PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **180**, 163–169 (1985)

Further Characterization of the Effect of Bacterial Lipopolysaccharide Preparations on Cyclic GMP Levels: The Importance of Macromolecular Synthesis (42159)

STANLEY E. GRABER AND MILLIE A. CLANCEY

Departments of Medicine, Veterans Administration Medical Center, and Vanderbilt University School of Medicine, Nashville, Tennessee 37203

Abstract. Bacterial lipopolysaccharides (LPS) greatly increase cGMP levels in short term cultures of rat fetal liver cells without affecting the concentration of cAMP. This effect is produced by very small (1 ng) amounts of LPS and is both dose and time dependent. The time dependence is characterized by an initial lag period of 60–120 min followed by a rapid, persistent increase in cGMP levels. Since this time course suggests that synthesis of an intermediate might play an important role in the cGMP elevation, a series of experiments was done to evaluate the effect of LPS on DNA, RNA, and protein (macromolecular) synthesis. LPS did not measurably effect total macromolecular synthesis. However, inhibitors of RNA and protein synthesis markedly reduced cGMP levels in LPS-treated cells, whereas inhibition of DNA synthesis did not. Addition of sodium nitroprusside to control and inhibitor-treated cultures produced large equivalent increases of cGMP levels in both cases, indicating that the cells present were fully capable of responding to a stimulus of guanylate cyclase. Taken together, this data suggests that expression of the LPS–cGMP response in fetal liver cells is dependent on synthesis of an intermediary protein(s) during the lag phase.

Bacterial lipopolysaccharides (LPS) greatly increase cGMP levels in short-term cultures of rat fetal liver cells and to a lesser extent in similar cultures of rat adult spleen cells without affecting the concentration of cAMP. This elevation is due to the Lipid A moiety of the LPS molecule, is potentiated by the phosphodiesterase inhibitor, isobutylmethylxanthine, and by small amounts of serum, and is both dose and time dependent (1–5). The time dependence is characterized by an initial lag period of 60–120 min followed by a rapid and very persistent increase in cGMP levels. Because this lag period suggested that synthesis of an intermediate or of guanylate cyclase, itself, might play an important role in the cGMP elevation produced by LPS, a series of experiments was undertaken to evaluate the effect of LPS on DNA, RNA, and protein synthesis. The results of these studies, which are reported here, indicate that both RNA and protein synthesis are involved in the cGMP effect and are consistent with the hypothesis that a cellular component is produced during the lag period that is necessary for the increase in cGMP levels to occur.

Materials and Methods. The methods were modified slightly from those previously described (4, 5). Cell suspensions in Waymouth's MB752-1 medium (Microbiological Associates, Bethesda, Md.) were prepared from rat fetal livers on Day 13 of gestation (Holtzman Company, Madison, Wis.). The concentration of nucleated cells was determined on a Coulter counter and adjusted to 1×10^7 cells per ml. Half milliliter aliquots of this suspension plus an additional 0.4 ml of Waymouth's medium were placed in polystyrene tubes (Falcon Plastics, Oxnard, Calif.), preincubated for 30 min, and then incubated with various concentrations of each inhibitor and/or LPS for 3–4 hr as specified for each experiment. All inhibitors and LPS were diluted in Hanks' balanced salt solution (HBSS) and added in a 0.1-ml vol that contained 6 μl of limulus lysate absorbed rat serum. At $2\frac{1}{2}$ or 3 hr, 0.25 μCi of both [^{14}C]thymidine and [^{3}H]uridine, or [^{3}H]leucine alone were added in a 50 μl vol of HBSS and, unless specified otherwise, the incubation was continued for another 30 min. At this point, the cultures were terminated by the addition of 1 ml of ice cold 10% trichloroacetic acid (TCA) followed by immediate sonication at 50 W for 5 sec. After centrifugation, the supernatants were extracted three

0037-9727/85 $1.50

times with 5 vol of water saturated ether, heated to 50°C for 25 min, diluted as necessary in pH 4.0 acetate buffer to a final concentration of 50 m*M*, acetylated, and assayed for cGMP by radioimmunoassay (6). Fresh standards were acetylated along with samples for each assay. Dilution analysis of a pooled sample with and without a fixed amount (16 fmole) of added standard showed correlation coefficients greater than 0.9 for both cases and y intercepts of 0.5 and 16.9 fmole, respectively. The pellets were washed three times, once with 2 ml of iced 10% TCA and twice with 2 ml of iced 10% perchloric acid (PCA). For analysis of DNA and RNA synthesis, the washed pellets were suspended in 5% PCA, heated for 15 min at 90°C to hydrolyze the RNA and DNA, rapidly cooled, and centrifuged. One ml of supernatant from each sample was mixed with 10 ml of Scintiverse (Fisher Scientific, Pittsburgh, Pa.) in a counting vial and ^{14}C and ^{3}H activities were measured in a liquid scintillation counter using a dual isotope counting technique adjusted so that no ^{3}H counts appeared in the ^{14}C channel. For analysis of protein synthesis, the pellets were incubated in 1 ml of 0.2 *N* sodium hydroxide at 37°C until completely dissolved. One-milliliter aliquots were then mixed with 10 ml of Scintiverse and ^{3}H activity was measured in a liquid scintillation counter.

Salmonella minnesota 9700 lipopolysaccharide prepared by the phenol extraction method of Westphal and Jann (7) was purchased from Difco Laboratories, Detroit, Michigan and used as supplied. Cytosine arabinoside and actinomycin D (Calbiochem-Behring Corp., La Jolla, Calif.) plus cycloheximide and cordycepin (Sigma Chemical Co., St. Louis, Mo.) were diluted in HBSS (Grand Island Biologicals, Grand Island, N.Y.) and added to cultures in 0.1-ml vol. [^{14}C]Thymidine, [^{3}H]uridine, and [^{3}H]leucine (New England Nuclear, Boston, Mass.) were diluted in HBSS and added to cultures in 0.05-ml vol. Guanosine 3′:5′-cyclic phosphoric acid 2′-*O*-succinyl-[^{125}I]iodotyrosine methylester (New England Nuclear) diluted in 50 m*M* acetate buffer, pH 4.0, was used in the radioimmunoassay for cGMP.

The data were evaluated by standard statistical methods including linear regression analysis and the unpaired t test (8).

Results. In an effort to evaluate whether or not any measurable changes in DNA, RNA, or protein synthesis were associated with the cGMP elevation produced by LPS, the effect of LPS on cGMP levels and on the incorporation of [^{14}C]thymidine, [^{3}H]uridine, and [^{3}H]leucine into their respective macromolecules was studied. Either 20 ng of LPS in HBSS or HBSS alone was added to the cultures at zero time, while the radioactive precursors were added after 3 hr. Cultures were then terminated at various times from 15 to 60 min later. As can be seen in Fig. 1, incorporation of all radioactive precursors into DNA, RNA, and protein was linear for the 60-min period studied. Furthermore, there was no difference in DNA, RNA, and protein synthesis between LPS and control cultures at all of the times tested. On the other hand, cGMP levels in the LPS-treated cultures (4876 ± 834 fmole) at these same times were 14-fold greater than in controls (341 ± 53 fmole). Thus, the large increase in cGMP concentration produced by LPS was not associated with any measurable changes in total DNA, RNA, or protein synthesis.

Since relatively large increases of a single or a few cellular components might be obscured in the measurement of total macro-

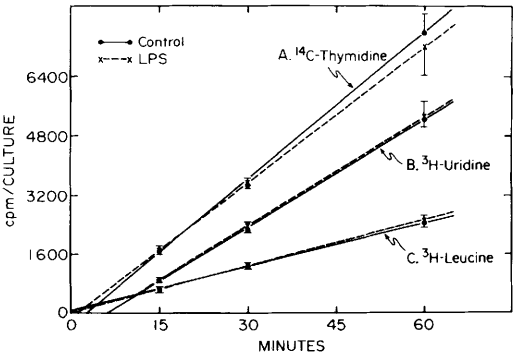

FIG. 1. Effect of LPS on synthesis of DNA, RNA, and protein in cultures of fetal liver cells. LPS (20 ng) or HBSS was added at zero time. Appropriate labeled precursors were added at 180 min and the cultures were terminated at 195, 210, and 240 min. All points represent the mean of triplicate cultures ± SD. A, DNA synthesis; B, RNA synthesis; C, protein synthesis.

molecular synthesis (9), the effects of several inhibitors of DNA, RNA, and protein synthesis were evaluated. A typical experiment utilizing cytosine arabinoside is shown in Table I. Cultures were treated at time zero with either HBSS alone, 20 ng of LPS alone, or LPS plus various concentrations of cytosine arabinoside. Synthesis of DNA, RNA, and protein was measured between 3 and $3\frac{1}{2}$ hr of culture and cGMP levels were determined at $3\frac{1}{2}$ hr. In addition, the effect of nitroprusside added at 3 hr on cGMP levels at $3\frac{1}{2}$ hr was measured in control cultures and in cultures receiving the highest dose of cytosine arabinoside. Cytosine arabinoside (10 μM) produced a mild 29% inhibition of the cGMP response, a marked 95% inhibition of DNA synthesis, and moderate to slight decreases in RNA and protein synthesis. Thus, almost complete inhibition of DNA synthesis did not markedly affect the increase in cGMP levels produced by LPS. Moreover, in cultures receiving the highest dose of cytosine arabinoside, sodium nitroprusside, a known activator of soluble guanylate cyclase (10), produced a large increase in cGMP levels equivalent to levels achieved in control cultures, indicating that the cells present were fully capable of responding to a stimulus of guanylate cyclase.

The data from a series of similar experiments utilizing other inhibitors of macromolecular synthesis (actinomycin D, cordycepin, and cycloheximide as well as cytosine arabinoside) are summarized in Table II. For each inhibitor, a single dose that produced the most discrimination between the various parameters is included in the table. The time course and the amount of LPS (20 ng per culture) are identical to the experiment in Table I. Actinomycin D, cordycepin, and cycloheximide all produced a marked inhibition of the cGMP response to LPS, whereas cytosine arabinoside did not. Although none of the inhibitors were completely specific, each had a predominant effect. Actinomycin D and cordycepin's major inhibition was on [^{3}H]uridine incorporation, while cycloheximide's was on [^{3}H]leucine incorporation, and cytosine arabinoside's was on [^{14}C]thymidine incorporation. Cycloheximide also produced a large decrease in [^{14}C]thymidine incorporation. However, since an even larger decrease was observed with cytosine arabinoside without a major effect on cGMP levels, it seems likely that the cGMP inhibitory effect of cycloheximide was related to its effect on protein synthesis and not DNA synthesis. The results of sodium nitroprusside controls done with each inhibitor experiment are shown in Table III. Nitroprusside produced large increases of cGMP levels which were comparable or greater in inhibitor-treated than in control cultures.

Discussion. We have previously shown that very small amounts of LPS produce a large increase of cGMP levels in rat fetal liver cells without affecting the cAMP concentration (1–3). The time course of this effect is characterized by a lag period of 60–120 min followed by a rapid and persistent elevation of the cGMP concentration (1, 2). This lag period, which is always present, suggests that synthesis of an intermediate or activator might be necessary for expression of the cGMP response. The data reported here and outlined below are consistent with this notion. First, although no effect of LPS on total DNA, RNA, or protein synthesis was observed, this does not rule out an effect on a specific messenger RNA that represents only a very small fraction of total synthesis. For example, in B lymphocytes treated with concanavalin A or LPS in mitogenic doses, *myc* gene messenger RNA is markedly elevated long before any effect on total RNA or DNA synthesis is observed (9). Second, the inhibitor studies show that blocking RNA synthesis with actinomycin D and cordycepin or blocking protein synthesis with cycloheximide virtually abolishes the cGMP effect of LPS, whereas cytosine arabinoside, an inhibitor of DNA synthesis, had very little inhibitory effect on cGMP levels. Third, actinomycin D in the doses used in our experiments is known to inhibit messenger RNA synthesis in other cell types (11). Fourth, cordycepin produces at least a part of its effect on RNA synthesis by interfering with the formation of polyadenylate sequences at the 3′-terminus of messenger RNA. This prevents the transfer of messenger RNA from the nucleus to the cytoplasm and thereby blocks the expression of the messenger RNA by the cell (12). Fifth,

TABLE I. EFFECT OF CYTOSINE ARABINOSIDE ON cGMP LEVELS AND DNA, RNA, AND PROTEIN SYNTHESIS IN CULTURES OF FETAL LIVER CELLS TREATED WITH LPS[a,b]

Additives	cGMP		[^{14}C]Thymidine		[^{3}H]Uridine		[^{3}H]Leucine	
	fmole	% LPS cultures[e]	cpm	% LPS cultures[e]	cpm	% LPS cultures[e]	cpm	% LPS cultures[e]
HBSS	128 ± 12[c]	3	3475 ± 157	103	2547 ± 160	99	1023 ± 8	103
20 ng LPS	4933 ± 799	100	3388 ± 48	100	2561 ± 54	100	996 ± 50	100
20 ng LPS 0.1 μM cytosine arabinoside	4638 ± 411	94	1776 ± 23[c]	52	2135 ± 163[c]	83	996 ± 60	100
20 ng LPS 1 μM cytosine arabinoside	4154 ± 319[c]	84	597 ± 23[c]	18	1904 ± 114[c]	74	959 ± 32	96
20 ng LPS 10 μM cytosine arabinoside	3496 ± 417[c]	71	152 ± 9[c]	5	1496 ± 55[c]	58	897 ± 53	90
100 μM nitroprusside	10,109 ± 3131[c]	205						
100 μM nitroprusside 10 μM cytosine arabinoside	10,679 ± 2840[c,d]	216						

[a] Values equal the mean of six replicate cultures ± SD for cGMP levels and three replicate cultures ± SD for [^{14}C]thymidine, [^{3}H]uridine, and [^{3}H]leucine cpm.

[b] LPS, cytosine arabinoside, and HBSS were added at time zero; nitroprusside, [^{14}C]thymidine, [^{3}H]uridine, and [^{3}H]leucine were added at 150 min. All cultures were terminated at 180 min.

[c] When compared to cultures treated with LPS alone, $P < 0.05$.

[d] When compared to cultures treated with nitroprusside alone, $P > 0.7$.

[e] % LPS cultures refers to the mean cGMP level or cpm in HBSS or LPS + inhibitor-treated cultures divided by the respective mean values in cultures receiving only LPS × 100.

TABLE II. EFFECT OF VARIOUS INHIBITORS ON cGMP LEVELS AND DNA, RNA, AND PROTEIN SYNTHESIS IN CULTURES OF FETAL LIVER CELLS[a,b]

Inhibitor (drug)	cGMP (fmole)			[^{14}C]Thymidine (cpm)			[^{3}H]Uridine (cpm)			[^{3}H]Leucine (cpm)		
	LPS	LPS + drug	% LPS[c]	LPS	LPS + drug	% LPS[c]	LPS	LPS + drug	% LPS[c]	LPS	LPS + drug	% LPS[c]
0.25 μM actinomycin D	5845 ± 1085	306 ± 44[d]	5	3716 ± 305	1848 ± 78[d]	50	3196 ± 174	321 ± 12[d]	10	1396 ± 89	1029 ± 61[d]	74
100 μM cordycepin	4554 ± 426	290 ± 76[d]	6	3500 ± 140	2399 ± 110[d]	69	2512 ± 163	1147 ± 39[d]	46	1056 ± 54	866 ± 64[d]	82
1 μM cycloheximide	3812 ± 753	77 ± 52[d]	2	3556 ± 230	1506 ± 22[d]	42	1974 ± 200	2305 ± 332	117	1293 ± 5	502 ± 26[d]	39
10 μM cytosine arabinoside	4933 ± 779	3496 ± 417[d]	71	3338 ± 48	152 ± 9[d]	5	2561 ± 54	1496 ± 55[d]	58	996 ± 50	897 ± 53	90

[a] Values equal the mean of six replicate cultures ± SD for cGMP levels and three replicate cultures ± SD for [^{14}C]thymidine, [^{3}H]uridine, and [^{3}H]leucine cpm.
[b] Inhibitors and/or LPS were added at time zero; [^{14}C]thymidine, [^{3}H]uridine, and [^{3}H]leucine were added at 150 min. All cultures were terminated at 180 min.
[c] % LPS refers to the mean cGMP level or cpm in LPS + inhibitor-treated cultures divided by the respective mean values in cultures receiving only LPS × 100.
[d] When compared to cultures receiving only LPS, $P < 0.05$.

although none of the inhibitors was absolutely specific, each produced a predominate effect on either DNA, RNA, or protein synthesis. Sixth, sodium nitroprusside, when added at the end of the incubation period to either control or inhibitor-treated cultures, produced large comparable increases of cGMP levels in both cases. Therefore, even after exposure to any of the inhibitors for the entire culture period, the cells were fully capable of responding to a stimulus of guanylate cyclase. Thus, taken together, our data support the hypothesis that expression of the LPS–cGMP response in fetal liver cells is dependent on transcription of messenger RNA and resultant synthesis of an intermediary protein(s) during the lag phase. It seems likely that production of this protein(s) represents only a small fraction of total protein synthesis occurring in the individual cells. Alternatively, it could represent a large proportion of protein synthesis in a small number of cells.

The nature of this hypothetical intermediary protein(s) is unknown. However, several possibilities are readily apparent and include synthesis of cell surface receptors for LPS, an enzyme which subsequently catalyzes formation of an activator of guanylate cyclase, an activator of guanylate cyclase, and guanylate cyclase itself. In addition, since the fetal liver cell suspensions are composed of several cell types, it is possible that more than one kind of cell is involved in the LPS response. LPS could bind to one cell type and cause release of an active substance (lymphokine, for example) which could then increase cGMP levels in another class of cells. Currently, only the synthesis of soluble guanylate cyclase seems unlikely. Since sodium nitroprusside, a known activator of soluble guanylate cyclase (10), produces huge increases of cGMP in cells not treated with LPS, the level of this enzyme in the cells does not appear to be rate limiting. Additional experiments including identification of the cell type or types involved in the reaction, measurement of binding of radioactive LPS to the cell surface, and the effect of adding LPS directly to broken cell preparations will have to be done in order to distinguish between these other possibilities.

Although the biological meaning of the cGMP response to LPS in fetal liver tissue is

TABLE III. EFFECT OF NITROPRUSSIDE ON cGMP LEVELS IN CONTROL AND INHIBITOR-TREATED CULTURES OF FETAL LIVER CELLS[a]

Additives	fmole cGMP[b]
HBSS	106 ± 22
0.25 μM actinomycin D	36 ± 5
HBSS + 100 μM nitroprusside	37,452 ± 570
0.25 μM actinomycin D + 100 μM nitroprusside	70,374 ± 1279[c]
HBSS	76 ± 8
100 μM cordycepin	50 ± 7
HBSS + 100 μM nitroprusside	6441 ± 120
100 μM cordycepin + 100 μM nitroprusside	7745 ± 346[c]
HBSS	80 ± 10
1 μM cycloheximide	30 ± 6
HBSS + 100 μM nitroprusside	7802 ± 751
1 μM cycloheximide + 100 μM nitroprusside	9593 ± 1793
HBSS	121 ± 8
10 μM cytosine arabinoside	125 ± 12
HBSS + 100 μM nitroprusside	10,109 ± 3131
10 μM cytosine arabinoside + 100 μM nitroprusside	10,679 ± 2840

[a] Inhibitors or HBSS were added at time zero; nitroprusside was added at 150 min and the cultures were terminated at 180 min.

[b] Values equal to the mean of three replicate cultures ± SD.

[c] When compared to cultures receiving HBSS + nitroprusside, $P < 0.05$.

unknown, several factors suggest that hematopoietic stem cells might be involved in the effect. First, fetal liver tissue obtained on the 13th day of gestation contains large numbers of hematopoietic stem cells and responds to LPS with very large increases in cGMP levels (4, 13, 14). Second, both the concentration of these stem cells within the fetal liver and the cGMP response to LPS decline with increasing gestational age (1, 2, 13). Third, LPS increases splenic hematopoiesis when injected into mice (15, 16) and elevates cGMP levels when added to cultures of rat adult spleen cells (3). Thus, it seems possible that the increase of cGMP levels produced by LPS is occurring in hematopoietic stem cells and may even be related to growth and differentiation of these cells. At present, there is no direct evidence to support this view. Nevertheless, it is an attractive hypothesis that deserves further evaluation.

1. Graber SE, Bomboy JD, Salmon WD, Krantz SB. Effect of erythropoietin preparations on cyclic AMP and cyclic GMP levels in rat fetal liver cell cultures. J Lab Clin Med **90**:162–170, 1977.
2. Graber SE, Bomboy JD, Salmon WD, Krantz, SB. Evidence that endotoxin is the cyclic 3′:5′-GMP-promoting factor in erythropoietin preparations. J Lab Clin Med **93**:25–31, 1979.
3. Bomboy JD, Graber SE. Stimulation of cyclic 3′:5′-guanosine monophosphate levels in rat spleen cells by lipopolysaccharide preparations. J Lab Clin Med **95**:654–659, 1980.
4. Graber SE, Hellerqvist CG. Direct evidence that bacterial lipopolysaccharides elevate cyclic GMP levels in rat fetal liver cells. J Biol Chem **257**:5414–5419, 1982.
5. Graber SE, Clancey MA. Further characterization of the effect of bacterial lipopolysaccharide preparations in cyclic 3′,5′-GMP levels: the importance of serum. J Cyclic Nucleotide Protein Phosphorylation Res **9**:155–164, 1983.
6. Brooker G, Harper JF, Terasaki WL, Moylan RD. Radioimmunoassay of cyclic AMP and cyclic GMP. Adv Cyclic Nucleotide Res **10**:1–33, 1979.
7. Westphal O, Jann K. Purification of bacterial lipopolysaccharide. Methods Carbohydr Chem **5**:83–90, 1965.
8. Colton T. Statistics in Medicine. Boston, Little, Brown, pp127–131, 191–204, 1974.
9. Kelly K, Cochran BH, Stiles CD, Leder P. Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35**: 603–610, 1983.
10. Bohme E, Graf H, Schultz G. Effects of sodium nitroprusside and other smooth muscle relaxants on cyclic GMP formation in smooth muscle and platelets. Adv Cyclic Nucleotide Res **9**:131–143, 1978.

11. Perry RP, Greenberg JR, Tartof KD. Transcription of ribosomal, heterogeneous nuclear, and messenger RNA in eucaryotes. Cold Spring Harbor Symp Quant Biol **35**:577–587, 1970.
12. Adesnik M, Salditt M, Thomas W, Darnell JE. Evidence that all messenger RNA molecules (except histone messenger RNA) contain poly(A) sequences and that the poly(A) has a nuclear function. J Mol Biol **71**:21–30, 1972.
13. Chui DHK, Liao SK, Walker K. Fetal erythropoiesis in steel mutant mice. III. Defect in differentiation from BFU-E to CFU-E during early development. Blood **51**:539–547, 1978.
14. Nicola NA, Metcalf D, vonMelchner H, Burgess AW. Isolation of murine fetal hemopoietic progenitor cells and selective fractionation of various erythroid precursors. Blood **58**:376–386, 1981.
15. Reissmann KR, Udupa KB, Labedzki L. Induction of erythroid colony forming cells (CFU-E) in murine spleen by endotoxin. Proc Soc Exp Biol Med **153**: 98–101, 1976.
16. Staber FG, Metcalf D. Cellular and molecular basis of the increased splenic hemopoiesis in mice treated with bacterial cell wall components. Proc Natl Acad Sci USA **77**:4322–4325, 1980.

Received March 6, 1985. P.S.E.B.M. 1985, Vol. 180.
Accepted May 14, 1985.