

MITOGENIC EFFECT BY LIPOPOLYSACCHARIDE
AND POKEWEED LECTIN ON DENSITY-INHIBITED CHICK
EMBRYO FIBROBLASTS

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Phytohemagglutinin (PHA),¹ concanavalin A (Con A), and the pokeweed mitogen (PWM) stimulate lymphocytes to transformation and mitosis (1, 2). Certain bacterial substances, the endotoxin lipopolysaccharides (LPS) and purified protein derivative of tuberculin (PPD), have a similar effect (3, 4). Some lectins also preferentially agglutinate tissue culture cells transformed spontaneously or by oncogenic viruses and chemical carcinogens (5). Mitogen cell surface interactions may elucidate the role of cell surface molecules in growth control and malignant transformation.

We have studied the role of cell surface functions in these phenomena using density-inhibited cultures of chick embryo fibroblasts that are stimulated by microgram quantities of insulin, trypsin, and neuraminidase (6, 7). We now report that these cells are stimulated by nanogram quantities of certain lymphocyte mitogens.

Materials and Methods

Density-inhibited cultures of chick embryo fibroblasts were prepared as described previously (6, 7). 10^6 chick embryo cells from trypsinized primary cultures were seeded in medium 199 containing 2% chicken serum and 2% tryptose phosphate broth in 50-mm plastic Petri dishes. After 48 h at 38.5°C the cultures were confluent and had a low rate of DNA synthesis and about 1.5×10^6 cells per dish.

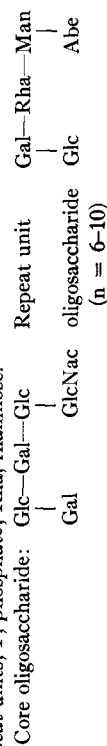
Lipopolysaccharides (LPS) were extracted from three *Salmonella* strains: a smooth (S) strain (SL 696) and a mutant of LPS chemotype Ra (SH 180), both *S. typhimurium* LT2, and a mutant of chemotype Re of *S. minnesota* (SH 320). LPS of strains SL 696 and SH 180 was extracted with the phenol water procedure according to Yuasa et al. (8); the petrol ether extraction (9) was used in the case of the strain 320 (10). The main features of these LPS's and the origin of the bacterial strains are shown in Table I. LPS's were solubilized by heating

¹ *Abbreviations used in this paper:* Con A, concanavalin A; LPS, endotoxin lipopolysaccharides; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; PPD, purified protein derivative of tuberculin; PWM, pokeweed mitogen.

TABLE I
Structure and Main Features of the *Salmonella* LPS Preparations

no.	Bacterial strain		Schematic structure	References
	Species	Chemo-type		
SL 696	<i>S. typhimurium</i> LT2	S	(Lipid A) — { KDO ₃ } — { P, EtN } — { hep ₂ } — { core oligosaccharide } — { repeat unit oligosaccharide } _n	11, 12
SH 180	<i>S. typhimurium</i> LT2	Ra	(Lipid A) — { KDO ₃ } — { P, EtN } — { hep ₂ } — { core oligosaccharide }	8, 12
SH 320	<i>S. minnesota</i>	Re	(Lipid A) — { KDO ₃ } — { P, EtN }	R 595 in 12

Abbreviations: Abe, abequeose; EtN, ethanolamine; Gal, galactose; Glc, glucose; GlcNac, *N*-acetylglucosamine; Hep, *L*-glycero-D-mannoheptose; KDO, 2-keto-3-deoxyoctonic acid; lipid A, glucosaminyl-1,6-glucosamine with fatty acid substituted to hydroxyl and amino groups (fatty acids found in lipid A are 3-hydroxymyristic acid, myristic acid, palmitic acid, and lauric acid); Man, mannose; n, unknown number of repeat units; P, phosphate; Rha, rhamnose.



concentrated aqueous suspension (1–10 mg/ml) for 3 min in a water bath at 100°C. All dilutions were made up in phosphate-buffered saline (PBS).

The sources and specific features of the other substances were as follows: pokeweed mitogen ([PWM] Grand Island Biological Co., Grand Island, N.Y.)—lyophilized crude extract of *Phytolacca americana*, reconstituted according to the manufacturer's instructions; purified tuberculin ([PPD] lot RT 32, Statens Serum Institut, Copenhagen, Denmark) 5×10^6 TU/mg; Bacto-Phytohemagglutinin M and P ([PHA-M and PHA-P] Difco Laboratories, Detroit, Mich.); crystalline leucoagglutinin purified from red kidney beans (13) kindly provided by Dr. C. T. Nordman, Minerva Foundation Institute for Medical Research, Helsinki, Finland; concanavalin A, lyophilized (Pharmacia, Uppsala, Sweden) or in saturated salt solution (Miles-Yeda, Rehovot, Israel); bovine albumin, crystalline (Sigma Chemical Co., St. Louis, Mo.); trypsin, crystalline, TPCK-grade (Worthington Biochemical Corp., Freehold, N.J.); insulin, crystalline (Novo Industri A/S, Copenhagen, Denmark; 24.9 IU/mg); neuraminidase, type VI (Sigma). In the stimulation experiments the above substances, diluted in PBS or Tris-buffered saline, were added to 2-day old density-inhibited cultures in volumes not exceeding 50 μ l.

For determination of [3 H]thymidine incorporation duplicate cultures were labeled for 60 min with 2 μ Ci, then washed twice with cold PBS, and extracted twice with cold 10% trichloroacetic acid, three times with cold ethanol, and air-dried. The cells were dissolved in 0.3 M NaOH and their radioactivity was determined. The number of cells per culture was determined after trypsinization in an electronic particle counter (Celloscope model 302; AB Lars Ljungberg, Stockholm, Sweden). Uptake of labeled substances was assayed according to Hatanaka et al. (14) with some modification (7). The cultures were washed twice with warm Hanks' balanced salt solution without glucose, then incubated for 10 min at 38.5°C with 2 ml of 2-deoxy-D-[1- 3 H]glucose, (24.4 Ci/mmol; The Radiochemical Centre, Amersham Buckinghamshire, England) or L[4,5- 3 H]leucine (58 Ci/mmol; The Radiochemical Centre) at 0.6×10^{-7} M. Cultures were then washed quickly four times with 10 ml of cold Hanks' solution, scraped into 1 ml of 1% sodium dodecyl sulfate, and homogenized by vibration. The radioactivity was determined, and protein assayed by Lowry's method. The uptake in control cultures was in these experiments about 1500 cpm/mg protein for 2-deoxyglucose and about 300,000 cpm/mg protein for leucine.

RESULTS

Addition of LPS, PWM, or PPD to density-inhibited cultures of chick embryo fibroblasts caused increased synthesis of DNA and cell division as evidenced by the increase in [3 H]thymidine incorporation and cell number (Table II). All three substances also caused an early increase in uptake of sugar. 3 h after the addition the uptake of 2-deoxy-D-[3 H]glucose had increased two to threefold but that of [3 H]leucine was unaltered. In Table II LPS, PWM, and PPD are also compared with substances known to initiate proliferation, such as insulin (7), trypsin (15), and neuraminidase (6). LPS, PWM, and PPD had a potent stimulatory effect on sugar uptake, but in initiating [3 H]TdR incorporation and cell division they were less effective than trypsin or insulin.

The smallest effective dose of LPS was, however, 1,000 times less than that of any of the substances previously known to reinitiate growth in stationary cell cultures (Table II). 30–100 pg/ml of LPS consistently stimulated sugar uptake, DNA synthesis, and cell division in density-inhibited chick embryo fibroblast cultures. Three different LPS preparations were used: LPS-S derived from wild-type strains, and LPS-Ra and LPS-Re from two rough strains

with increasing incompleteness of the polysaccharide portion of their LPS (for details see Table I). The dose response curves of all three LPS preparations were quite similar (Fig. 1). They were active in picogram amounts and were not toxic apart from LPS-S that at high doses, $\geq 10 \mu\text{g/ml}$, caused microscopic changes other than those characteristic of stimulated cells. At these doses the stimulatory effect of LPS-S preparation was already considerably

TABLE II
Stimulation by Lymphocyte Mitogens of Density-Inhibited Chick Embryo Fibroblasts

Substance added	Concentration ($\mu\text{g/ml}$) or dilution	Rate of sugar uptake at 3 h (cpm/mg protein, ratio to control)	Rate of [^3H]TdR incorporation at 12 h (cpm per culture)	Cell number at 40 h $\times 10^{-6}$
Control	—	1.0	940	1.84
Bovine albumin	100	1.0	990	1.91
Lipopolysaccharide Ra	0.001	2.8	10280	2.94
Pokeweed mitogen (0.31 mg/ml protein)	1:1000	2.5	9320	2.96
Tuberculin	1	2.4	7430	2.34
Leucoagglutinin	1	1.1	710	1.53
Phytohemagglutinin P 14 mg/ml protein	1:1000	1.0	730	1.63
Phytohemagglutinin M 3.2 mg/ml protein	1:1000	0.9	850	1.85
Concanavalin A	1	1.1	870	1.82
Neuraminidase	1	1.9	8470	2.79
Trypsin	5	2.5	19240	3.63
Insulin	1	2.7	18590	3.69

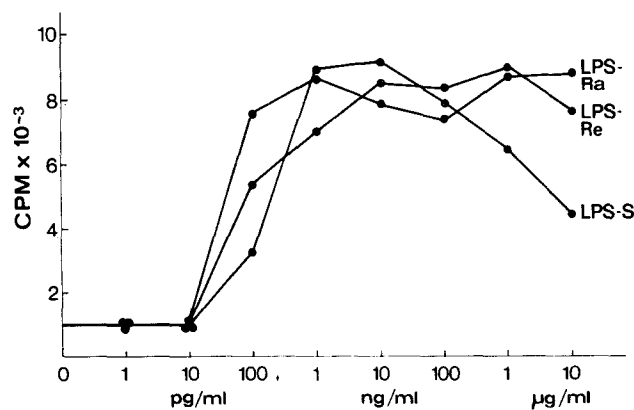


FIG. 1. Dose response curve for three different LPS preparations in stimulation of [^3H]thymidine incorporation in density-inhibited chick embryo fibroblasts. [^3H]TdR incorporation was assayed using a 60 min pulse 12 h after addition of the test substances.

reduced (Fig. 1). LPS-Ra, on the other hand, gave practically identical stimulatory effects at 100 pg/ml and 100 μ g/ml.

In view of the known amphipathic properties of LPS (12, 16), nonionic (Triton X-100) and ionic detergents (sodium dodecyl sulfate and *N*-cetyl-*N*-*N*-*N*-trimethylammonium bromide) were tested in doses ranging from 1 ng/ml to 1 mg/ml. In no dose were they stimulatory, whereas the higher concentrations were toxic.

Of the other mitogens PPD was active in high doses only (Table III). The crude pokeweed mitogen extract was, however, active in dilutions up to 10^{-4} (Table II, Fig. 2). Three lectin preparations derived from red kidney beans (*Phaseolus vulgaris*) were studied. The crude PHA-M ("muco-phytohemagglu-

TABLE III
Comparison of the Dose Ranges of Certain Substances in Stimulation of [³H]Thymidine Incorporation in Density-Inhibited Chick Embryo Fibroblasts

Substance added	Smallest effective dose	Minimum dose for full effect
	ng/ml	ng/ml
LPS-Ra	0.03	0.1
PWM-crude	10*	100*
Neuraminidase	30	300
Insulin	30	1,000
Tuberculin	100	3,000
Trypsin	300	3,000

The substances were added in half-log dilutions. [³H]TdR incorporation was assayed using a 60 min pulse 12 h after addition of the test substances. The minimum dose gives \geq 2-fold increase above the control level. The limit dose of maximal effect gives $>80\%$ of the maximum.

* The final doses of PWM crude are given as concentrations of protein.

tinin from which the polysaccharide has been removed") induce l detachment and clumping of cells at high doses ($\leq 1 : 300$), weak stimulation of cells in some experiments at about $1 : 1,000$, and no effect at lower doses. The pure crystalline leucoagglutinin caused microscopic alterations in cells at 10 μ g/ml but had no stimulatory effect at any dose. The two preparations of Con A gave similar results. High doses ($> 100 \mu$ g/ml) were toxic; lower doses were slightly inhibitory or had no effect (Table II).

DISCUSSION

Our data indicate that three different mitogens, lipopolysaccharide, the pokeweed lectin, and tuberculin, induce proliferation in density-inhibited (contact-inhibited) cultures of chick embryo fibroblasts. This demonstrates that the stimulatory effect of these substances, generally implied as lymphocyte mitogens, is not restricted to lymphoid cells. In fact in activation of the chick embryo fibroblasts, the doses needed for optimal effect are 10,000 times

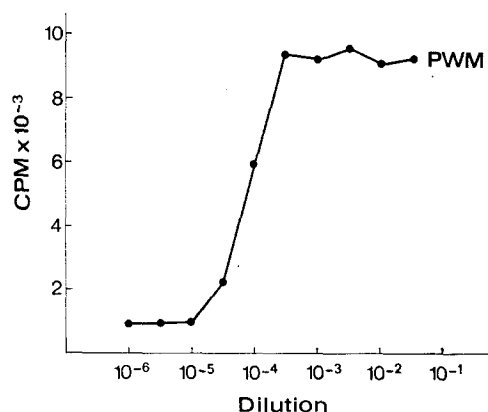


FIG. 2. Dose response curve for a crude pokeweed mitogen preparation (see Materials and Methods) in stimulation of [³H]thymidine incorporation in density-inhibited chick embryo fibroblasts. [³H]TdR incorporation was assayed using a 60 min pulse 12 h after addition of the test substances.

(LPS) or 10 times (PWM, PPD) lower than those giving full activation of lymphocytes (3, 4). Activation of lymphocytes by mitogen through the interaction of surface immunoglobulins has been discussed (17) as one of the possible mechanisms. The stimulation of nonlymphoid cells by these same mitogens does not support this hypothesis.

Certain mitogens (PHA, Con A) induce blast transformation and DNA synthesis in murine lymphocytes of thymic origin (3, 18), while others such as PWM may activate both thymus-dependent (T) and thymus-independent (B) lymphocytes (19). The nonantigenic (mitogenic) effects of LPS and PPD are restricted to B cells (3, 4). It is of interest that only B-cell mitogens, LPS, PWM, and PPD, activated chick embryo fibroblasts. This tends to suggest that their cell surface, a presumed initial target of mitogen, shares certain functional or molecular properties expressed in B but not in T lymphocytes.

The present report seems to be the first time that activation of nonlymphoid cells by lipopolysaccharides has been described. The concentration of LPS (endotoxin) maximally stimulating chick embryo fibroblasts was remarkably low (about 100 pg/ml). This is in the fairly wide range of concentrations expected to prevail in tissues of different animals after effective or toxic doses of bacterial endotoxin (16). Activation of various cell populations *in vivo* could well be the basis for some of the many host-reactive properties of LPS.

The results obtained with the three LPS preparations were very similar, although the LPS's differ greatly in the structure of their hydrophilic carbohydrate part (Fig. 1). They all have the same lipophilic lipid A end; the LPS-Re has only a trisaccharide attached to it, the oligosaccharide chain of LPS-Ra has in addition two heptoses and six hexoses, while the LPS-S has 40 more

hexoses. This indicates that the size or the structure of the carbohydrate chain is not important for the mitogenic effect of LPS. This is in agreement with the recent finding by Andersson et al. (17) that the lipid A moiety was responsible for the total mitogenic effect produced by intact LPS on lymphocytes. It also agrees with the amply documented evidence that most of the biological effects of LPS are caused by the lipid A moiety (20, 21).

Due to its high content of lipid A, LPS-Re could have been expected to be more effective than the other two LPS preparations. However, none of them are water soluble. The higher activity of LPS-Re may be opposed by its very poor water solubility when compared with the more hydrophilic LPS-Ra and LPS-S.

The receptor or the site LPS binds to and reacts with during mitogenic stimulations is unknown. The cytoplasmic membrane would, however, be a plausible target. Amphipathic LPS is known to interact with various components of biological membrane, and it can penetrate into artificial phospholipid bilayers altering their stability (22, 23). Brailovsky et al. (24) have found that (a) transformed but not normal rat embryo fibroblasts were inhibited by high concentrations (10 $\mu\text{g}/\text{ml}$) of LPS, and (b) transformed cells, as well as cells treated by trypsin or neuraminidase, bound LPS much more effectively than normal fibroblasts. LPS is thus likely to adsorb or penetrate into the (outer) cytoplasmic membrane of fibroblasts, mainly by hydrophobic binding of lipid A with phospholipids. The extremely high activity of LPS observed in this study suggests the possibility of a high degree of specificity, possibly even involving specific sites of interaction.

SUMMARY

The B lymphocyte mitogens, bacterial lipopolysaccharide (LPS), pokeweed lectin, and tuberculin, induced proliferation in density-inhibited monolayer cultures of chick embryo fibroblasts. The stimulation was seen both as an early increase in sugar uptake and cell volume and later as an increase in thymidine incorporation and cell number. The concentration of LPS maximally stimulating fibroblasts was remarkably low, about 0.1–1 ng/ml. LPS preparation with very different sugar chains gave quite similar results indicating that the architecture of the hydrophilic carbohydrate part is not critical for the mitogenic effect.

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