Bacterial Endotoxin Effects on Several Cell Lines in Culture

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Introduction

The *in vivo* biological effects of bacterial endotoxin (lipopolysaccharide, LPS) are well known and include fever, tissue damage, endotoxic shock and lethality (14). The basic mechanisms of cellular injury caused by endotoxin which lead to disseminated tissue damage and possible death of the host are not well known. *In vivo* studies have shown, for example, that the complement system is activated by endotoxin, but the significance of its possible role in inducing cellular injury is not clear (13).

Many of the *in vitro* studies of endotoxin effects on cellular structure or function have involved short-term cell cultures. For example, endotoxin inhibited adenosine triphosphatase activity of rabbit or human leukocytes (22). *In vitro* studies on the effects of endotoxin on guinea pig, rabbit and human macrophages migrating from organ explants also revealed endotoxin cytotoxicity (10). Bona (2) showed pinocytotic uptake of endotoxin by guinea pig macrophages and its transfer to autologous lymphocytes. In murine macrophages the stimulation of synthesis of both lysosomal and non-lysosomal enzymes was reported by Allison et al. (1), and Spitzer (20) found that isolated canine fat cells exposed to endotoxin *in vitro* show higher norepinephrine-stimulated lipolytic responses and higher cyclic adenosine monophosphate levels compared to control cells. There are a number of reports on endotoxin stimulation of murine bone marrow-derived lymphocytes (7,9,11,16). Interaction of endotoxin with human erythrocytes and the isolation and partial characterization of receptor material was reported by Springer et al. (21), although the relationship of the red blood cell receptor to the biological activities described above is unclear. Brailovsky et al. (3) used endotoxins with short core oligosaccharides (endotoxic glycolipids) and found *in vitro* growth inhibition of spontaneously or virally transformed rat embryo fibroblasts, but not untransformed fibroblasts. However, others failed to find cytotoxic effects when primary (adult and embryonic) cell cultures and established cell strains from rabbit, monkey, mouse and man were treated with up to 1 mg/ml endotoxin (8). In the present studies we demonstrate, using established murine and hamster cell lines, that endotoxin can markedly affect growth of some, but not all, of these cell lines in culture.

Materials and Methods

Cell cultures. Cell lines used in this study were obtained from several sources. Murine 3T3, SV3T3 and MSV3T3 were originally obtained from Dr. S. Aaronson, Viral Carcinogenesis Branch, National Cancer Institute. B16 melanoma (B16-F1) came from Dr. I.J. Fidler, NC1-Frederick Cancer Research Center, Frederick, Maryland. The S49 (S49-1) and CHO-K1-PRO lines were obtained from Dr. R. Hyman, The Salk Institute for Biological Studies, San Diego, California. BHK and PyBHK came from Dr. W. Eckhart of the Salk Institute. The RAW 117 line (RAW 117-P) was obtained from Dr. P. Ralph at the Salk Institute; the RAW 117-H10 variant line was selected from the parental strain (4). Cells were grown in Dulbec-

co's modified Eagle's medium (19) supplemented with 10% fetal bovine serum or 10% donor calf serum, nonessential amino acids and antibiotics as necessary (5) in plastic tissue culture or petri dishes.

Endotoxin. The gram-negative bacterial endotoxin used in our experiments was a lipopolysaccharide prepared by phenol-water extraction from *Salmonella typhosa* 0901 (Difco Laboratories, Detroit, Michigan). Biological activities of the endotoxin preparation were shown to be similar to purified lipopolysaccharide obtained from *Salmonella* (6,12) and were effective *in vitro* for inhibition of growth of certain tumor cells (17,18). Endotoxin (5 mg/ml) was heated in phosphate-buffered saline for 1 hr at 100°C, and aliquots were stored frozen at -20°C until just prior to thawing and dilution for use in the experiments.

Results

Several available cell lines were screened for obvious growth inhibition or morphological changes in the presence of endotoxin (Table I). No effects due to endotoxin were detected in most of the cell lines examined, even with the addition of up to 500 μg LPS/ml culture. With the murine lymphoma cell line S49 (a T-cell lymphoma), for example, the growth curve in the presence of LPS was essentially identical to growth of the cells in the control culture (no LPS added), as shown in Figure 1.

Murine cell lines markedly affected by endotoxin in these experiments were the lymphosarcoma cell line RAW 117-P and a malignant variant line RAW 117-H10 (Table I). Growth of the lymphosarcoma cell lines was inhibited early and the effect lasted for about three days in culture. There was an LPS dose-dependent effect (data not shown), and only 10 mcg LPS/ml inhibited growth of the cell lines 50% or more compared to control cultures (no LPS added) as shown in Figure 2. As seen in Figure 2, RAW 117-H10 cells were inhibited more by LPS (upper panel) than were the parental RAW 117-P cells (lower panel). Interestingly, after exposure to 10 mcg LPS/ml for three days, a "rebound" effect occurred in cultures of both cell lines, such that by the fourth day cultures in the presence of LPS had almost overtaken the control cultures.

The only hamster cell line affected by LPS was the Chinese hamster cell line CHO•K1•PRO; growth of this cell line was markedly inhibited by endotoxin (Table

TABLE I. *Endotoxin-mediated growth inhibition and morphological changes*

Cell Line	Animal Strain	Transforming Agent	Growth Inhibition	Mophological Changes
Mouse				
3T3	BALB/c	Untransformed	–	–
SV3T3	BALB/c	Simian Virus 40	–	–
MSV3T3	BALB/c	Murine Sarcoma Virus	–	–
B16 MELANOMA	C57BL/6J	Spontaneous	–	–
S49-1	BALB/c	Mineral Oil	–	–
RAW 117-P	BALB/c	Abelson Leukemia Virus	+	+/–
RAW 117-H10	BALB/c	Abelson Leukemia Virus	+	+/–
Hamster				
BHK		Untransformed	–	–
PyBHK		Polyoma Virus	–	–
CHO•K1•PRO		Untransformed	+	+

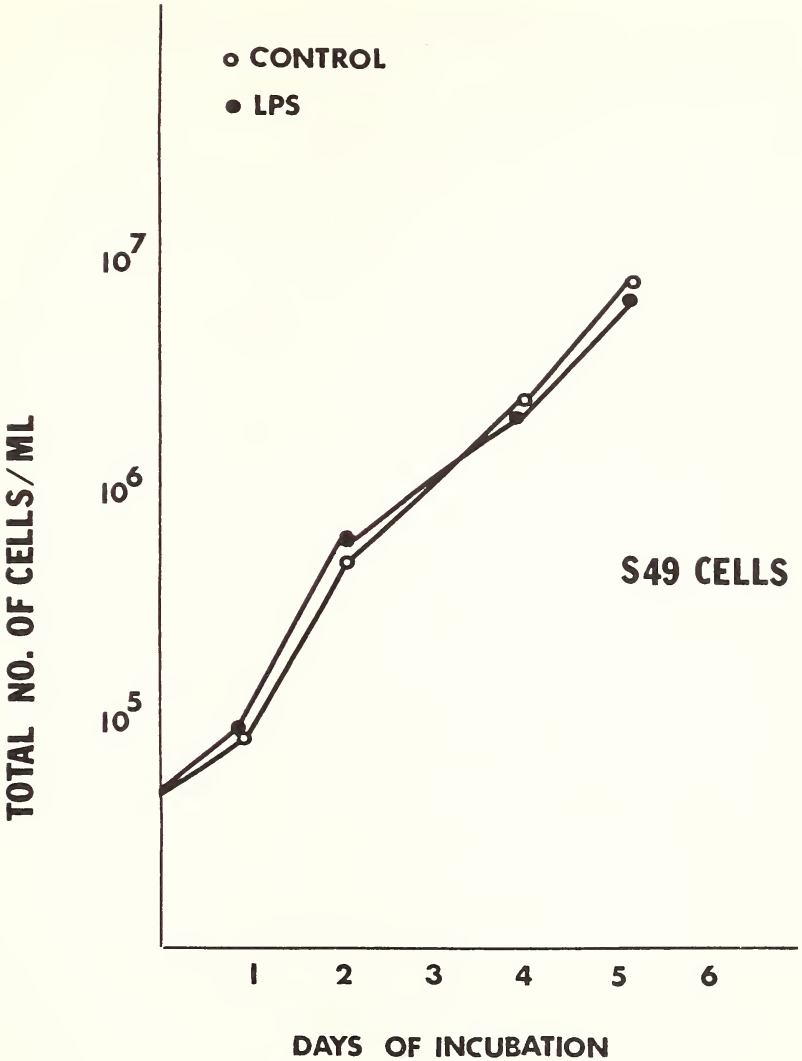

FIGURE 1. *Cells were plated at 5x10⁴/ml in a 60 mm petri dish and incubated in either 100 μg LPS/ml (final concentration), or no LPS was added (control). Each point represents the average of triplicate determinations of the number of cells/ml.*

I). Obvious endotoxin effects were not noted until after about four days' incubation at which time cell growth was almost completely inhibited (Figure 3). That this effect is due solely to cytotoxicity is unlikely, since cell populations remained greater than 90% viable by trypan blue dye exclusion after 7 days of endotoxin exposure. Interestingly, low concentrations (5 μg/ml) of endotoxin caused slight growth stimulation (Figure 3). Cells incubated in the presence of higher concentrations of

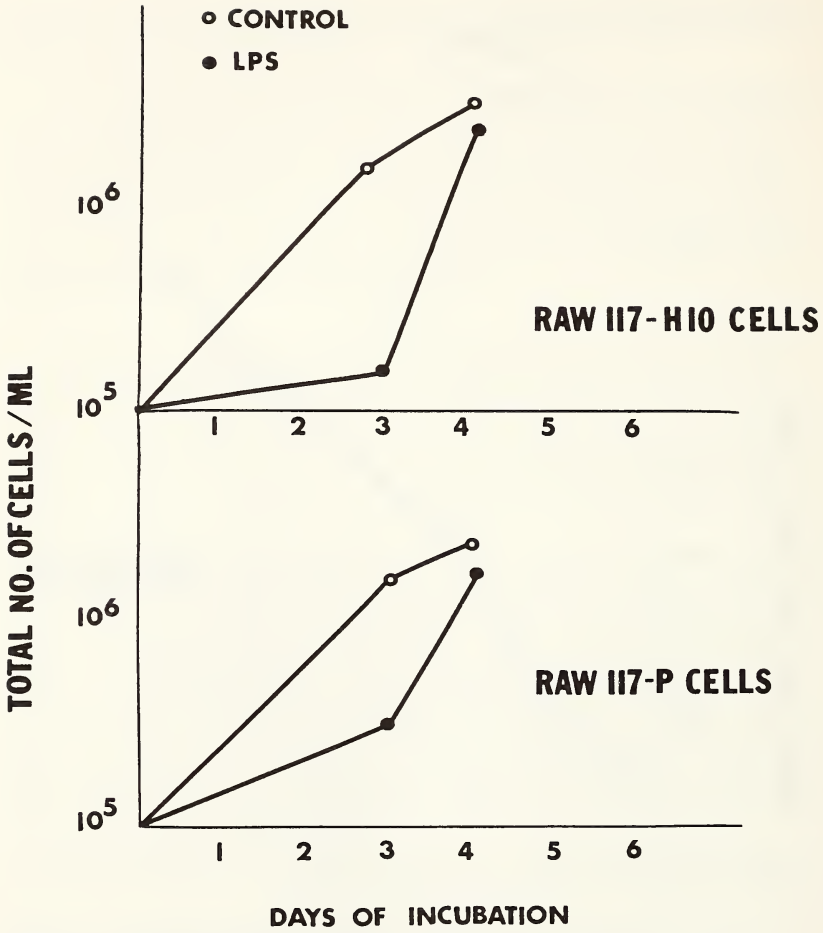

FIGURE 2. *Cells were plated at 1x10^5/ml in a 60 mm petri dish and incubated in 10 μg LPS/ml (final concentration) or no LPS was added (control). Each point represents the average of triplicate determinations of the number of cells/ml.*

endotoxin (50 or 500 mcg/ml) were not stimulated and grew at about the same rate as control cells (no endotoxin added) until about 4 days' incubation, after which the endotoxin-treated cells exhibited growth inhibition (Figure 3).

CHO•K1•PRO cells incubated at a high endotoxin concentration (500 mcg/ml) for 7 days contained many large, light-refractile vesicles and had markedly altered cell shape and increased spreading (Figure 4) compared to control cells (Figure 5). Untreated cultures grew to confluency, while cells exposed to endotoxin remained quite sparse with the presence of several rounded cells (Figure 4). When the CHO•K1•PRO cells exposed to high levels of endotoxin were examined ultrastructurally, modifications were seen in the cytoplasms of the treated cells (data not shown). After 4 days' exposure to endotoxin, vesicles began to form in toxin-exposed cells which were not apparent in control untreated cells (data not shown).

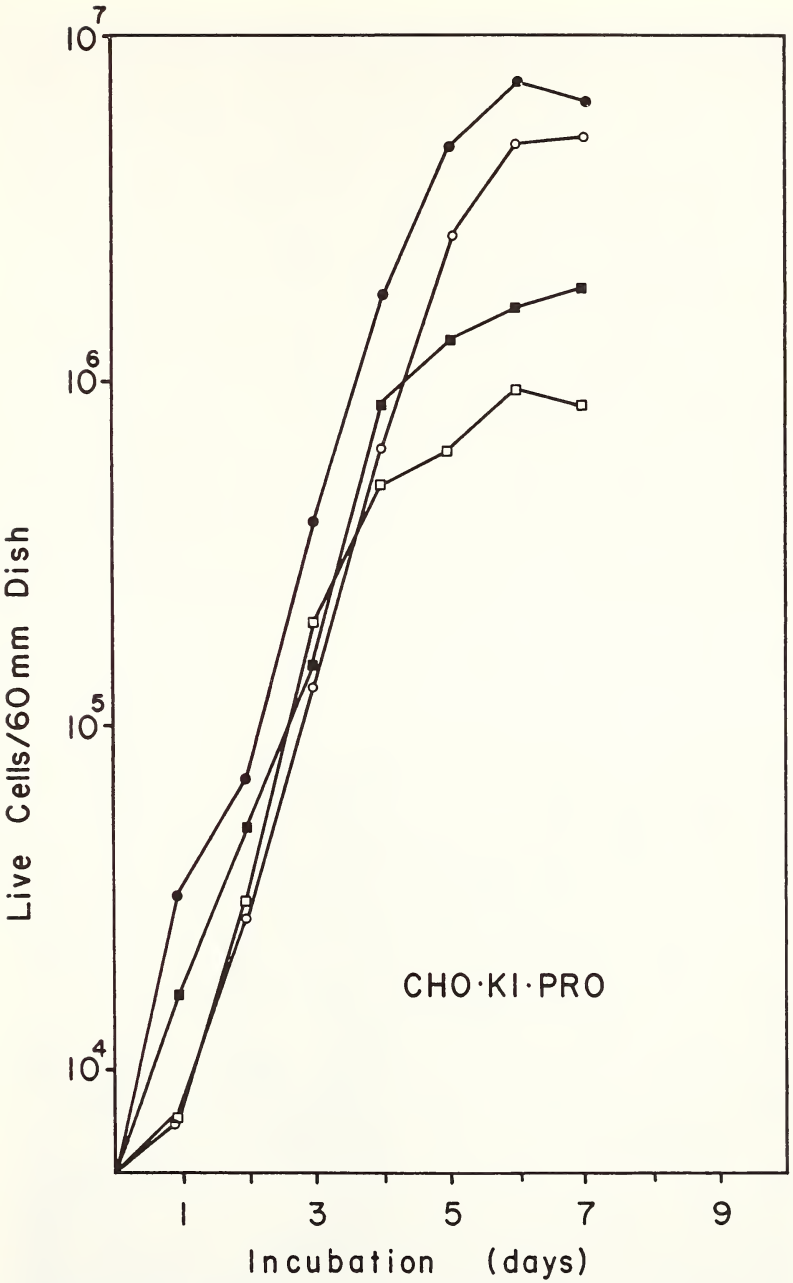

FIGURE 3. *Cells were plated at 5x10³/60 mm tissue culture dish and incubated in the indicated final concentrations of LPS. Each point represents the average of triplicate determinations of the number of cells.* ○ *controls, no LPS added;* • *5 μg LPS/ml;* ■ *50 μg LPS/ml;* □ *500 μg LPS/ml.*

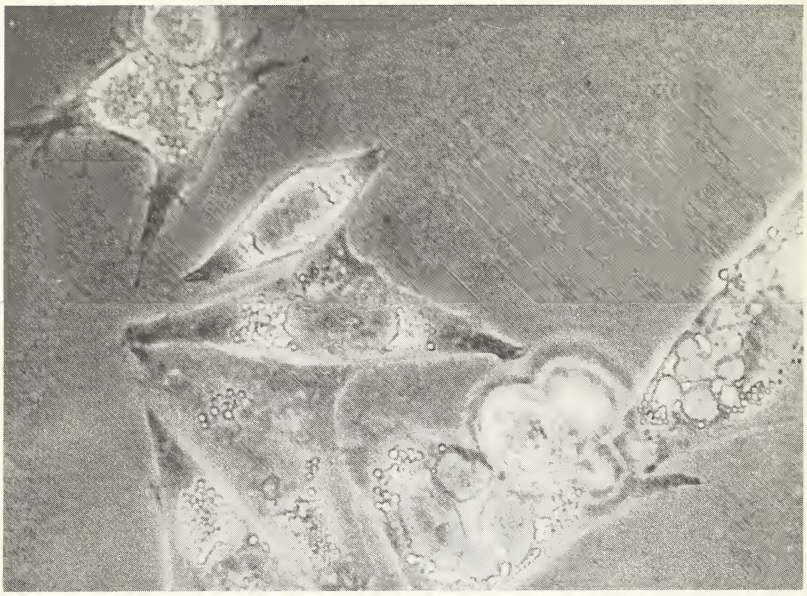

FIGURE 4. *CHO•K1•PRO cells incubated for 7 days, 500 μg LPS/ml. Phase contrast x 510.*

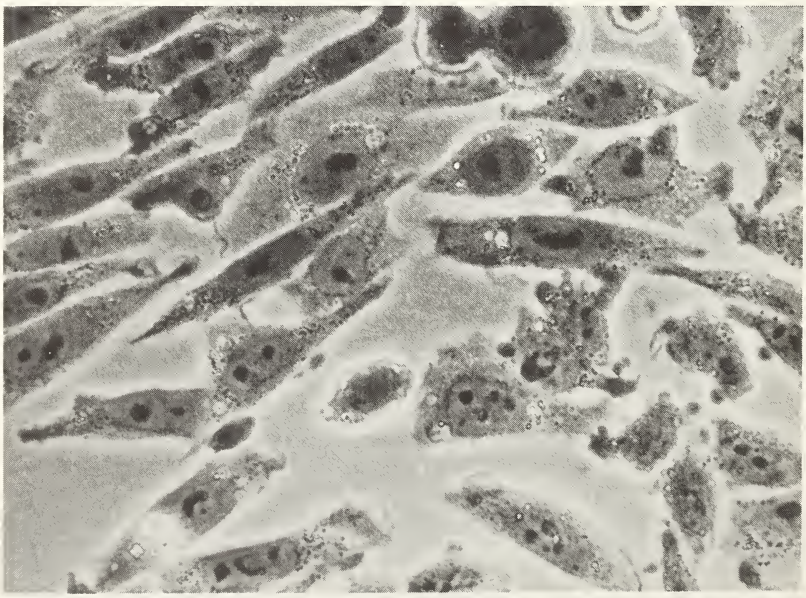

FIGURE 5. *Control (no LPS added) CHO•K1•PRO cells incubated for 7 days. Phase contrast x 510.*

By 7 days very large vacuoles were seen in ultrathin section of cells treated with endotoxin compared to control cells. The cellular modifications seen with light microscopy could thus be confirmed using electron microscopy; the ultrastructural studies will be the subject of a separate report.

Discussion

Although stimulatory (mitogenic) for mouse bone-marrow derived lymphocytes (B cells) *in vitro* (9), endotoxin is inhibitory for some murine B lymphoid tumor cells (18). In our studies with the RAW 117 lymphosarcoma cells, although growth was inhibited by LPS initially, a later period of growth stimulation was noted. Further work will be needed to see what may be involved in the stimulatory period. A number of possibilities exist, such as LPS depletion, emergence of an LPS inhibition-resistant population, or production of stimulatory products.

In other *in vitro* studies of cell growth inhibition by endotoxin the most marked growth inhibition of transormed rat embryo fibroblasts began after 3-4 days' incubation (3) much like our observations with CHO•K1•PRO cells. Similar to our investigations, cytotoxic effects were not seen; however, morphological alterations were not observed in the endotoxin-treated fibroblast cells (3). Cell shape changes (rounding) have been reported for Y-1 adrenal tumor cells exposed to a variety of gram-negative bacterial endotoxins (23), although endotoxin incubation times (13-16 hours) were less than those in the present report. It would be interesting if endotoxin also inhibits the growth of Y-1 adrenal tumor cells *in vivo*.

The *in vitro* endotoxin effects on inhibition of growth, induction of vacuolization and alteration of morphology of Chinese hamster ovary cells occurs only after prolonged endotoxin incubation with CHO•K1•PRO cells. These effects do not appear to be related to neoplastic transformation, because other untransformed/transformed hamster and mouse lines (Table I) are unaffected by similar concentrations of endotoxin.

Further work will be necessary to determine the mechanism(s) involved in growth inhibition of the CHO•K1•PRO and RAW 117 cell lines by LPS. It is interesting that "lag periods" similar to that observed in our studies between addition of LPS to CHO•K1•PRO cells and maximal cellular effects such as growth inhibition (3) or lymphocyte stimulation (4) have been observed. Additionally, mouse macrophages were shown to develop vacuoles after incubation with endotoxin (15), similar to what we observed in the CHO•K1•PRO cells. Thus, the possibility exists that LPS may exert analoguous effects *in vitro* on a variety of cell types.

Acknowledgments

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