

## B-CELL ACTIVATION BY LIPOPOLYSACCHARIDE

### Distinct Pathways for Induction of Mitosis and Antibody Production\*

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Bacterial lipopolysaccharide (LPS)<sup>1</sup> has been shown to substitute for helper T cells in the production of humoral antibody. For instance, cultures of T cell-depleted murine spleen cells produce antibody to sheep erythrocytes when LPS is added (1) and LPS facilitates the formation of antibody to haptens on nonimmunogenic carriers such as autologous erythrocytes (2) or amino acids (3), for which helper T cells do not exist. Recently we have reported that injection of LPS to bacille Calmette-Guérin-infected mice causes the release of a factor into the serum which also substitutes for helper T cells (4). Because serum produced in this fashion causes acute necrosis of subcutaneous tumor grafts, it is termed tumor necrosis serum (TNS) (5), and its tumor-necrotizing component is referred to as tumor necrosis factor (TNF) (6). TNF is contained in a glycoprotein fraction (mol wt 150,000) which migrates with the  $\alpha_2$  macroglobulins (6). Although the factor that substitutes for T cells is also found in that fraction, it is not known if the two are identical. The cellular source of TNS has not yet been identified but some of the findings point to the macrophage (5).

On the basis of these recent results, we have examined the hypothesis that macrophages play a critical role in the LPS-induced augmentation of the B-cell response, and that TNS contains mediators elaborated by macrophages. Essential features of our experiments are (a) the use of a mouse strain unresponsive to LPS (C3H/HeJ) (7-10), and its histocompatible responsive counterpart (C3HeB/FeJ), and (b) the use of an antigen, 2,4,6-trinitrophenol (TNP)-conjugated mouse erythrocytes (MRBC-TNP), which is not immunogenic when given alone but becomes immunogenic when given with LPS (2). We report here that (a) LPS enables spleen cells from C3HeB/FeJ but not C3H/HeJ mice to produce antibody to MRBC-TNP in tissue culture, (b) TNS enables spleen cells from both mouse strains to produce antibody to MRBC-TNP, (c) LPS fails to support antigen-dependent production of antibody by macrophage-depleted spleen cells from C3HeB/FeJ mice, (d) TNS supports antigen-dependent production in the same setting, and (e) addition of C3HeB/FeJ macrophages permits C3H/HeJ spleen cells to produce antibody to MRBC-TNP in the presence of LPS.

\* Supported in part by The National Cancer Institute grants CA 08748 and CA 17673, and by The American Cancer Society grants IM 82 and IM 87.

<sup>1</sup> Abbreviations used in this paper: LPS, lipopolysaccharide; MRBC, murine erythrocytes; PFC, plaque-forming cells; TNF, tumor necrosis factor; TNP, 2,4,6-trinitrophenol; TNS, tumor necrosis serum.

## Materials and Methods

**Animals and Antigens.** Male C3HeB/FeJ mice and male C3H/HeJ mice, supplied by The Jackson Laboratory (Bar Harbor, Maine) were used. Sheep and horse erythrocytes were obtained from the Colorado Serum Co. (Denver, Colo.). Mouse erythrocytes (MRBC) were obtained from the same mice from which the spleen cells were prepared. Erythrocytes were conjugated with TNP by the method of Rittenberg and Pratt (11), as modified by Kettman and Dutton (12).

**Cell Culture.** Spleen cells were cultured according to the method of Mishell and Dutton (13) except that 2-mercaptoethanol  $5 \times 10^{-5}$  M was added to the culture medium unless stated otherwise. Adherent peritoneal cells as a source of macrophages (14) were obtained by incubating peritoneal cells from normal mice for 2 h in plastic dishes (model no. 3001, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and removing nonadherent cells by repeated washing. Spleen cells were then added to the peritoneal cells that remained. Macrophages were removed from spleen cell suspensions by passing them through Sephadex G-10 columns as described by Ly and Mishell (15). One volume Sephadex G-10 was washed five times in five volumes distilled water and twice in saline. 30-ml aliquots of Sephadex G-10 in saline were autoclaved at 110°C for 30 min and stored at room temperature. Columns were prepared by loading sterile 10-ml plastic syringe barrels with 8 ml Sephadex G-10, warmed up to 42°C, and washing the Sephadex with 20 ml balanced salt solution (13), also at 42°C.

**Assay for Plaque-Forming Cells (PFC).** Cells producing antibody to TNP were assayed by the method of Rittenberg and Pratt (11) as modified by Kettman and Dutton (12), using TNP conjugated with HRBC.

**LPS, Lipoprotein, and TNS.** LPS-sodium salt was prepared by phenol-water extraction of *Salmonella abortus-equi* and subsequent electro dialysis ( $s = 100S$ ). Lipoprotein from the outer cell wall of *Escherichia coli* was prepared as previously described (16). TNS prepared as described previously, was provided by L. J. Old and E. Carswell, Memorial Sloan Kettering Cancer Center.

## Results

**Effects of TNS and LPS on the Production of Antibody to MRBC-TNP by Spleen Cells from LPS-Responder and Nonresponder Strains.** In the first set of experiments, the effects of TNS and LPS on the production of anti-hapten antibody by spleen cell cultures immunized with haptened autologous erythrocytes were compared. Spleen cells were obtained from C3HeB/FeJ mice (responsive to LPS) and C3H/HeJ mice (unresponsive to LPS). Autologous erythrocytes were conjugated with TNP as described before (11, 12). Aliquots of approximately  $10^8$  MRBC were incubated with increasing amounts of 2,4,6-trinitrobenzene sulfonic acid (Fig. 1) to obtain different hapten densities on the erythrocyte surface. 1 million MRBC-TNP were added to spleen cells which were cultured according to the method described by Mishell and Dutton (13). Antibody formation was measured in terms of the number of PFC. Anti-TNP PFC were counted after a 4-day culture period (12). Antibody production in the absence of antigen will be referred to as polyclonal response (17), antibody production resulting from exposure to MRBC-TNP as antigen-dependent response.

In the absence of TNS or LPS, addition of MRBC-TNP failed to induce a significant number of anti-TNP PFC in cultures of spleen cells from both C3H substrains (Fig. 1). Addition of LPS caused an antigen-dependent response as well as a polyclonal response in cultures of C3HeB/FeJ spleen cells, but not (over a wide dose range) in cultures of C3H/HeJ spleen cells. Addition of TNS, on the other hand, facilitated the antigen-dependent response in cultures of spleen cells from both substrains (but not, as noted previously, the polyclonal response). Optimal antigenicity of MRBC-TNP was obtained by incubating

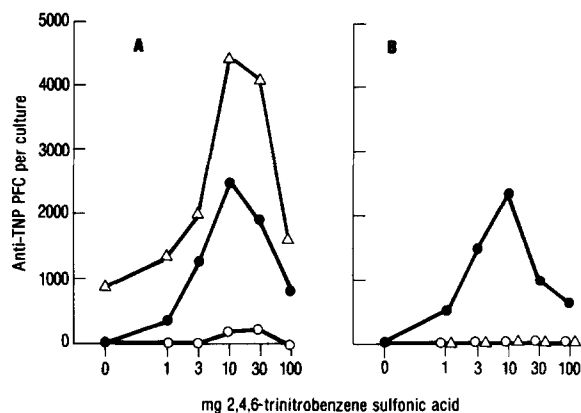


FIG. 1. Facilitation of anti-TNP response by LPS or by TNS.  $10^7$  spleen cells from C3HeB/FeJ mice (A) or C3H/HeJ mice (B) were immunized with  $10^6$  MRBC conjugated with TNP at different densities. Amount of 2,4,6-trinitrobenzene sulfonic acid per 10 ml buffer containing  $10^8$  MRBC is indicated on the abscissa. Additions to cultures: ○, none; ●, TNS 1%; △, LPS 10  $\mu$ g/ml.

MRBC in a solution that contained 10 mg trinitrobenzene sulfonic acid per 10 ml buffer. More heavily conjugated erythrocytes were less immunogenic unless T cells (presumably suppressor cells) were removed from the spleen cell suspension by treatment with anti-Thy-1 serum and complement before culture (not shown here).

*Responder Macrophages Support the LPS-Induced Production of Antibody to MRBC-TNP in Nonresponder Mice.* To determine the role of macrophages in the antigen-dependent as well as polyclonal production of antibody, macrophages were removed from spleen cell suspensions by passage through Sephadex G-10 columns (15). LPS did not support the antigen-dependent production of antibody in macrophage-depleted C3HeB/FeJ spleen cell cultures, although it continued to induce the polyclonal response. By contrast, TNS supported the antigen-dependent production of antibody by C3HeB/FeJ spleen cells even in the absence of macrophages (Table I). The effectiveness of LPS in facilitating the antigen-dependent production of antibody by macrophage-depleted cultures of C3HeB/FeJ spleen cells was fully restored by adding purified peritoneal C3HeB/FeJ macrophages, but not C3H/HeJ macrophages (Fig. 2 A). Spleen cells from C3H/HeJ mice (unresponsive to LPS), responded to LPS in the presence of C3HeB/FeJ macrophages and produced antibody to MRBC-TNP.

*Responder Macrophages Fail to Support the LPS-Induced Mitotic Response of Nonresponder B Cells.* Peritoneal macrophages from C3HeB/FeJ mice (which facilitate formation of antibody to MRBC-TNP by C3H/HeJ spleen cells in response to LPS), failed completely to support the mitogenic response of C3H/HeJ B cells to LPS (Table II).

*Effects of Lipoprotein Extracted from Gram-Negative Bacteria.* As there are reports that proteins extracted from the outer membrane of gram-negative bacteria induce mitosis of C3H/HeJ B cells (10, 16) and phenotypic maturation of C3H/HeJ T- and B-cell precursors (18), not seen with pure LPS preparations in this strain, we tested a lipoprotein prepared from the outer membrane of *E. coli* for its capacity to induce antigen-dependent antibody production in cultures

TABLE I  
 Role of Macrophages in the LPS-Induced Production of Antibody to MRBC-TNP

Source of spleen cells ( $5 \times 10^6$ /culture)	Addition to culture	Anti-TNP PFC on day 4			
		Unfractionated spleen cells		Macrophage-depleted spleen cells (Sephadex G-10)	
		Without antigen	With antigen	Without antigen	With antigen
C3HeB/FeJ	—	92	79	65	63
	TNS 1%	83	890	75	1,230
	LPS 10 $\mu$ g/ml	910	6,200	2,050	2,130
C3H/HeJ	—	40	38	80	75
	TNS 1%	45	620	45	750
	LPS 10 $\mu$ g/ml	42	44	95	88

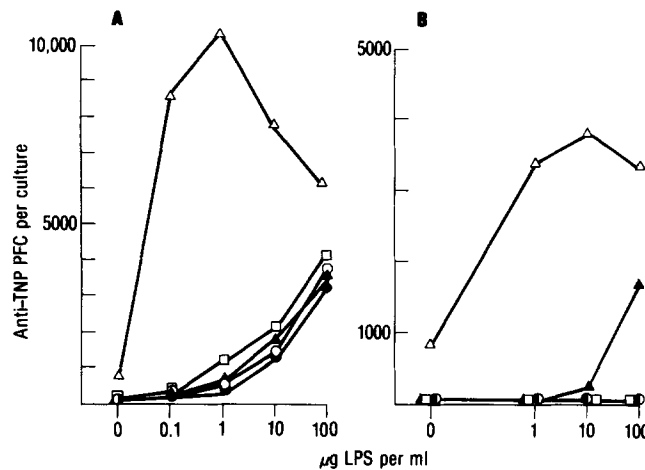


FIG. 2. Facilitation of antigen-dependent and polyclonal antibody production, in the presence of LPS, by macrophages from an LPS-responsive mouse strain. (A) response of macrophage-depleted C3HeB/FeJ spleen cells. (B) response of macrophage-depleted C3H/HeJ spleen cells. Additions to cultures: ●, none; ○, MRBC-TNP; ▲,  $6 \times 10^4$  C3HeB/FeJ macrophages; △,  $6 \times 10^4$  C3HeB/FeJ macrophages + MRBC-TNP; □,  $10^5$  C3H/HeJ macrophages + MRBC-TNP.

of C3H/HeJ spleen cells. We found that the effects of this material on C3H/HeJ spleen cells were in fact similar to the effects of LPS on C3HeB/FeJ spleen cells (Fig. 3). Spleen cells from C3H/HeJ mice immunized with MRBC-TNP generate anti-TNP PFC efficiently in the presence of lipoprotein; only few PFC were induced at the highest dose of lipoprotein when macrophages had been removed. Thus, lipoprotein activates macrophages from both C3H substrains.

### Discussion

We reported earlier that TNS, induced by injecting BCG-infected mice with LPS (5), replaces helper T cells in the PFC response to heterologous erythrocytes *in vitro* (4), and induces phenotypic and functional maturation of B cells (19).

TABLE II  
*Failure of LPS to Stimulate Mitosis of C3H/HeJ B Cells*

Source of spleen cells	Source of macrophages	Addition	
		None	LPS 1 $\mu\text{g/ml}$
<i>(10<sup>6</sup>/ml)</i>	<i>(5 <math>\times</math> 10<sup>4</sup>/ml)</i>	<i>(<sup>3</sup>H/Thymidine cpm)</i>	
C3HeB/FeJ	—	215	17,225
	C3HeB/FeJ	395	16,310
	C3H/HeJ	410	16,800
C3H/HeJ	—	405	525
	C3HeB/FeJ	480	570
	C3H/HeJ	455	465

Macrophage-depleted spleen cells were cultured ( $2 \times 10^5$  cells/0.2 ml) with or without LPS, and with or without macrophages, for 48 h, and subsequently pulsed with [<sup>3</sup>H]thymidine (0.1  $\mu\text{Ci}$ ) for 2 h. Incorporated [<sup>3</sup>H]thymidine was counted in a liquid scintillation counter.

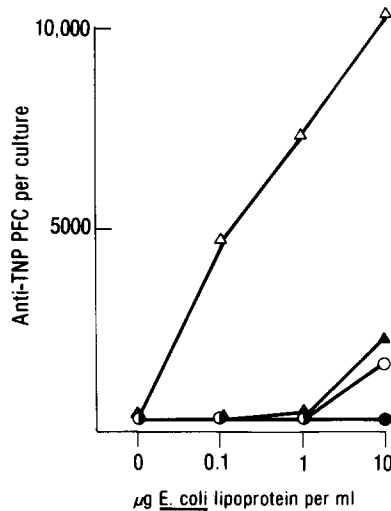


FIG. 3. Effect of bacterial lipoprotein on antigen-dependent and polyclonal antibody production by C3H/HeJ spleen cells. Addition to macrophage-depleted C3H/HeJ spleen cells ( $5 \times 10^6/\text{ml}$ ): ●, none; ○, MRBC-TNP; ▲,  $10^5$  C3H/HeJ macrophages; △,  $10^5$  C3H/HeJ macrophages + MRBC-TNP.

The latter is of particular interest in that TNS, unlike LPS, does not induce maturation of T-cell precursors and is thus the first agent known to induce selective differentiation of murine B cells (19). The findings with TNS add support to the notion that LPS exerts various biological effects through endogenous mediators. We have pursued this question further in experiments with two syngeneic C3H substrains, one responsive to LPS and the other not (7-10), and an antigen which is immunogenic in the presence of LPS but not when given alone. Schmidtke and Dixon showed first that mice immunized with MRBC-TNP produce antibody to TNP only when treated with LPS (2). They attributed the lack of response in the absence of LPS to the lack of MRBC-reactive helper T cells. We confirm and extend this observation. Cultures of

unfractionated spleen cells from C3HeB/FeJ mice, exposed to MRBC-TNP, produce antibody to TNP when LPS is added. Spleen cells from C3H/HeJ mice (unresponsive to LPS) cannot be induced with LPS to produce antibody to TNP. LPS-induced TNF, on the other hand, facilitates production of antibody to TNP in response to MRBC-TNP in cultures of spleen cells from both strains, a finding which adds further support to the contention that the active principle in TNS is not residual LPS itself.

In considering possible cellular sources of TNF, the massive proliferation of macrophages caused by the priming agents used in its production has directed attention to the macrophage (5). Consequently, we examined whether there are similarities between immunological effects of macrophages and those of TNS. We found that macrophages are indispensable for the facilitation of antibody production to MRBC-TNP by LPS in spleen cell cultures. In this reaction, macrophages from a mouse strain which is unresponsive to LPS cannot substitute for macrophages from an LPS-responsive strain even though the lymphocytes are from an LPS-responsive strain. Splenic lymphocytes from unresponsive mice, on the other hand, become responsive to MRBC-TNP in the presence of LPS when small numbers of macrophages from an LPS-responsive strain are added. Thus, in terms of the antigen-dependent production of antibody, the defect that makes spleen cells of C3H/HeJ mice unresponsive to LPS can be traced to the macrophage. In experiments of this sort, the failure of macrophages to respond to LPS is demonstrable only when highly purified preparations of LPS are used, and not with lipoprotein-containing bacterial extracts.

Our findings suggest that macrophages play a critical role in mediating biological effects of LPS. This view is also supported by Schrader's observation that cultured spleen cells from athymic nu/nu mice produce antibody to fowl gamma globulin when LPS is added, provided that activated peritoneal macrophages are present (20). In his experiments, the macrophage effect was mediated by a trypsin-sensitive factor released as a consequence of the interaction between macrophages and LPS. Wood and Gaul described a B-cell activating factor which is released *in vitro* by human monocytes in response to LPS (21).

Macrophages from LPS-responder mice, as well as LPS-induced TNF, facilitate the production of antibody by nonresponder spleen cells in the presence of LPS, but fail to support mitosis of nonresponder B lymphocytes in response to LPS (reference 9, and confirmed by us). Our findings, summarized in Table III, indicate that the two responses represent separate pathways of B-cell activation by LPS, one representing direct interaction between LPS and B cells (mitosis), the other depending on macrophages or macrophage-produced factors (antigen-dependent production of antibody). A dissociation between the adjuvant activity of LPS and its mitogenic effect on B cells has also been suggested by Jacobs and Morrison (22) who reported that the cationic polypeptide antibiotic, polymyxin B, abrogates the mitogenic effect of LPS but enhances the immune response to TNP conjugated with LPS.

### Summary

The role played by macrophages in two effects of lipopolysaccharide (LPS) on the immune system of the mouse — substitution for helper T cells and induction

TABLE III  
*Different Requirements of Induction of Antibody Production or B-Cell Mitosis by LPS*

	Antigen-dependent production of anti- body	Mitosis
Unfractionated spleen cells		
Responder + LPS	+	+
Nonresponder + LPS	-	-
Responder + TNS	+	-
Nonresponder + TNS	+	-
Macrophage-depleted spleen cells		
Responder + LPS	-	+
Nonresponder + LPS	-	-
Responder + TNS	+	-
Nonresponder + TNS	+	-
Nonresponder spleen cells + responder macro- phages and LPS	+	-
Responder macrophage-depleted spleen cells + nonresponder macrophages and LPS	-	+

of B-cell mitosis – has been investigated. C3H/HeJ mice are unresponsive and do not produce (as other strains do) antibody to 2,4,6-trinitrophenol (TNP) conjugated with autologous mouse erythrocytes (MRBC-TNP) in the presence of LPS. We found that C3H/HeJ spleen cells produce antibody to MRBC-TNP when (a) LPS and macrophages from LPS-responsive C3HeB/FeJ mice or (b) tumor necrosis serum ([TNS] induced by LPS in responsive mice) are added. The mitotic response was not restored. The findings suggest that adjuvanticity and mitogenicity represent distinct pathways of B-cell activation by LPS, subject to different regulatory mechanisms.

We thank Ms. Anita Micali, Ms. Elaine Williams, and Ms. Angela Calandrella for their excellent help.

*Received for publication 23 August 1977.*

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