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Synergistic Action of Bacterial Lipopolysaccharides on Serum-Stimulated DNA Synthesis in Mouse Embryo Fibroblasts (39507)

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It is widely recognized that agents which stimulate DNA synthesis and multiplication of animal cells in culture exert their activity through interaction with specific components of the cell surface. Examples include the lymphocyte mitogens Concanavalin A and phytohemagglutinin, insulin, and a class of growth-regulating protein hormones called somatomedins (1, 2). The biochemical nature of this interaction between growth-promoting substances and cell surfaces remains obscure. However, compounds which are known to cause alterations in cell membranes and which modulate the response of cells to specific mitogenic agents may prove to be useful tools with which to probe the complex mechanisms that regulate cell proliferation.

Vaheri *et al.* (3) have previously reported that lipopolysaccharides (LPS) from gram-negative bacteria exert a dramatic mitogenic effect on stationary chicken embryo fibroblasts in culture. This effect was seen using extremely low concentrations of LPS (0.1–1 ng/ml). Although to our knowledge this has been the only report of such an effect of bacterial endotoxins on fibroblasts, LPS is widely recognized as a B cell specific mitogen (4–7). In view of the above mentioned report (3), demonstrating a striking effect of LPS on the stimulation of DNA synthesis and growth of fibroblasts, it was of interest to further examine the multiplication-stimulating potential of LPS on another serum-dependent cell type, the mouse embryo fibroblast.

This report describes the effect of purified bacterial LPS on growth-related parameters of early passage mouse embryo fibroblasts. In contrast to the results reported previously by Vaheri *et al.* (3) on chicken cells, LPS has little or no effect by itself on cultured mouse cells. However, in the presence of calf serum, LPS exerts a potent synergistic effect on the stimulation of DNA synthesis in stationary confluent mouse fibroblasts. This permissive effect of LPS on serum-induced DNA synthesis may provide unique insights into the mechanism by which serum growth factors interact with cell surfaces. Implications of this observed synergism between LPS and serum on fibroblast growth and its possible role in infections and wound healing are discussed.

Materials and methods. Primary mouse embryo cell cultures were prepared from 14- to 16-day-old embryos of outbred Swiss albino mice. Embryos were decapitated, minced, and disaggregated with 0.25% trypsin in phosphate-buffered saline (PBS). Cell suspensions were centrifuged and cells were resuspended in Dulbecco's modified Eagle's medium (D-MEM) containing 10% calf serum. Cells were plated in 100-mm Lux plastic tissue culture dishes and incubated at 37° in a humidified CO_2 incubator under an atmosphere of 5% CO_2 and 95% air.

Secondary cultures were prepared by trypsinization of primary cultures. Cells were centrifuged and replated in 100-mm dishes to maintain a stock cell supply or in 35- and 60-mm dishes for experimentation. Cells were only used for experimentation from the first through the third transfer to maintain growth patterns characteristic of cells *in vivo*.

To assay for the stimulation of cell growth, mouse embryo fibroblasts were plated in D-MEM with 10% calf serum (5×10^5 cells per 60-mm dish with 5 ml of medium). After allowing the cells to attach to the bottom of the culture dish, medium was replaced with D-MEM containing no or 10% serum with and without LPS. Cell counts were determined at subsequent time intervals in a hemocytometer after individual trypsinization of duplicate cultures.

To assay for the stimulation of DNA synthesis, mouse embryo fibroblasts were

plated in D-MEM with 10% calf serum (2×10^5 cells per 35-mm dish to 2 ml of medium). The next day the medium was replaced with 2 ml of D-MEM containing 0.5% calf serum. Cells prepared in this manner entered a stationary resting phase in which little DNA synthesis occurred. Four or five days after the final medium change, cultures were used to assay for serum stimulation of DNA synthesis by replacing the medium with 2 ml of fresh medium with or without 10% serum and the various test substances. DNA synthesis was measured 18 hr after stimulation during the peak of "S" phase, by removing the medium and incubating the cultures for 1 hr in 1 ml of D-MEM containing 0.2 μCi of [^{3}H]thymidine. Thymidine incorporation under these conditions was shown to be linear for periods greater than 2 hr and a linear relationship between incorporation and numbers of cells in S phase as determined by autoradiography was obtained (data not shown). At the end of this 1-hr pulse, the radioactive medium was removed, cultures were washed once with cold PBS and twice with cold 10% trichloroacetic acid (TCA) and fixed in ethanol:ether (3:1). Cultures were then air-dried, cells were dissolved in 0.2 *N* NaOH, and aliquots were added to 10 ml of aqueous scintillation fluid for determination of incorporated radioactivity. All points were done in duplicate, and duplicate samples did not vary by more than 10%.

Serum stimulation of cells prepared in this manner resulted in an approximate 10-fold increase in thymidine incorporation over the no serum controls (Fig. 2). Final cell density in the stationary cultures was about 8×10^4 cells/cm^2 and the monolayer had just reached confluence. In these stationary cultures less than 2% of the cells were shown to be in S phase at any one time as determined by autoradiography (data not shown) after a 1-hr pulse of [^{3}H]thymidine.

To determine the percentage of labeled nuclei, cells were labeled as described above with a 1-hr pulse of [^{3}H]thymidine. Cultures were washed twice with cold 10% TCA, fixed in ethanol:ether (3:1), and air-dried. Cultures were then coated with Kodak nuclear track emulsion NTB3 and exposed for 14 days at 4°. Autoradiographs were developed and the percentage of labeled nuclei was determined using phase contrast microscopy. At least 1500 cells were counted per culture.

Calf serum and Dulbecco's modified Eagle's medium (catalog No. H-21 HG) were purchased from Grand Island Biological Co. Tritiated thymidine (40 Ci/mmole) was purchased from New England Nuclear. Bacterial lipopolysaccharides were purchased from Difco Laboratories. Lipopolysaccharide W from *Escherichia coli* 055:B5 was used unless indicated otherwise.

Results. The effects of lipopolysaccharides from *E. coli* on the growth rate of early passage mouse embryo fibroblasts were examined. As is evident from Fig. 1, the presence of LPS (20 μg/ml) produced a significant increase in cell number over those cells grown in the presence of 10% calf serum alone. This increase in growth rate was particularly evident during the first 48 hr and this margin of difference was maintained throughout the course of the experiment. Under these conditions the cells become confluent at a density of approximately 2.2×10^6 cells per dish. It it therefore evident that LPS is effective in enhancing the growth of both sparse and confluent cells.

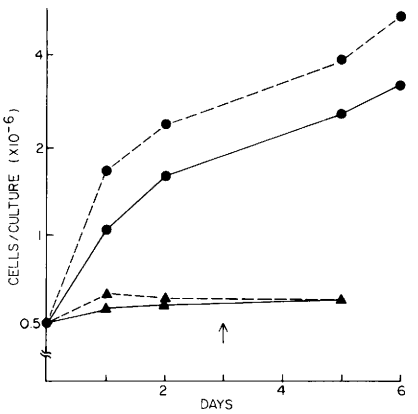

FIG. 1. Effect of LPS on the growth rate of mouse embryo fibroblasts. Cells (5×10^5) were seeded per 60-mm culture dish in 5 ml of DMEM containing 10% calf serum. After allowing the cells to attach, medium was removed and replaced with DMEM containing 0 (▲) or 10% (●) serum with (– –) or without (——) 20 μg/ml of *E. coli* 055:B5 LPS W. Medium was changed on Day 3 as indicated by the arrow, and cell counts were performed at the times indicated.

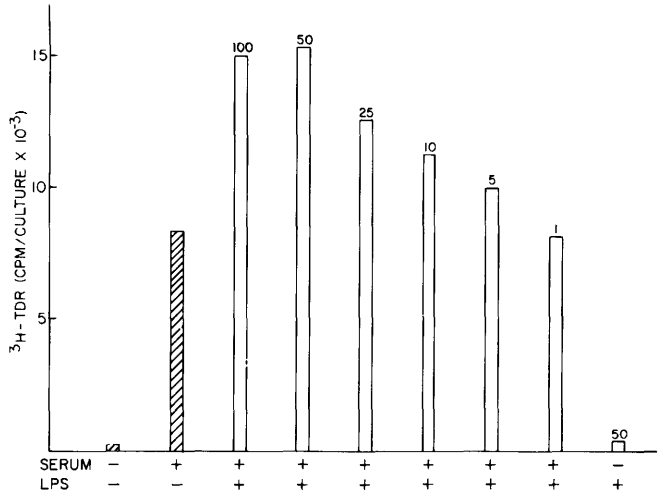

FIG. 2. Effect of LPS on the serum stimulation of DNA synthesis. Stationary cultures of mouse embryo fibroblasts were prepared by maintenance in low serum containing medium as outlined in Materials and Methods. Culture fluid was changed to medium containing 0 or 10% serum with and without various concentrations of LPS. Numbers at the top of each bar indicate μg of LPS per culture (2 ml). Rates of DNA synthesis in the cultures were measured 18 hr later using a 1-hr pulse of [^{3}H]thymidine as described in Materials and Methods.

Controls show that in the absence of calf serum, cells do not grow and LPS is inactive alone. LPS was not toxic to the cells and did not result in a loss of cells from the monolayer. Additional experiments using various concentrations of LPS did not yield results significantly different from those shown in Fig. 1. Concentrations less than 1 μg/ml were without effect and LPS was not active in the absence of calf serum.

In view of this moderate, but significant, enhancement of the growth rate by LPS, it was of interest to investigate whether LPS could also enhance the incorporation of [^{3}H]thymidine by stationary cells stimulated to synthesize DNA by changing the culture fluid to fresh medium containing 10% calf serum with or without various concentrations of LPS (Fig. 2). Rates of DNA synthesis were determined during the peak of S phase using a 1-hr pulse of [^{3}H]thymidine. The data shown demonstrate that while LPS has no apparent stimulatory effect in itself, it enhances the serum stimulation of DNA synthesis almost twofold. This maximum stimulatory effect is seen using 50 μg of LPS/ml. Lower or higher concentrations of LPS yield a diminishing result. Thus, a synergism is seen when LPS is present in addition to calf serum resulting in an increase in incorporation over that seen in the presence of either supplement alone. The results of the DNA synthesis experiment are therefore consistent with the early effects of LPS on the growth rate of mouse fibroblasts and seem to suggest that LPS is exerting a permissive effect on the cells and allowing more cells to enter the replicative cycle in a given period.

In order to verify this, autoradiographic experiments were performed to determine if the increase in [^{3}H]thymidine incorporation seen in Fig. 2 using LPS was due to an increase in the actual number of cells in S phase or due to increased rates of thymidine incorporation by the same number of cells. It is conceivable that the enhanced thymidine incorporation in the presence of LPS is due to an effect on thymidine transport allowing an increased uptake of the precursor by cells already stimulated by serum. However, the results shown in Table I clearly indicate that the twofold increase in [^{3}H]thymidine incorporation seen in the presence of LPS is due to a corresponding twofold increase in the number of cells actually engaged in DNA synthesis. It is therefore clear that the presence of LPS in addition to serum results in an increased efficiency of stimulation in that more cells are

TABLE I. EFFECT OF LPS ON THE PERCENTAGE OF LABELED NUCLEI.[a]

Stimulating medium	[^{3}H]Thymidine incorporation (cpm/culture)	Percentage of labeled nuclei
No serum	210	3.3
No serum + LPS (50 μg/ml)	300	4.6
10% serum	1920	28.5
10% serum + LPS (50 μg/ml)	4200	61.0

[a] Stationary cultures of mouse embryo fibroblasts were prepared as described in Materials and Methods. Each system consisted of four identical cultures that were stimulated, pulsed, and processed in exactly the same manner. Two cultures were hydrolyzed and used for determination of total incorporated radioactivity and two cultures were processed for autoradiography and determination of the percentage of labeled nuclei.

capable of responding to serum by synthesizing DNA if LPS is also present in the medium. Preliminary experiments (data not shown) verified that the results seen were not due to a shift in the time of S phase by LPS. The peak of DNA synthesis occurred at 18 hr after addition of serum with or without LPS.

To further characterize this cellular response to LPS, the effect of serum concentration was examined. The dose-response curves illustrated in Fig. 3 show that the synergistic effect of LPS is evident at all serum concentrations used and that the LPS has no effect in the absence of serum.

In order to determine whether the LPS is exerting its effect directly on the cell or on a component of the serum, the experiment described in Table II was performed. Cells were pretreated for 6 hr with LPS in serum-free medium prior to stimulation. Cell cultures were washed twice with PBS to remove all traces of LPS that were not cell associated. The data show that pretreatment with LPS in the absence of serum also results in a synergistic effect on the serum stimulation of DNA synthesis. While this experiment does not rule out the possibility that cell-bound LPS is reacting directly with serum growth factors at the cell surface, it strongly suggests that the mode of action of LPS is to cause an alteration in the cell membrane which results in a more efficient utilization of growth factors by the cell.

Discussion. The results reported indicate that LPS from the gram-negative bacterium *E. coli* is capable of exerting a striking synergistic effect on the DNA synthetic response of stationary early passage mouse embryo fibroblasts to serum. Data suggest that the LPS, which is ineffective by itself, is acting directly on the cell and is not altering a serum component. It appears that LPS exerts a permissive effect on the cells, enabling them to respond more efficiently to serum stimulation. Actually, twice as many cells respond to serum if LPS is also present in the medium or if the cells have been pretreated with LPS.

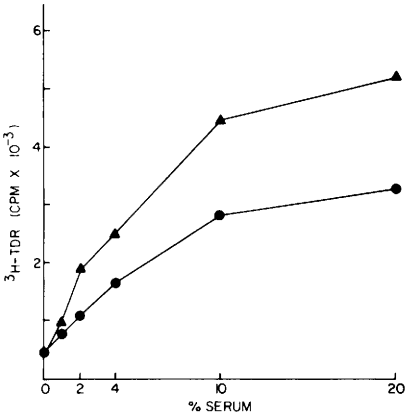

FIG. 3. Effect of serum concentration on the synergistic action of LPS. Stationary cultures of mouse embryo fibroblasts were prepared as outlined in Materials and Methods. Cells were stimulated by changing the medium to fresh DMEM containing 0 (●) or 25 μg/ml of LPS (▲) and various concentrations of serum. Rates of DNA synthesis were determined 18 hr later with a 1-hr pulse of [^{3}H]thymidine.

TABLE II. EFFECT OF LPS PRETREATMENT.[a]

Stimulating medium	LPS pretreatment	[^{3}H]Thymidine incorporation (cpm/culture)
No serum	–	820
No serum	+	930
No serum + LPS	–	890
10% serum	–	4260
10% serum	+	8640
10% serum + LPS	–	9780

[a] Stationary cultures were prepared as previously described. Some cultures were treated with 25 μg/ml of LPS for 6 hr in the absence of serum prior to stimulation.

Endotoxins (LPS) from gram negative bacteria are well-known mitogens of B lymphocytes. However, reports of the stimulation of nonlymphoid cells by LPS are very rare (3). Vaheri *et al.* (3) report that LPS from *Salmonella* sp. is a potent stimulator of DNA synthesis in chick embryo fibroblasts. The results shown in the present report are basically different in that LPS has no activity alone, but only acts synergistically with serum to promote the DNA synthetic response. This effect on confluent mouse embryo fibroblasts is only exerted at concentrations of LPS greater than 10 μg/ml. This difference is probably due to the cell type used since chick fibroblasts are generally more sensitive to growth-promoting agents such as serum or insulin than are mouse cells. However, the experiments reported by Vaheri *et al.* (3) were carried out in the presence of depleted serum and the results could have been due to increased utilization of residual serum factors brought about by the addition of LPS.

Whether or not this interaction between LPS and serum is an operative biological process cannot be determined at this time. However, it is interesting to speculate that the presence of bacteria in wounds and during infections may provide a natural environment which leads to synergistic stimulation of fibroblast proliferation by serum and bacterial endotoxins or other products.

The mode of action for this permissive effect of LPS is not clear; however, an obvious site of interaction between serum growth factors and LPS would be the cell surface. LPS are amphipathic molecules consisting of a highly polar polysaccharide region and a nonpolar lipid portion (lipid A). It is known that LPS interacts with cell membranes and with artificial lipid bilayers (8, 9). LPS may be acting in our system by causing alterations or pertubations in the cell membrane which might lead to an uncovering of receptor sites for growth factors in serum. The insertion of the lipid portion of LPS into the membrane may also affect membrane fluidity, a phenomenon which correlates with the mitogenic activity of Concanavalin A (10, 11).

It is suggested that the synergistic response seen in this report provides a unique opportunity to study serum-induced cell proliferation. Since the structure of LPS is generally known and techniques are available for alteration of that structure, effects of LPS on serum-induced DNA synthesis may provide a means of elucidating basic aspects of that interaction. Vaheri *et al.* (3) have demonstrated that the carbohydrate portion of LPS is not important for the mitogenic effect of LPS on chick cells. This is in agreement with additional evidence that the biological activity of bacterial endotoxins resides in the lipid A moiety (12–14). Experiments are currently in progress to determine the mode of action of the LPS effect on DNA synthesis and to establish if this effect is common to all bacterial endotoxins and other similar bacterial products.

In addition, it should be noted that while LPS is a well-known B cell mitogen (4–7) and a T cell independent antigen, bacterial endotoxin has been demonstrated to have the ability to synergistically enhance the response of T cells to the mitogen Concanavalin A (15).

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