitogenic, adjuvant and immunogenic activities. *J. Immunol.* **114**:770.
4. Chiller, J. M., H. J. Skidmore, D. C. Morrison, and W. O. Weigle. 1973. Relationship of the structure of bacterial lipopolysaccharide to its function in mitogenesis and adjuvant activity. *Proc. Natl. Acad. Sci. U. S. A.* **70**:2129.
5. Skidmore, B. J., J. M. Chiller, W. O. Weigle, R. Riblet, and J. Watson. 1976. Immunologic properties of bacterial lipopolysaccharide (LPS). III. Genetic linkage between the *in vitro* mitogenic and *in vivo* adjuvant properties of LPS. *J. Exp. Med.* **143**:143.
6. Watson, J., and R. Riblet. 1974. Genetic control of responses to bacterial lipopolysaccharide in mice. *J. Exp. Med.* **140**:1147.
7. Louis, J., J. M. Chiller, and W. O. Weigle. 1973. The ability of bacterial lipopolysaccharide to modulate the induction of unresponsiveness to a state of immunity. *J. Exp. Med.* **138**:1481.
8. Schmidtke, J. R., and F. J. Dixon. 1972. Immune response to a hapten coupled to a nonimmunogenic carrier. Influence of lipopolysaccharide. *J. Exp. Med.* **136**:392.
9. Hoffmann, M. K., C. Galanos, S. Koenig, and H. F. Oettgen. 1977. B-cell activation by lipopolysaccharide. Distinct pathway for induction of mitosis and antibody production. *J. Exp. Med.* **146**:1640.
10. Sultzer, B. M., and B. S. Nilssen. 1972. PPD tuberculin a B-cell mitogen. *Nat. New Biol.* **240**:198.
11. Coutinho, A., E. Gronowicz, and B. M. Sultzer. 1975. Genetic control of B-cell responses. I. Selective unresponsiveness to lipopolysaccharide. *Scand. J. Immunol.* **4**:139.
12. Allison, A. C., and A. J. S. Davies. 1971. Requirement of thymus-dependent lymphocytes for potentiation by adjuvants of antibody formation. *Nature (Lond.)*. **233**:330.
13. Hamaoka, T., and D. H. Katz. 1973. Cellular site of action of various adjuvants in antibody responses to hapten-carrier conjugates. *J. Immunol.* **111**:1554.
14. Armerding, D., and D. H. Katz. 1974. Activation of T and B lymphocytes *in vitro*. I. Regulatory influence of bacterial lipopolysaccharide (LPS) on specific T-cell helper function. *J. Exp. Med.* **139**:24.
15. Newburger, P. E., T. Hamaoka, and D. H. Katz. 1974. Potentiation of helper T cell function in IgE antibody responses by bacterial lipopolysaccharide (LPS). *J. Immunol.* **113**:824.

16. Shinohara, N., and M. Kern. 1976. Differentiation of lymphoid cells: B cell as a direct target and T cell as a regulator in lipopolysaccharide-enhanced induction of immunoglobulin production. *J. Immunol.* **116**:1607.
17. Norcross, M. A., and R. T. Smith. 1977. Regulation of B-cell proliferative responses to lipopolysaccharide by a subclass of thymic T cells. *J. Exp. Med.* **145**:1299.
18. Unanue, E. R., B. A. Askonas, and A. C. Allison. 1969. A role of macrophages in the stimulation of immune responses by adjuvants. *J. Immunol.* **103**:71.
19. Spitznagel, J. K., and A. C. Allison. 1970. Mode of action of adjuvants: effects on antibody responses to macrophage associated bovine serum albumin. *J. Immunol.* **104**:128.
20. Rosenstreich, D. L., and S. B. Mizel. 1978. The participation of macrophages and macrophage cell lines in the activation of T lymphocytes by mitogens. *Immunol. Rev.* **40**:102.
21. Farrar, J. J., and W. J. Koopman. 1979. Characterization of macrophage and lymphocyte-derived mitogenic factors and their effect on the antibody response *in vitro*. In *Biology of the Lymphokines*. S. Cohen, E. Pick, M. Landy and J. J. Oppenheim, editors. Academic Press, Inc., N. Y. In press.
22. Koopman, W. J., J. J. Farrar, J. J. Oppenheim, J. Fuller-Bonar, and S. Dougherty. 1977. Association of a low molecular weight helper factor(s) with thymocyte proliferative activity. *J. Immunol.* **119**:55.
23. Farrar, J. J., and J. Fuller-Bonar. 1976. The xenogenic effect. II. Requirement for unactivated murine T cells during restoration of immune responsiveness with xenogeneic reconstitution factor. *J. Immunol.* **117**:274.
24. McIntire, F. C., H. W. Sievert, G. H. Barlow, R. A. Finley, and A. Y. Lee. 1967. Chemical, physical, and biological properties of a lipopolysaccharide from *Escherichia coli* K-235. *Biochemistry.* **6**:2363.
25. Morrison, D. C., and L. Leive. 1975. Fractions of lipopolysaccharide from *E. coli* 0111: B4 prepared by two extraction procedures. *J. Biol. Chem.* **250**:2911.
26. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126**:423.
27. Ly, I. A., and R. I. Mishell. 1974. Separation of mouse spleen cells by passage through columns of Sephadex G-10. *J. Immunol. Methods.* **5**:239.
28. Julius, M. F., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3**:645.
29. Handwerker, B. S., and R. H. Schwartz. 1974. Separation of murine lymphoid cells using nylon wool columns: recovery of the B cell-enriched population. *Transplantation (Baltimore).* **18**:544.
30. Falkoff, R., and J. Kettman. 1972. Differential stimulation of precursor cells and carrier-specific thymus-derived cell activity in the *in vivo* response to heterologous erythrocytes in mice. *J. Immunol.* **108**:54.
31. Rosenstreich, D. L., A. Nowotny, T. Chused, and S. E. Mergenhagen. 1973. *In vitro* transformation of mouse bone-marrow-derived (B) lymphocytes induced by the lipid component of endotoxin. *Infect. Immun.* **8**:406.
32. Rittenberg, M. B., and K. L. Pratt. 1969. Anti-trinitrophenol (TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate immunogen. *Proc. Soc. Exp. Biol. Med.* **132**:575.
33. Rosenstreich, D. L., and L. M. Glode. 1975. Difference in B cell mitogen responsiveness between closely related strains of mice. *J. Immunol.* **115**:777.
34. Glode, L. M., and D. L. Rosenstreich. 1976. Genetic control of B cell activation by bacterial lipopolysaccharide is mediated by multiple distinct genes or alleles. *J. Immunol.* **117**:2061.
35. Goodman, M. G., D. E. Parks, and W. O. Weigle. 1978. Immunologic responsiveness of the C3H/HeJ mouse. Differential ability of butanol-extracted lipopolysaccharide (LPS) to evoke LPS-mediated effects. *J. Exp. Med.* **147**:800.

36. Parks, D. E., M. V. Doyle, and W. O. Weigle. 1977. Effect of lipopolysaccharide on immunogenicity and tolerogenicity of HGG in C57BL/6J nude mice: evidence for a possible B cell deficiency. *J. Immunol.* **119**:1923.
37. Koenig, S., M. K. Hoffman, and L. Thomas. 1977. Induction of phenotypic lymphocyte differentiation in LPS unresponsive mice by an LPS-induced serum-factor and by lipid A-associated protein. *J. Immunol.* **118**:1910.
38. Narayanan, P. R., and G. Sundharadas. 1978. Differential effects of polyadenylic: polyuridylic acid and lipopolysaccharide on the generation of cytotoxic T lymphocytes. *J. Exp. Med.* **147**:1355.
39. Glode, L. M., A. Jacques, S. E. Mergenhagen, and D. L. Rosenstreich. 1977. Resistance of macrophages from C3H/HeJ mice to the *in vitro* cytotoxic effects of endotoxin. *J. Immunol.* **119**:162.
40. Poe, W. J., and G. J. Michael. 1976. Separation of the mitogenic and antigenic responses to bacterial lipopolysaccharide. *Immunology.* **30**:241.
41. Frank, S., S. Specter, A. Nowotny, and H. Friedman. 1977. Immunocyte stimulation *in vitro* by nontoxic bacterial lipopolysaccharide derivatives. *J. Immunol.* **119**:855.
42. Farrar, J. J., W. J. Koopman, and J. Fuller-Bonar. 1977. Identification and partial purification of two synergistically acting helper mediators in human mixed leukocyte culture supernatants. *J. Immunol.* **119**:47.