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Polycationic Lipids Translocate Lipopolysaccharide into HeLa Cells


ABSTRACT

We have investigated the ability of LIPOFECTAMINE™, a polycationic lipid reagent used in DNA transfection, to translocate E. coli lipopolysaccharide (LPS) into HeLa cells. Although HeLa cells did not spontaneously take up fluorescein isothiocyanatelabelled LPS (FITC-LPS) from the culture medium, the cells that were co-incubated with greater than 1 g/mL FITC-LPS and LIPOFECTAMINE showed punctate fluorescence. Virtually all cells were loaded on incubation with 100 μg/mL FITC-LPS. Confocal scanning laser microscopy showed extensive FITC-LPS loading in the cytoplasm of HeLa cells, but no label was evident in the nuclear regions of these cells. Loading with LPS for up to six hours had no effect on the viability of HeLa cells, beyond the 30% reduction in live cells that is attributable to the toxic effect of LIPOFECTAMINE itself. In contrast to cells treated with etoposide for six hours, LPS-loaded cells did not display apoptotic bodies. Exposure of cells to 4β-phorbol 12-myristate 13-acetate led to the induction of the immediate early gene c-fos and resulted in an enhanced c-Fos signal, detected by Western blot analysis. In contrast, LPS loading did not alter the c-Fos expression in HeLa cells. The loading of LPS into HeLa cells by means of polycationic lipids results in relatively low acute toxicity, as judged from cell viability, morphology and c-fos expression. Therefore, our method appears well suited to the study of acute actions of LPS in the intracellular compartment of mammalian cells.

INTRODUCTION

Lipopolysaccharide (LPS, endotoxin) consists of an O-specific polysaccharide chain, a core oligosaccharide and a fatty acid-bearing component termed Lipid A, which displays most of the endotoxic properties of the complete molecule (12). LPS alters mammalian cell function by binding to cell surface receptors such as the CD14 molecule. Following LPS binding, extensive signal transduction occurs, including activation of the extracellular signal-regulated kinase (ERKs) superfamily of enzymes (4). Phosphorylated ERKs translocate to the nucleus and activate transcription factors such as the ternary complex factor, Elk-1. Elk-1 is important in the pathway leading to transcription of many immediate early (IE) genes, including c-fos (14). However, LPS can directly activate two important intracellular enzymes, ERK-2 and protein kinase C (PKC), even in a cell-free system devoid of known endotoxin receptors (3). Furthermore, LPS accumulates inside cells during the replication of invasive bacteria (7) and can enter some cell types by transmembrane diffusion (10).

These studies suggest that LPS may also influence cell function when present in the intracellular compartment of mammalian tissues. To test this hypothesis, techniques permitting the reliable translocation of endotoxin into a well-characterized mammalian cell line are desirable. Here, we describe the successful use of polycationic lipids, widely used for DNA transfection, for the translocation of E. coli LPS into the cytoplasm of HeLa cells. Because LPS activates ERK-2 and PKC in vitro, and both of these enzymes are known regulators of c-fos expression, the ability of translocated LPS molecules to enhance levels of c-Fos in HeLa cells was also examined.

MATERIALS AND METHODS

HeLa cells were cultured in a 37°C, 5% CO₂ environment using DMEM...
with 10% heat-inactivated fetal bovine serum (FBS), (Life Technologies, Gaithersburg, MD, USA). Cells were sub-cultured to about 70% confluency in six-well plates containing DMEM + 10% FBS and then were serum-starved for 36–48 h by incubation in serum-free DMEM. LIPOFECTAMINE reagent is a 3:1 (wt/wt) mixture of the lipids 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminium-trifluoroacetate (DOSPA) and dioleoyl phosphatidylethanolamine (DOPE) (Life Technologies). For loading of LPS into cells, 7 µL LIPOFECTAMINE were added to DMEM, mixed with 0.5, 1, 10 or 100 µg of LPS (E. coli 0127:B8; Sigma, St. Louis, MO, USA) in DMEM and incubated at 21°C. This mixture was then added to the existing serum-free DMEM bathing the cells. Cells were incubated at 37°C for 30 min, 1, 2, 4 or 6 h before the washout of reagents and fixation or protein isolation. The following controls were performed: (i) cells treated with the tumor promoter and known c-fos inducer, 4β-phorbol 12-myristate 13-acetate (PMA, 100 ng/mL) as a positive control, (ii) serum-free DMEM as the control medium, (iii) LPS alone and (iv) LIPOFECTAMINE alone.

For the visualization of LPS loading, cells on 18 mm coverslips were incubated at 37°C in control DMEM, LIPOFECTAMINE alone, fluorescein isothiocyanate LPS (FITC-LPS) alone or LIPOFECTAMINE plus FITC-LPS for periods of 2.5, 5 or 7 h. The cells were washed, fixed in 4% paraformaldehyde and examined by indirect epifluorescence microscopy and confocal scanning laser microscopy (Bio-Rad Laboratories, Hercules, CA, USA). Three-dimensional reconstructions of scanned cells were obtained using the NIH-Image and Adobe® Photoshop® software packages.

Standard Western blotting methods were used to detect transcription of c-fos in control and endotoxin-loaded cells. Electroblotted nitrocellulose membranes were incubated with anti-c-Fos rabbit antibody (SC-52G; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1 000 dilution. The blots were incubated for 1 h with anti-rabbit, peroxidase-conjugated secondary antibody and visualized using an ECL™ detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Cell viability was measured using the MTT dye reduction assay (8). Two hours before the completion of the loading, 492 µg/mL MTT in PBS were added to each well. Cells were lysed in 1 mL of lysis buffer [20% (wt/vol) SDS in a 1:1 solution of N,N-dimethyl formamide and distilled water, pH 4.7] and incubated overnight at 37°C before measuring the absorbances at 570 nm. Cell morphology was recorded by phase-contrast microscopy using an Olympus PM-6 camera at 300× magnification.

RESULTS

Cells incubated in control medium exhibited a low level of fluorescence (Figure 1A). This background signal was not altered by exposure of the cells to LIPOFECTAMINE alone (Figure 1B).
or to 100 µg/mL FITC-LPS alone (Figure 1C). This result agrees with previous reports that HeLa cells do not normally take up LPS from the external medium (7). Cells co-incubated with LIPOFECTAMINE and 0.5–1.0 µg/mL FITC-LPS also displayed little evidence of fluorescence above background levels. However, when the concentration of FITC-LPS was increased to 10 µg/mL, a punctate pattern of enhanced intracellular fluorescence was observed (Figure 1D). When 100 µg/mL FITC-LPS were applied, virtually all of the cells exhibited enhanced fluorescence. This high rate of translocation is consistent with the efficient entry of plasmids into HeLa cells exposed to the reagent (15).

Following 2.5 h incubation in 100 µg/mL FITC-LPS and LIPOFECTAMINE, cells exhibited a largely submembranous distribution of enhanced fluorescence, as detected by confocal-scanning laser microscopic methods (Figure 2a). However, following 5 and 7 h incubation under these conditions, enhanced staining was evident throughout the cytoplasm of treated HeLa cells (Figures 2b and 3b). Vertical re-slicing of cells loaded for 2.5–7 h showed the apparent exclusion of FITC-LPS from the cell nuclei, despite the extensive labeling present in the cytoplasm (Figure 2, a–c, inserts).

No significant differences in cell viability were seen between cells treated with serum-free DMEM, LIPOFECTAMINE, 100 µg/mL LPS (E. coli 0127:B8), LIPOFECTAMINE + LPS or the DNA synthesis inhibitor etoposide (100 µM) for 2 h (Figure 3a). After incubation for 4 h, treatment with LIPOFECTAMINE or with LIPOFECTAMINE + LPS reduced viability by 35% ± 3% and 33% ± 3%, respectively, relative to treatment with serum-free DMEM (Figure 3b). These reductions were not significantly different. After incubation for 6 h, the reductions in cell viability from treatment with LIPOFECTAMINE or LIPOFECTAMINE + LPS remained very similar to each other and showed no further increase over values obtained at 4 h (Figure 3c).

Cell death resulting from the treatment of HeLa cells with etoposide also became significant after 6 h incubation in this agent (Figure 3c). Treatment with etoposide also triggered the appearance of apoptotic bodies in many cells (Figure 4b). In contrast, this feature was not characteristic of cells treated with LIPOFECTAMINE, LPS or LIPOFECTAMINE + LPS for 6 h. Rather, a minority of cells in these cultures (Figure 4, d and e) exhibited an increase in vacuolization, cell rounding and blebbing, relative to untreated controls (Figure 4a).

HeLa cells were responsive to c-fos up-regulation, as shown by the enhanced c-Fos signal seen after 2 h in the presence of 100 ng/mL PMA (Figure 5b). However, loading of cells with LPS for periods of 0.5, 2 or 4 h failed to enhance c-Fos levels over values obtained by adding control serum-free DMEM alone (Figure 5). Similarly, neither LIPOFECTAMINE nor LPS applied alone caused a significant change in c-fos expression, when compared to the effects of the control medium (Figure 5).

**DISCUSSION**

Primary cells transfected with DNA samples contaminated by LPS exhibit low transfection efficiency, blebbing or apoptosis (2). These effects have been attributed to the entry of LPS into the host cell cytosol during transfection (1). However, the possibility that LPS could not be ruled out in these studies. The present results show that LPS is ef-

![Figure 2. Confocal scanning laser micrographs showing the intracellular distribution of FITC-LPS loaded into HeLa cells. Cultures were incubated in 100 µg/mL FITC-LPS + LIPOFECTAMINE for (a) 2.5 h, (b) 5 h and (c) 7 h. Lines indicate where stacks were vertically re-sliced to show the distribution of FITC-LPS label in the vertical plane surrounding cell nuclei (inserts).](image-url)
ficiently translocated into mammalian cells by cationic lipid reagents.

Polycationic lipid/DNA complexes are taken up by receptor-mediated endocytosis, appear in early endosomes and are released into the cytoplasm (6). LPS can adopt a micellar form that is similar in size and negative charge distribution to large DNA molecules (1). Therefore, it is conceivable that LPS translocation may also be primarily by means of receptor-mediated endocytosis. Polycationic lipids also fuse with the nuclear membrane, giving rise to extensive intranuclear reticula (6). Here, however, no evidence for delivery of LPS into the nuclei of HeLa cells was observed.

Liposomal vectors made from cationic lipids and the fusogenic agent DOPE are toxic to a variety of phagocytes (5) and alter metabolism in other cell types (11). Here, HeLa cell viability declined significantly after a 4 h exposure to LIPOFECTAMINE. However, no additional toxicity was evident when LPS was present in either the extracellular or intracellular compartment. The lack of cell death in the presence of extracellular LPS was consistent with the fact that HeLa cells lack CD14 endotoxin recep-

Figure 4. Phase contrast photomicrographs of HeLa cells incubated for 6 h. (a) Serum-free DMEM (Control), (b) 100 µM etoposide, (c) 100 µg/mL LPS, (d) LIPOFECTAMINE and (e) 100 µg/mL LPS + LIPOFECTAMINE.

Figure 5. c-Fos levels in LPS-loaded HeLa cells. Cultures were incubated for (a) 0.5 h, (b) 2 h or (c) 4 h after addition of the following media: 100 ng/mL PMA (PMA); serum-free DMEM (SF-D); LIPOFECTAMINE (LFA); 100 µg/mL LPS (LPS); 100 µg/mL LPS + LIPOFECTAMINE (LL100); 10 µg/mL LPS + LIPOFECTAMINE (LL10). Western blot densities were normalized to the highest PMA signal obtained within each trial. Values shown are mean ± SEM for 3–7 independent trials. Asterisks in Figure 5b indicate PMA treatment significantly enhanced c-fos expression compared to treatment with control medium (P < 0.05, ANOVA).
ors (7). The inability of intracellular LPS molecules to enhance c-fos expression in HeLa cells was unexpected.

Two principal mechanisms may account for this result. First, intracellular LPS may remain complexed to polycationic lipids or may be unable to escape from the endosomal compartment of HeLa cells. Second, LPS shows marked toxicity, as judged from cell viability, morphology and c-fos expression, the latter being commonly activated by many stress pathways (14). Therefore, our method appears well suited to the study of acute actions of LPS in the intracellular compartment of mammalian cells.

REFERENCES


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Simple Method for Preparation of Fluor/ Hapten-Labeled dUTP


ABSTRACT

Many projects, such as multiplex-fluorescence in situ hybridization (M-FISH) karyotyping, require the use of relatively large amounts of multiple fluor- or hapten-labeled nucleotides for the preparation of DNA probes. Such a requirement makes these experimental approaches prohibitively expensive for many researchers. The cost of such nucleotides can be reduced approximately 99% by purchasing the chemical precursors, fluor or hapten succinimidyl esters and 5-(3-aminoallyl)-2′-deoxyuridine 5′triphosphate (AA-dUTP), and performing the simple coupling/purification described here. It is possible to finish four to ten different fluor/hapten dUTP preparations of 2.5 μM scale within a 24 h period. The reagent cost for each preparation ranges from $33–$237 per μM, depending on the fluor/hapten. This laboratory uses such nucleotide preparations to prepare FISH probes by nick translation or PCR amplification.

INTRODUCTION

Before the mid-1980s, nucleic acid probes were labeled isotopically and detected after hybridization by autoradiography. The use of non-isotopically labeled probes has, for numerous reasons beyond the safety issue (2), replaced isotopically labeled probes has, for numerous reasons beyond the safety issue (2), replaced isotopically labeled probes for hybridization. In particular, the ability of fluor-labeled probes to differentiate a number of different hybridization targets simultaneously has proven to be extremely powerful because each fluor can be differentiated by its spectral signature. Using multiple fluoros, karyotypes of human metaphase chromosomes can be obtained with a single hybridization (3,4).

The choice of fluor/hapten for labeling depends on the following factors. They must be available as succinimidyl esters or their homologues. Each fluor used in a given experiment must have excitation and emission spectrum...