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# Lipopolysaccharide Effects on Sensitive and Resistant Variant Chinese Hamster Ovary Cell Lines

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Chinese hamster ovary (CHO·K1·PRO) cell growth was inhibited by addition of a gram-negative bacterial lipopolysaccharide (LPS) to the cell culture medium. Growth inhibition began after three or four days of incubation, was dose-dependent up to a maximum at an LPS concentration of 500 μg/ml and was accompanied by cell shape changes and enhanced cytoplasmic vacuolization. Formation of bizarre CHO·K1·PRO cell shapes and vacuole formation were most pronounced after seven days of incubation with LPS and could be observed by light and electron microscopy. An LPS-resistant cell population was obtained by intermittent in vitro exposure to high levels of LPS; these variant cells or clones derived from them failed to display growth inhibition in the presence of LPS. A clone from the LPS-resistant variant population showed altered cell properties compared to the parental cell line which included changes in cell morphology, adhesion, and endocytosis. Parental cells were markedly density-inhibited, whereas the variant clone exhibited considerable growth after confluency. The LPS-resistant variant cells showed a more elongated morphology than the parental line. No significant differences were observed between rates of detachment of parental and variant cells when sparse cultures of either line were removed from tissue culture dishes by ethylenediaminetetracetate (EDTA). However, at confluency approximately 100% of the variant cells versus 35% of the parental cells were removed by EDTA in one hour. Measurements of $^{125}$I-ferritin uptake by parental and variant cells showed approximately twenty-fold and twofold increases, respectively, in uptake induced by LPS when compared to untreated control cultures.

**Key words: adhesion, endotoxin, lipopolysaccharide, variant cell line**

Endotoxin or lipopolysaccharide (LPS) from cell walls of gram-negative bacteria produces a variety of pathophysiologic effects when administered to experimental animals or man [1]. LPS-induced pathophysiologic changes can be due to distinct complement-

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dependent or complement-independent reactions [2]. For example, some of these effects may be mediated by LPS interaction with complement serum components, since LPS can activate complement via the alternate pathway [3]. However, it has been shown that the more severe LPS-caused events such as hypotensive shock, disseminated intravascular coagulation, and death of experimental animals following injection of lethal amounts of endotoxin are not abrogated by prior complement depletion [2]. This suggests that the direct action of LPS on target cells may be important in determining LPS effects. Cellular targets for LPS include peripheral blood leukocytes [4], macrophages [5], spleen lymphocytes [6], and both parenchymal and nonparenchymal liver cells [7].

The interaction of LPS with target cells in vitro correlates well with in vivo LPS-cell action. Zlydaszyk and Moon [7] demonstrated that radiolabeled endotoxin interacts with hepatoma cells in tissue culture, and this correlates well with in vivo experiments involving the association of labeled endotoxin with nonparenchymal liver cells. Also, the capacity of LPS to induce morphologic transformation, cell division, and immunoglobulin synthesis by bone marrow-derived (B) lymphocytes in vitro has been shown to parallel these same effects in vivo [8]. Thus, in vitro studies of LPS effects on mammalian cells may help to elucidate some pathophysiologic events that occur due to LPS administration to experimental animals, and they may help explain certain clinical symptoms and effects due to infections with gram-negative bacteria. We report here the growth inhibiting and morphologic effects of LPS on a sensitive Chinese hamster ovary (CHO·K1·PRO) cell line in tissue culture and describe some properties of an LPS-resistant variant cell line derived from the LPS-sensitive cells.

## MATERIALS AND METHODS

### Cells and Toxin

The Chinese hamster ovary cell line CHO·K1·PRO [9] was obtained from Dr Robert Hyman of The Salk Institute, San Diego, California. Cells were grown in plastic tissue culture dishes (Falcon Plastics) in Dulbecco's modified Eagle's medium (DMEM) [10] supplemented with 10% fetal bovine serum, nonessential amino acids, and antibiotics. Gram-negative bacterial endotoxin used in our experiments was the lipopolysaccharide prepared by hot phenol water extraction [11] from Salmonella typhosa 0901 (LPS-W) and was obtained from Difco Laboratories (Detroit, Michigan). The biological activities of this toxin preparation are similar to that of a variety of other endotoxin preparations [12, 13]. Before use endotoxin at a concentration of 5 mg/ml in phosphate-buffered saline (PBS) was heated for 1 h at 100°C; then aliquots were frozen at −20°C until thawing prior to use. Dilutions were made in PBS and added to cell cultures to yield the appropriate final endotoxin concentrations; diluent alone was added to control cell cultures. To obtain LPS-resistant variant cell populations, parental cells were grown in LPS (500 μg/ml) for two weeks, then fed fresh DMEM (supplemented) without endotoxin. Surviving adherent cells were allowed to grow, and these were transferred once in supplemented DMEM, and at the second transfer were incubated again with LPS for two weeks. Thirteen cycles of intermittent endotoxin exposure were required to yield the LPS-resistant population. The resistant culture was cloned by limiting dilution, and a clonal cell line (CHO·K1·PRO-LPS13·3) was chosen for further study.

### Inhibition of Cell Proliferation

Cell growth inhibition was calculated from numbers of cells after exposure to LPS for seven days, compared to control cultures without added endotoxin. The percentage inhibition of cell proliferation was calculated from the formula $100 - (T/C \times 100)$, where T = number of cells in the presence of endotoxin and C = number of cells in control cultures without added endotoxin.

### Microscopic Studies

Cells were grown in the presence or absence of endotoxin (500 μg/ml) for four or seven days. For light microscopy cells were grown in tissue culture dishes or glass slide culture chambers (Lab-Tek, Miles), and after the appropriate period of incubation, photographed using a phase microscope. For electron microscopic studies cells were grown in plastic dishes and fixed with 1.5% glutaraldehyde in cacodylate buffer. After washing twice with cacodylate buffer, the samples were postfixed in 1% cacodylate-buffered 1% osmium tetroxide. Postfixed cell samples were then dehydrated through ethanol-propylene oxide and embedded in Epon 812, and thin sections were stained with 2% uranyl acetate and observed in a Hitachi model HU-12 electron microscope at 75 kV.

### Cell Detachment Assay

Growth medium was aspirated from sparse or confluent cell cultures in 35-mm tissue culture dishes, and prewarmed 2 mM ethylenediaminetetracetate (EDTA) in ($Ca^{2+}$-$Mg^{2+}$)-free PBS was added to each dish. The dishes were placed on a gyratory platform in the warm room (37°C) and rotated at approximately 1 Hz. Triplicate samples were carefully removed at timed intervals and both released and adherent cells (removed with EDTA) were counted in a particle counter (Electrozone/Celloscope, Particle Data).

### Ferritin Uptake

Ferritin was radioiodinated by the $^{125}$I-monochloride method of McFarlane [14]. The iodinated protein was dialyzed and diluted in PBS to yield a specific activity of $(0.5–1.5) \times 10^6$ cpm/mg. Endocytosis of $^{125}$I-ferritin (final concentration 6 mg/ml in DMEM) was measured in cultures of parental or LPS-resistant cells in the presence or absence of LPS for seven days. At the end of the incubation period, growth medium and free $^{125}$I-ferritin was aspirated, dishes were washed once with DMEM, and cells were fixed with 1.5% glutaraldehyde in PBS. The fixed cells were carefully scraped from the dish with a rubber policeman into PBS rinsing buffer and washed three times in PBS before counting in a gamma counter or further preparation for electron microscopy.

## RESULTS

### Growth Inhibition and Altered Morphology

Parental cells were growth-inhibited beginning at the fourth day of incubation with endotoxin, and the inhibition of growth occurred in a LPS dose-dependent fashion, as previously reported [15]. Low concentrations of LPS (5 μg/ml) caused a slight stimulation of growth rate, while 50 μg/ml LPS showed inhibition, and maximal inhibition occurred at 500 μg/ml (Table I). While cell cultures containing an LPS concentration of 500 μg/ml were

quite sparse after seven days incubation, control cell populations attained confluency during this period. The growth inhibiting effect of LPS was not due to cytotoxicity, since the toxin-exposed and control cell populations both displayed greater than 90% viability (assessed by trypan blue dye exclusion) after incubation for seven days in the absence or presence of gram-negative bacterial endotoxin. Alterations in cell morphology were first noticeable at four days in 500 μg/ml LPS (Fig. 1) and became the most pronounced when

**TABLE I. Inhibition of CHO·K1·PRO Cell Proliferation by LPS**

| Cell type | LPS (μg/ml) | Number of cells | % Inhibition |
|---|---|---|---|
| Variant | 0 | $8.1 \times 10^5$ | – |
| | 500 | $8.4 \times 10^5$ | 0 |
| Parental | 0 | $1.2 \times 10^6$ | – |
| | 500 | $8.4 \times 10^4$ | 93 |
| | 50 | $5.0 \times 10^5$ | 59 |
| | 5 | $1.6 \times 10^6$ | – 33 |

Cells were plated at $5 \times 10^3$ per 60-mm dish in triplicate with or without indicated amounts of LPS. After incubation at 37°C for seven days, cells were removed from the dishes with 2 mM EDTA for counting in a hemacytometer.

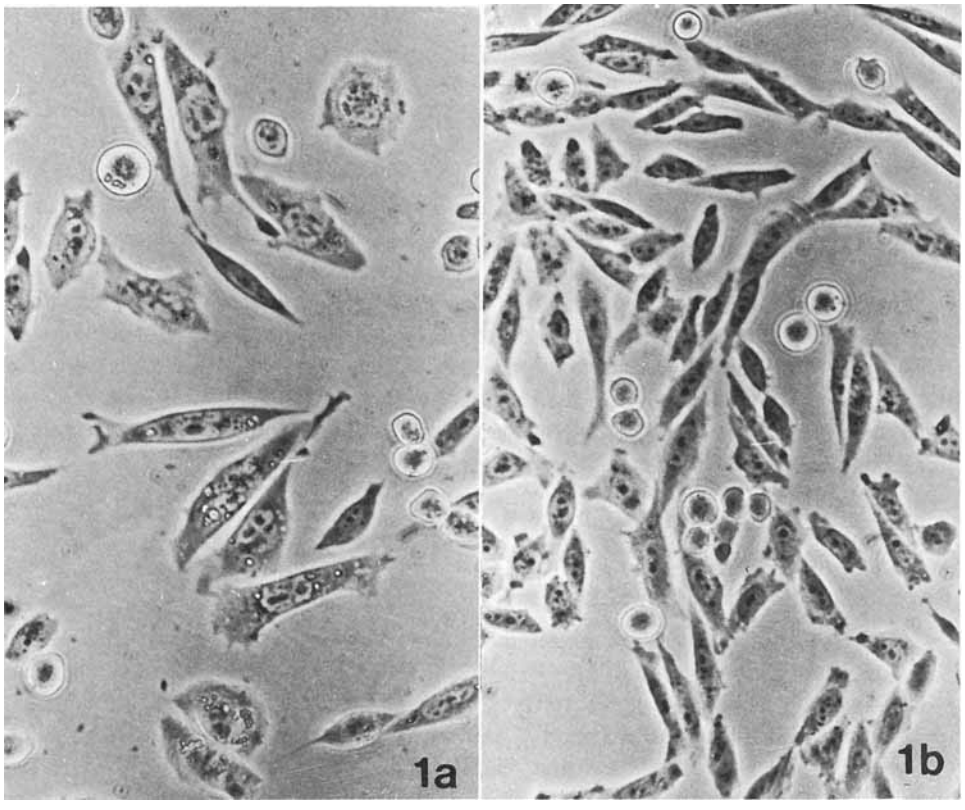


Fig. 1. Parental CHO·K1·PRO cells incubated in the presence (a) or absence (b) or 500 μg/ml LPS for four days. Phase contrast, × 200.

cells were incubated for seven days with 500 μg/ml LPS (Fig. 2). Aberrant cell shapes and numerous large cytoplasmic vacuoles could be seen in endotoxin-treated cells (500 μg/ml LPS) compared to control cells without endotoxin (Fig. 2). Ultrastructural differences, especially cytoplasmic vacuole formation, were induced in the parental CHO·K1·PRO cells during the seven-day exposure to LPS compared to untreated control cells (Fig. 3).

### Selection of an LPS-Resistant Variant Line

An LPS-resistant population was selected from parental CHO·K1·PRO cells by repeated cycles of LPS (500 μg/ml). Survivors were grown out after each selection, and after 13 cycles of LPS exposure, a resistant population was obtained that failed to respond to 500 μg/ml LPS. A clone (CHO·K1·PRO-LPS13·3) obtained from the LPS-resistant population was not growth-inhibited in the presence of 500 μg/ml LPS, while growth of the parental cells (CHO·K1·PRO) was inhibited 93% by LPS compared to untreated cells (Table I). The morphologic changes associated with LPS-mediated growth inhibition of the parental cells were not seen with the LPS-resistant clone (Figs. 4 and 5). LPS-resistant variant cells showed a more elongated morphology compared to the parental cell line (cf, Figs. 1 and 4) and exhibited postconfluent vertical growth (Fig. 6).

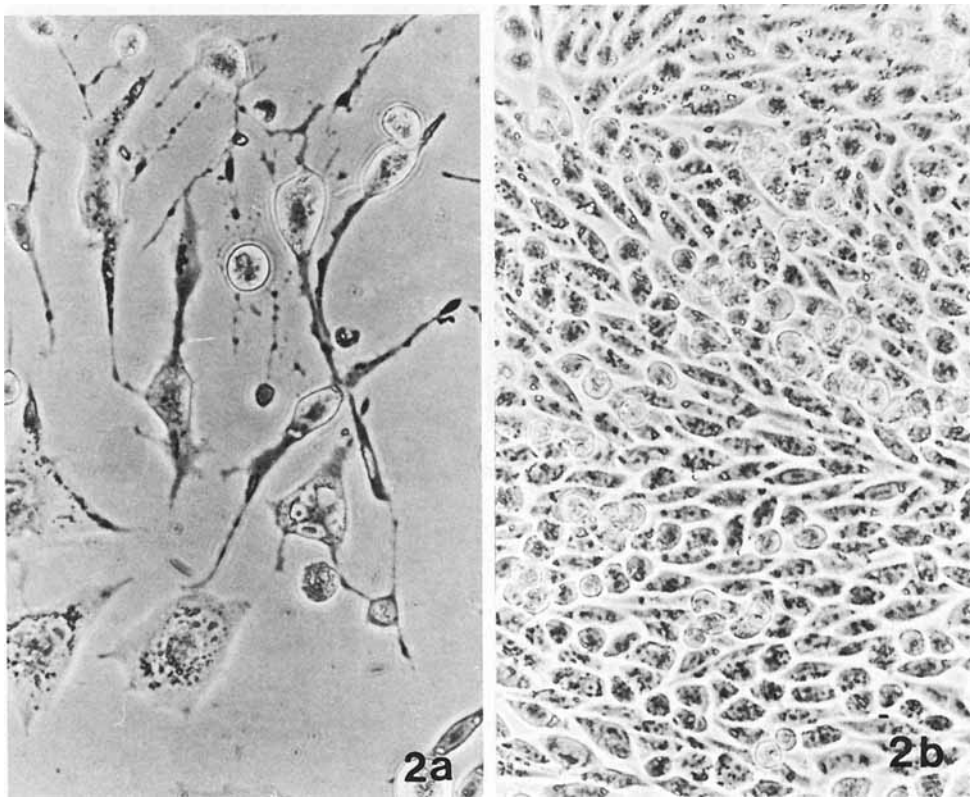


Fig. 2. Parental CHO·K1·PRO cells incubated in the presence (a) or absence (b) of 500 μg/ml LPS for seven days. Phase contrast, × 200.

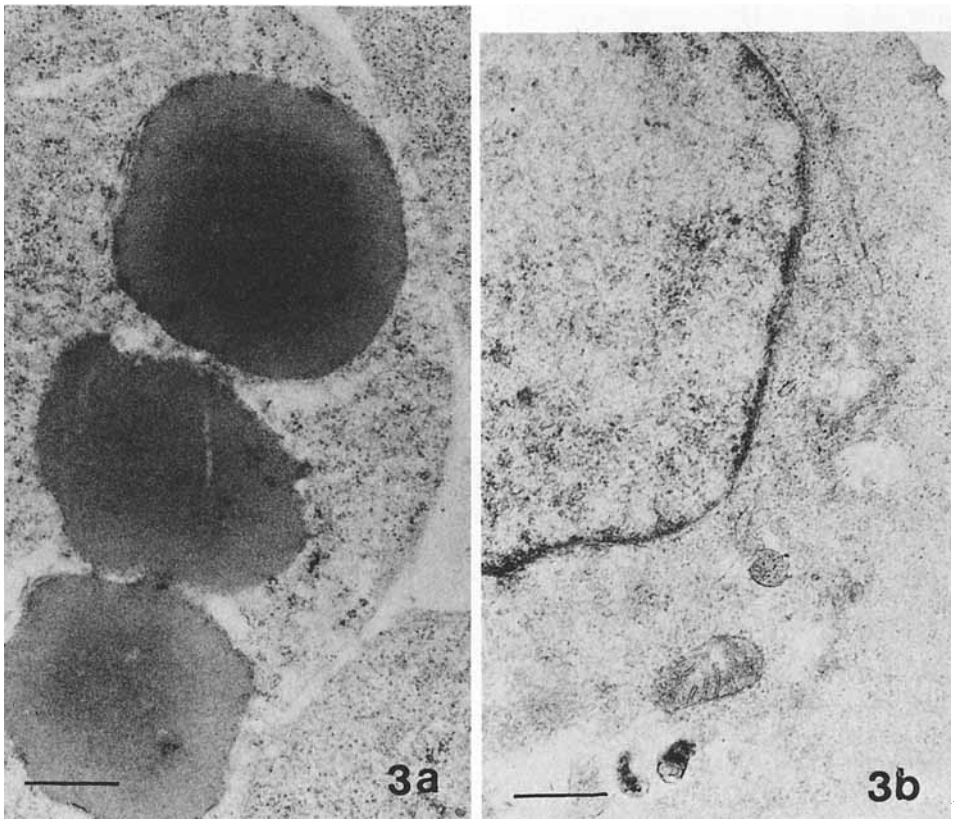


Fig. 3. Ultrastructure of parental CHO·K1·PRO cells incubated in the presence (a) or absence (b) of 500 μg/ml LPS for seven days. Bar equals 0.5 μm.

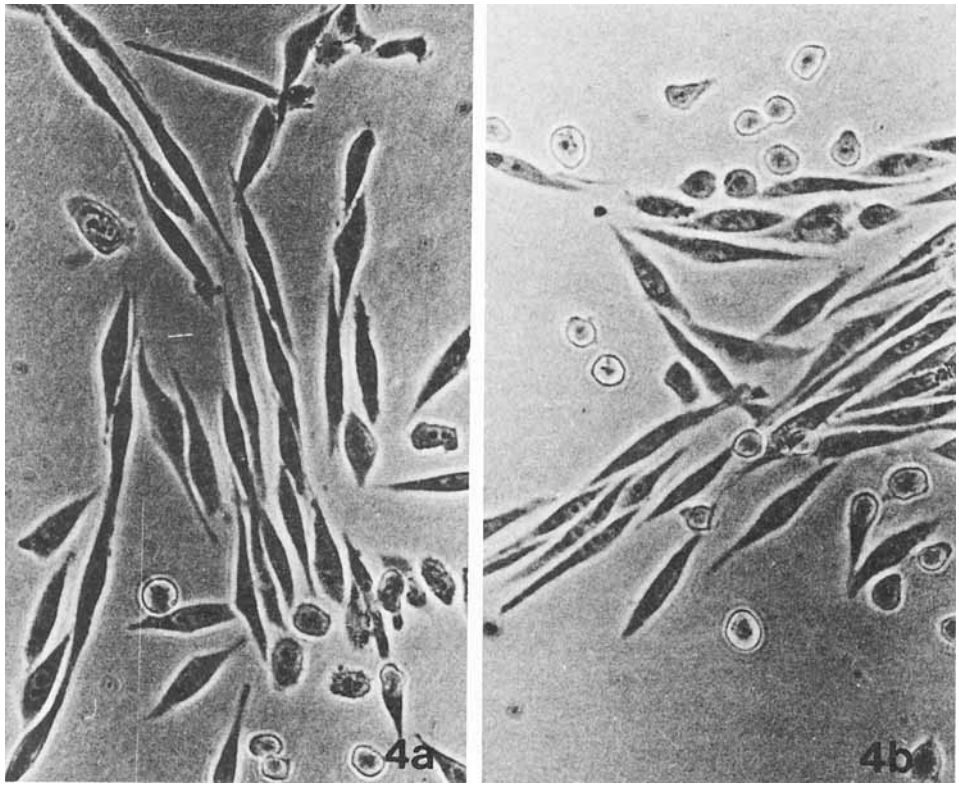


Fig. 4. LPS-resistant CHO•K1•PRO-LPS13•3 cells incubated in the presence (a) or absence (b) of 500 μg/ml LPS for four days. Phase contrast, × 200.

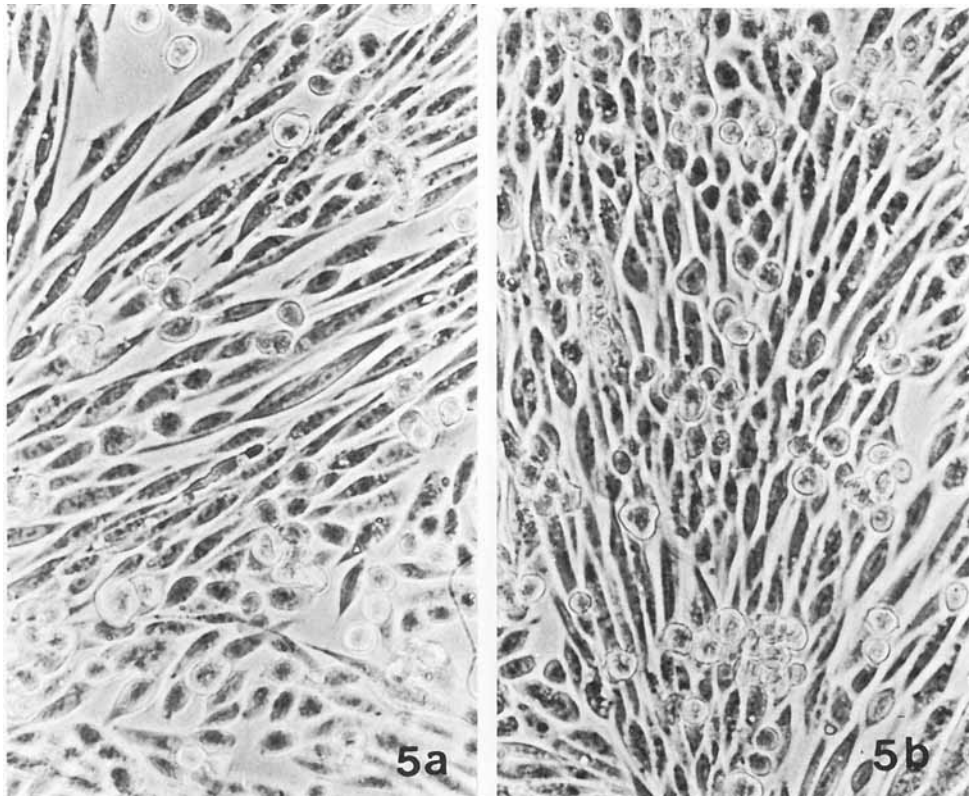


Fig. 5. LPS-resistant CHO•K1•PRO-LPS13•3 cells incubated in the presence (a) or absence (b) of 500 μg/ml LPS for seven days. Phase contrast, X 200.

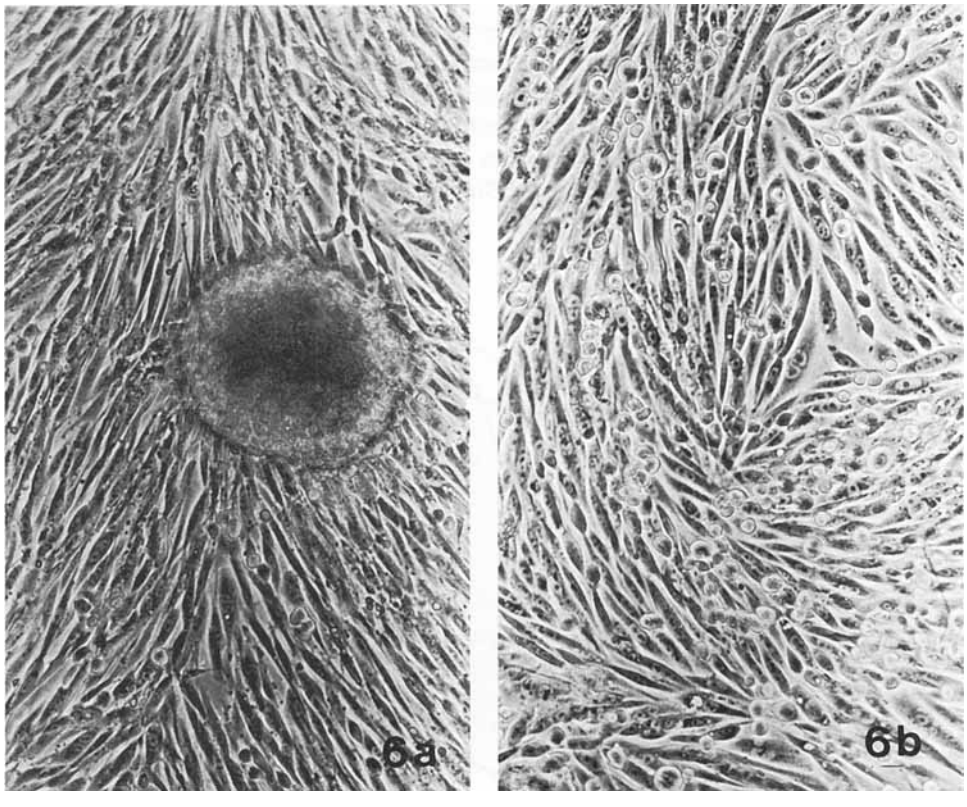


Fig. 6. Confluent cultures of LPS-resistant variant CHO•K1•PRO-LPS13-3 cells exhibiting vertical growth (a) and parental CHO•K1•PRO cells (b). Phase contrast, × 100.

### Cell Detachment

No significant difference in EDTA detachment rates were noted between sparse cultures of parental and LPS-resistant CHO·K1·PRO cells. Removal of either sparse parental or LPS-resistant variant cells was almost complete (75–85%) in 10 min, and essentially all the sparse cells were removed from the dish in 20 min (Fig. 7). Detachment of confluent cultures of LPS13·3 variant cells was similar to the removal of sparse cultures. This was in marked contrast to detachment of confluent cultures of the parental cells, where only about 35% were removed after a 1-h exposure to EDTA (Fig. 7).

### Ferritin Uptake

The endocytotic activity of parental or variant CHO·K1·PRO-LPS13·3 cell lines was quite similar in untreated control cultures, as measured by $^{125}$I-ferritin uptake (Table II). Marked differences were noted, however, in cultures containing LPS. While $^{125}$I-ferritin uptake was increased over 20-fold in parental CHO·K1·PRO cells exposed to LPS compared to controls, there was only an approximately twofold difference in uptake with LPS in the LPS-resistant cell cultures. Similar LPS/control uptake ratios were noted

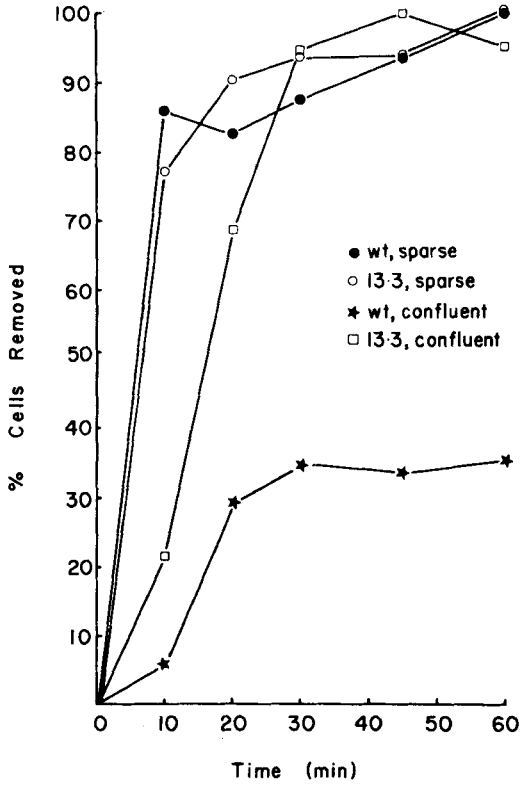


Fig. 7. EDTA detachment of parental CHO·K1·PRO and LPS-resistant CHO·K1·PRO-LPS13·3 cells. Growth medium was aspirated from sparse or confluent cell cultures grown in 35-mm tissue culture dishes, and 2 ml prewarmed 2 mM EDTA in ($Ca^{2+}$-$Mg^{2+}$)-free PBS was added to each dish. Dishes were placed on a gyratory platform at 37°C and rotated at 1 Hz. Samples were removed at various time intervals and suspended cells counted in a particle counter. Each point represents the average of triplicate determinations. Standard deviations were less than 10%.

TABLE II. $^{125}$I-Ferritin Endocytosis

| Cell type | $^{125}$I-ferritin incubation time (h) | LPS | Average cpm | Average cells | cpm/$10^6$ cells | LPS/ control |
|---|---|---|---|---|---|---|
| Parental | 3 | + | 11,757 | $2.2 \times 10^5$ | 53,441 | 27.7 |
| | | − | 6,555 | $3.4 \times 10^6$ | 1,928 | |
| | 6 | + | 13,396 | $2.2 \times 10^5$ | 60,841 | 27.3 |
| | | − | 7,596 | $3.4 \times 10^6$ | 2,234 | |
| Variant | 3 | + | 18,580 | $3.3 \times 10^6$ | 5,630 | 2.1 |
| | | − | 9,952 | $3.8 \times 10^6$ | 2,619 | |
| | 6 | + | 27,475 | $3.3 \times 10^6$ | 8,326 | 1.9 |
| | | − | 16,245 | $3.8 \times 10^6$ | 4,275 | |

Parental or variant cells were grown in the presence or absence of LPS (500 μg/ml) for seven days and $^{125}$I-ferritin (~ $1 \times 10^6$ cpm/mg) was added to cell cultures to give a final concentration of 6 mg/ml culture medium. At the end of the incubation period medium and excess ferritin were aspirated, dishes were washed once with DMEM and cells were fixed with 1.5% glutaraldehyde in phosphate buffer. Fixed cells were scraped from the dish into phosphate rinsing buffer and washed three times before counting.

whether cells were incubated for 3 h or 6 h in the presence of $^{125}$I-ferritin. That the unreleased $^{125}$I-ferritin was endocytosed was confirmed by electron microscopy. Essentially no $^{125}$I-ferritin remained cell surface bound after the three PBS washes, but ferritin was easily identified in endocytotic vesicles.

## DISCUSSION

Our results show that gram-negative bacterial LPS can have a profound cellular effect on the sensitive CHO·K1·PRO line in tissue culture. The LPS effects on CHO· K1· PRO growth rate and cell morphology that we observed are by no means universal for established lines in tissue culture. We [15] and others [16] have found that a number of tissue culture lines appear to be unresponsive to LPS in vitro.

The rather long lag period (three to four days) between addition of LPS to CHO·K1·PRO cultures and discernible cellular effects may be similar to other reports of LPS action in vitro. For example, Brailovsky et al [17] employed endotoxic glycolipids and reported in vitro growth inhibition of spontaneously or virally transformed rat embryo fibroblasts. The most marked growth inhibition was seen after incubation with endotoxin for three to four days. Kabir and Rosenstreich [6] have recently pointed out the necessity of incubating LPS and murine B lymphocyte spleen cells together for at least 72 h for maximum lymphocyte activation. It is interesting that while LPS stimulates proliferation of normal mouse spleen cells (B lymphocytes), it is inhibitory for some murine B lymphoid tumor cell lines [18].

The lag period between addition of LPS and cellular effects such as growth stimulation or inhibition, which has now been observed in several systems, probably represents more than mere binding of LPS to cells for activation. Indeed, it is known that thymus-derived (T) lymphocytes bind LPS but are not activated [5, 19]. This result may be similar to the converse finding that B lymphocytes bind concanavalin A and phytohemagglutinin but are not mitogenically stimulated [20]. Also, the lack of LPS-mediated mitogenic

activation of spleen cells from C3H/HeJ mice, which are resistant to LPS mitogenic effects, is not due to deficiency in LPS binding [5, 21].

The cell receptor(s) of biological importance for endotoxin binding is not known. A lipoglycoprotein has been isolated from human erythrocyte membranes which binds LPS, and only the protein component was shown to be involved in LPS interaction [22]. In other model systems cell surface lipids may be more important in mediating LPS effects. There has been presented [23] and tested [24] a theoretical model for endotoxin binding which predicts that insertion of the lipid portion (lipid A) of the LPS molecule into the lipid bilayer of cells is responsible for perturbation of the membrane. Recently presented evidence has shown interaction of endotoxin with phosphatides from leukocytes [25]. While attachment of LPS to cell surface receptors may be a necessary prerequisite for subsequent cellular effects of LPS, binding alone is insufficient, and there is some evidence that endotoxin association with cell nuclei of HeLa, hepatoma, and normal liver cells could be important in its action [7].

Our present report describes some of the differences found between LPS-sensitive and LPS-resistant CHO·K1·PRO lines, such as change in cell morphology and adhesive properties. Further work will be needed to directly relate these changes in the variant cells to endotoxin resistance, but the two cell lines should be useful for investigations of the mechanism of LPS effects on target cells in vitro.

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