Regulation of the Immune Response to Bacterial Lipopolysaccharide by Adherent Cells

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Immune response to bacterial lipopolysaccharide is usually short lived, but it often reappears without additional stimulus in a cyclic fashion. Activated adherent cells, presumably macrophages, were found to have a role in the reduction of the immune response to *Escherichia coli* O127 lipopolysaccharide. The suppressive activity of the adherent cells was abrogated before renewal of the responsiveness.

Bacterial lipopolysaccharide (LPS) is a stable antigen that persists in circulation for extended periods of time providing a long-lasting antigenic stimulus (5). Immune response to LPS, as measured by counting plaque-forming cells (PFC), is short lasting and cyclic: a single injection of LPS induces formation of splenic PFC for several days, with cyclical reappearance after a latency period (4, 6). The cyclic response to LPS has been interpreted as representing feedback suppression of active antibody synthesis by immunoglobulin M (IgM) antibodies. The biologically stable antigen is presumed to be unable to stimulate antibody synthesis after reacting with initially produced antibodies. In the absence of further stimulation, antibody-producing cells decay, leaving antigen free to initiate a second cycle of antibody synthesis. This hypothesis was supported by the finding that antiserum treatment at the time of the first peak of responsiveness suppressed the appearance of a second peak. It has been, therefore, proposed that immune regulation of humoral responses is controlled via a dynamic equilibrium among circulating antibody, antigen, and antibody-antigen complexes throughout the extracellular compartment. This concept was challenged by Romball and Weigle who demonstrated that rabbits injected with aggregated human IgG show a well-defined polycyclical immune response, with intervals of decreased responsiveness of splenic and peripheral blood PFC (14). The cycles in responsiveness occurred regardless of the dose of antigen or the amount of circulating antibody formed. Thus, in addition to feedback suppression, other factors must be operational in immune response regulation to LPS. Bacterial LPS, besides being a powerful antigen, stimulates lymphocytes and macrophages nonspecifically, and these stimulated cells have the potential to modify the immune response. Results of our study indicate that immunization with LPS

leads to activation of adherent cells which profoundly suppress immune response to LPS.

MATERIALS AND METHODS

Animals. Six- to eight-week-old normal female CB/J mice were obtained from Jackson Laboratories, Bar Harbor, Maine. Congenitally athymic (nude) mice were bred in our animal laboratory facilities from matings of BALB/c (nu/nu) homozygous males and BALB/c (nu/+) heterozygous females.

Reagents. *Escherichia coli* O127 bacteria were obtained from the American Type Culture Collection, Washington, D.C. The bacteria were grown in brain heart infusion broth overnight and heat killed by incubation at 70°C for 30 min, washed three times in sterile distilled water, and aliquoted into sterile bottles for lyophilization. Specified amounts of the lyophilized bacteria were then suspended in phosphate-buffered saline (PBS) and frozen at 20°C. From these frozen stocks, dilutions were made and added to Mishell Dutton cultures.

LPS. *E. coli* O127:B8 and *Salmonella typhosa* O901 lipopolysaccharide were obtained from Difco Laboratories, Detroit, Mich. LPS was used either as an in vivo antigenic stimulus or as the mitogenic stimulus in blastogenic assays. In either case, specified amounts of LPS were suspended in PBS (pH 7.2) and incubated in a 37°C water bath for 30 min. The LPS solution was then sterilized with a Gelman Acrodisc (0.45 µm) and stored at -20°C.

Elimination of T lymphocytes. Anti-theta antiserum was obtained from Litton Bionetics. A final concentration of 1:80 in minimal essential medium was used for T-cell cytotoxicity. The effectiveness of this reagent in eliminating T lymphocytes was indicated by the killing of 40% of splenic lymphocytes and 95% of thymocytes upon their exposure to the anti-theta antiserum plus guinea pig complement (diluted 1:30).

Drugs. Carrageenan in the form of Seakem 9 was obtained from Marine Colloids, Rockland, Maine. Carrageenan was suspended in sterile PBS, pH 7.2.

Preparation of cells for assay. (i) **Splenocytes.** Single-cell suspensions were prepared by pressing mouse spleens through a no. 80 fine-mesh stainless steel screen. Erythrocytes in suspensions were eliminated by treatment with 0.85% NH₄Cl. The cells were then washed three times in Hanks balanced salt solu-

tion and suspended in appropriate volumes of minimum essential medium plus 10% calf serum.

(ii) **Nonadherent splenocytes.** Spleen cell suspensions (5×10^6 cells per ml) were added to plastic culture bottles (Corning; 75-cm² surface area). After 3.5 h of incubation, the nonadherent cells were removed and allowed to adhere a second time for 1 h in fresh bottles. The cells which did not attach to the plastic were considered nonadherent splenocytes.

(iii) **Adherent splenocytes.** Spleen cells (2×10^6) were added to plastic culture wells (Falcon tissue culture plates no. 3008), and after 3.5 h of incubation (37°C, 5% CO₂), nonadherent cells were removed by washing the wells with warm sterile PBS. After nonadherent cells were removed, anti-theta serum (1:80 dilution) was added with guinea pig complement and allowed to incubate with the cells, followed by washing with PBS. The cells that did wash away were considered to be adherent splenocytes free of T lymphocytes.

(iv) **Peritoneal Exudate Cells (PEC).** PEC were removed from mice by peritoneal lavage with Hanks balanced salt solution. The cells thus collected were washed three times and resuspended in appropriate volumes of minimal essential medium. We observed that the ratio between adherent and nonadherent cells in the peritoneum remained the same in untreated and immunized mice.

Assay and culture techniques. (i) **Jerne plaque assay (modified).** Mouse spleen cells were suspended in medium 199 and mixed with sheep erythrocytes or LPS-coated sheep erythrocytes into 0.5 ml of 0.6% agarose on coated slides (0.1% agarose in distilled water). All slides were done in triplicate. The slides were flooded with medium 199 containing guinea pig complement (1:30) and incubated for 3 h (5% CO₂, 37°C). In this way, direct (IgM) plaques could be counted. By subtracting plaques formed with uncoated sheep erythrocytes from plaques formed with coated sheep erythrocytes (*E. coli* O127 LPS), specific anti-LPS antibody-producing cells were quantitated. Each experiment was repeated at least two times.

(ii) **Cultures.** Splenic cells (5×10^6) were placed into Falcon tissue culture plates (no. 3008), and antigen or PBS was added. All samples were cultured in triplicate. The cultures were incubated on a rocking platform under a three-gas mixture (83% N, 10% CO₂, 7% O₂) at 37°C. After 5 days, the cells were harvested, and samples were incorporated into a Jerne plaque assay to enumerate PFC. The survival of the cells was determined by trypan blue exclusion. The percentage of viable cells was similar in all cultures (35 to 40%).

(iii) **Blastogenic assay.** A total of 5×10^6 cells was placed into Costar tissue culture plates (no. 3596), and LPS or PBS was added. Six cultures per each sample were done. The cultures were incubated for 48 h before they were pulsed with 1 circurcure of tritiated thymidine. After 20 h of further incubation, the cultures were harvested with a cell harvester onto glass fiber filters. The filters were placed into plastic scintillation vials, 3 ml of liquid scintillation fluid was added, and radioactivity was counted in a Packard scintillation counter (5-min counts).

RESULTS

Immune response to LPS, measured by PFC count, usually peaks on day 4 or 5 and then

rapidly declines (1). The response may return in a cyclic fashion 10 to 12 days later (4, 6). This pattern of responsiveness has been observed by us in both athymic and euthymic mice (12). Splenocytes from mice immunized with LPS were cultured while they were stimulated by the same antigen. Splenocytes from mice immunized 4 days earlier with LPS showed a significant decrease in responsiveness to LPS culture (Table 1). The unimpaired response was again observed on day 9, followed by renewed suppression. Splenocytes cultured without the LPS antigen were only minimally responsive, and the response was always less than 5% of the antigen-stimulated culture. All reported data were adjusted by subtracting the control values. The suppression was not antigen specific: reduction in responsiveness was achieved with homologous (*E. coli* O127) or heterologous, non-cross-reactive LPS (*S. typhosa* O901). The results obtained after treatment with both preparations were similar, and therefore data obtained only with the homologous antigen (*E. coli* O127) are presented.

The reduction in the ability of the splenocytes to respond in vitro could be due to (i) lesions in B cells themselves or (ii) activity of suppressive cells that inhibit B cell functions.

To consider these possibilities, peritoneal exudates rich in adherent cells (PEC) from LPS-treated mice were added to splenocyte cultures from untreated mice, and blastogenic and immune response to LPS were measured.

A 10% supplement of PEC from LPS-treated mice added to normal splenocytes drastically reduced responsiveness (Table 2). Maximal suppression was obtained with addition of PEC originating from mice immunized with LPS 4 days earlier. PEC from mice pretreated with

TABLE 1. LPS-induced immunosuppression^a

Day after immunization	Immunosuppression	
	Blastogenic response to <i>E. coli</i> O127 LPS (cpm)	Immune response to <i>E. coli</i> O127 LPS (PFC/10 ⁶)
Untreated controls	103,353 ± 8,784	582 ± 66
1	62,136 ± 4,981	664 ± 209
2	61,585 ± 6,759	488 ± 51
3	40,984 ± 3,119	178 ± 47
4	34,366 ± 5,296	38 ± 18
5	72,509 ± 4,500	451 ± 43
9	98,756 ± 7,950	631 ± 77
11	57,830 ± 3,710	319 ± 38
13	44,172 ± 1,660	220 ± 12

^a The concentration of LPS in blastogenic cultures was 150 µg/ml, and that of bacteria in immune cultures was 10 µg/ml. Suppression on days, 3, 4, and 13 was significant ($P < 0.05$) compared with controls.

TABLE 2. *Effect of peritoneal cells from LPS-treated mice on the blastogenic and immune responses to LPS^a*

Group	Cultures of normal spleen cells plus PEC from the following mice:	Effect of peritoneal cells from LPS-treated mice on:	
		Blastogenic response to <i>E. coli</i> O127 LPS (cpm)	Immune response in culture to <i>E. coli</i> O127 LPS (PFC/ 10^6)
A	Controls (no PEC)	87,888 $\pm$ 10,255	418 $\pm$ 106
B	Normal	70,804 $\pm$ 5,475	547 $\pm$ 124
C	2-day immunization	56,023 $\pm$ 5,870	166 $\pm$ 18
D	3-day immunization	59,886 $\pm$ 7,718	68 $\pm$ 88
E	4-day immunization	29,594 $\pm$ 7,536	50 $\pm$ 47
F	5-day immunization	51,356 $\pm$ 3,470	310 $\pm$ 48
G	9-day immunization	81,380 $\pm$ 8,566	588 $\pm$ 171
H	13-day immunization	34,620 $\pm$ 4,320	98 $\pm$ 12

^a PEC were added to the cultures at 10% of the spleen cell concentration. Suppression in groups C, D, E, and H was significant ($P < 0.05$) compared with controls.

LPS 9 days earlier and from normal mice had no appreciable effect on the cultures. Fewer PEC were found to be ineffective. In a series of experiments presented in Table 3, co-cultures of adherent and nonadherent splenocytes were prepared from normal and LPS-immunized mice and tested. The data clearly show that only adherent cells from mice immunized with LPS are suppressive when added to normal, untreated nonadherent splenocytes. Adherent cells from untreated animals and nonadherent cells from treated animals had neither enhancing or suppressive properties. Adherent cell preparations were treated with anti-theta serum and complement before their inclusion in co-cultures.

Carrageenan was shown to be uniquely toxic for macrophage (2), and therefore we studied the effect of this substance on adherent cells. When splenocytes from mice immunized with LPS were cultured in the presence of carrageenan, the suppression of blastogenic and PFC responses did not occur, indicating that the suppressive cells were probably macrophages and sensitive to carrageenan treatment (Table 4). Cultures with added carrageenan after overnight incubation lacked adherent cells.

DISCUSSION

In this study, the mechanism of immunosuppression after immunization with LPS was investigated. Britton et al. first demonstrated

that the number of PEC declines rapidly in LPS-immunized mice soon after the stimulus, followed by recovery of the response, and it has been assumed that some type of suppressive mechanism(s) becomes operational shortly after immunization (4). Britton et al. suggested that endogenously produced antibodies against LPS act in a feedback system to regulate immune response and that the interaction between these antibodies and the antigen is responsible for the cyclical fluctuation of the immune response. We suspected that macrophages may also have a role in this reduction of the immune response to LPS for two reasons: (i) LPS activates macrophages; and (ii) activated macrophages have been implicated to enhance or suppress a variety of immune systems (1). Kirchner et al. reported that splenocytes from mice bearing Moloney sarcoma virus-induced tumors inhibited the proliferation of syngeneic normal spleen cells to mitogenic stimuli. The suppressive cells were radioresistant, inactivated by carrageenan, and removed by a Sephadex G-10 adherence column (8, 9). Suppressor macrophages have also been observed in other tumor systems (7, 10, 15).

Becker and Rudbach have recently shown enhanced primary antibody response to LPS in macrophage-impaired mice. The macrophage impairment was accomplished by treatment with microparticulate silica or carrageenan (3).

Lemke et al. did not observe any stimulatory effect of adherent cells in the LPS-induced lymphocytic transformation. They did observe, however, a profound suppressive effect which was dependent both on the number of macrophages and on the LPS concentration (11).

We propose that *E. coli* O127 LPS injected intravenously stimulates both B-cells and macrophages. B-cells differentiate into PFC, with a peak in response within 4 days. Macrophages stimulated by LPS became suppressive as soon

TABLE 3. *Contributions of adherent and nonadherent splenic cells to immunosuppression^a*

Group	Spleen cells in culture	Supplement	PFC/ 10^6
A	Normal mice		1,649 $\pm$ 32
B	Normal mice	Normal NA	1,418 $\pm$ 344
C	Normal mice	4-Day-immunized NA	1,592 $\pm$ 42
D	Normal mice	Normal A	1,333 $\pm$ 256
E	Normal mice	4-Day-immunized A	387 $\pm$ 118
F	Normal NA mice		583 $\pm$ 92
G	Normal NA mice	Normal A	544 $\pm$ 244
H	Normal NA mice	4-Day-immunized A	152 $\pm$ 73

^a NA, Nonadherent cells. A, Adherent cells. Suppression in groups E and H was significant ($P < 0.05$) compared with the appropriate controls. Supplement was added to the cultures at a 10% concentration of spleen cells in the culture.

TABLE 4. *Effect of carrageenan on immunosuppression*^a

Group	Spleen cells in culture	Supplement added	Effect of carrageenan on:	
			Blastogenic response (cpm)	Immune response (PFC/10 ⁶)
A	Normal	None	25,858 ± 8,683	916 ± 304
B	4-Day immunized	None	3,989 ± 3,914	212 ± 16
C	Normal	Carrageenan	31,891 ± 8,393	1,370 ± 472
D	4-Day immunized	Carrageenan	26,653 ± 5,803	1,144 ± 131

^a Cultures were supplemented with 500 µg of carrageenan per ml. Suppression in group B was significant ($P < 0.05$) compared with controls.

as 24 h after immunization, reaching the maximum level 2 to 3 days later. Suppression, however, became alleviated at days 7 to 10, and reappeared at a later day. The mechanism for this apparent cyclic form of suppression is presently unknown.

Results of our study do not exclude other contributors to suppression, such as antibody feedback inhibition and B suppressor cells. Additional work is necessary to further unravel the complexity of immunoregulation in a "simple" thymus-independent immune system.

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