Bacterial Lipopolysaccharide Suppresses the Production of Catalytically Active Lysosomal Acid Hydrolases in Human Macrophages

David W. H. Riches and Peter M. Henson

Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206. Address correspondence to Dr. Riches.

Abstract. Sub-microgram quantities of bacterial lipopolysaccharide (LPS) have been found to substantially reduce the intracellular catalytic activities of three representative lysosomal enzymes (namely, acid phosphatase, hexosaminidase, and β-glucuronidase) in human monocyte-derived macrophages. This response was not associated with a concurrent increase in enzyme catalytic activity in the culture supernatant, and hence, could not be explained by mobilization of preformed material. By conducting experiments in the presence and absence of indomethacin, a cyclooxygenase inhibitor, the reduction in lysosomal enzyme catalytic activities was shown not to be dependent on the ability of LPS to induce prostaglandin E_2 production. The response was not found to be the result of a more generalized LPS-dependent reduction in the ability of the cells to synthesize protein, since the presence of LPS in macrophage cultures did not appreciably affect the amount of [^{35}S]methionine incorporated into total cellular proteins. A kinetic analysis of the effect of LPS on the down-regulation of enzyme catalytic activities indicated that this was an early response of the cells to LPS exposure. An investigation of the effects of blockade of enzyme catabolism (using the lysosomotropic weak-base, methylamine) indicated that the reduction of catalytic enzyme activities in response to LPS was probably due to a decreased rate of production of active product, rather than an enhanced rate of enzyme catabolism. This suggestion was confirmed by experiments in which the synthesis of pro-hexosaminidase (measured by biosynthetic labeling with [^{35}S]methionine and specific immunoprecipitation of labeled pro-hexosaminidase) was found to be reduced by 42% after a 24-h exposure to LPS (although the synthesis of complement component C3 was stimulated by a factor of 4.5). It is suggested that the ability of LPS to regulate the functional expression of protein products contributes to changes in the overall functional status of these cells in response to this bacterial product.

THE effects of bacterial lipopolysaccharides (LPS)[1] on cells of the mononuclear system are many and varied. At a general level, these include the induction of cytotoxicity towards tumor cells (1, 5, 9), and the stimulation of Fc receptor-mediated erythrophagocytosis (7). More specifically, LPS has been shown to stimulate the production of prostaglandin E_2 (15), to prime macrophages for increased stimulus-dependent superoxide anion production (23), as well as increasing the synthesis of a number of protein products such as tissue transglutaminase (19), interleukin 1 (29), fibroblast growth factor(s) (10, 17), collagenase (37), a pro-coagulant activity (28), and interferon (14). However, some of these responses (notably, the induction of collagenase secretion and a cellular pro-coagulant activity) have been shown on the basis of antagonism of these responses to LPS by the cyclooxygenase inhibitor indomethacin to be dependent, at least in part, on the ability of LPS to induce prostaglandin E_2 synthesis.

Since all of these changes in the protein synthetic phenotype of the macrophage occur as a result of exposure to LPS, it has been suggested by several investigators that the expression of these protein products is, in some as yet poorly defined way, frequently required for the heightened functional activities that LPS-stimulated macrophages are engaged in. By the same token, however, it can also be argued that the acquisition of heightened functional states might be associated with the repression of functional activities of proteins that are not required, or are inhibitory to the activity of the LPS-stimulated macrophage. In a previous communication (25), we provided evidence based on the separation of biosynthetically labeled macrophage proteins on SDS polyacrylamide gels, that the synthesis of a number of unidentified proteins was down-regulated during exposure of the cells to LPS. Werb and Chin (38) have provided data that apolipoprotein E

1. *Abbreviations used in this paper:* LPS, lipopolysaccharide; TCA, trichloroacetic acid.

represents one of the proteins whose synthesis is strongly inhibited in LPS-stimulated macrophages. In this paper, we will show that LPS also down-regulates the functional expression of a number of lysosomal acid hydrolases in LPS-stimulated human monocyte-derived macrophages. Furthermore, we will show that mechanistically this process is not dependent on prostaglandin production.

Materials and Methods

Materials

Lipopolysaccharides *Escherichia coli* 0111:B4 and 055:B5 were obtained as Westphal extracts from List Biological Labs Inc., Campbell, CA. *E. coli* K235, purified by the McKintyre procedure (18a). was a gift from Dr. D. C. Morrison, Emory University, Atlanta, GA. Percoll, *p*-nitrophenyl phosphate, *p*-nitrophenyl-β-D-glucuronide, *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucosamine, lactic acid, NAD^+, and pyruvic acid were obtained from Sigma Chemical Co., St. Louis, MO. EN^{3}HANCE, and L-[^{35}S]methionine were purchased from New England Nuclear, Boston, MA. Medium 199, Hanks' balanced salt solution and RPMI 1640-Selectamine Kits were obtained from GIBCO, Santa Clara, CA. 24-well tissue culture plates (3424 Mark II) were obtained from Costar, Cambridge, MA. In our experience, these were the only tissue culture plates to which the human monocyte–derived macrophages remained strongly adherent throughout the experimental period. Immunoprecipitin, acrylamide, and *bis*-acrylamide were purchased from Bethesda Research Laboratories, Gaithersburg, MD, while anti-human C3 IgG was obtained from Atlantic Antibodies, Scarborough, ME.

Cells

Human monocyte-derived macrophages were obtained using a variation of the method described by Musson and Henson (20). Citrated whole blood from healthy volunteers was centrifuged at 300 *g* for 20 min and the platelet-rich plasma removed. The mixed leukocytes and erythrocytes were resuspended to the original volume, and dextran (500,000-mol-wt) was added to a final concentration of 0.6% (wt/vol). After sedimentation at unit gravity for 30 min, the leukocyte-rich supernatant was aspirated and the cells were collected by centrifugation at 300 *g* for 10 min. After resuspension in platelet-poor plasma, the mononuclear cells were separated from the granulocytes and residual erythrocytes by centrifugation through a discontinuous plasma-Percoll gradient (13). The mononuclear cells were collected, washed three times in phosphate-buffered saline, and suspended in Hanks' balanced salt solution containing 0.1% (vol/vol) autologous serum. Mononuclear cells were distributed into 16-mm diameter wells of 24-well tissue culture plates at a density of 4×10^6 cells/well in a volume of 1 ml. After 1–2 h of adhesion at 37°C, the non-adherent cells (mainly lymphocytes) were removed by gently rinsing the monolayers three times with Hanks' balanced salt solution. The monocytes were then cultured in Medium 199 containing 5% (vol/vol) autologous serum for 5 d to facilitate their maturation into macrophages. The medium was changed once on day 3 and additional detached cells were removed.

Before exposure to LPS, the macrophage monolayers were rinsed twice with Hanks' balanced salt solution before being exposed to LPS in serum-free Medium 199. At the end of the experiment, the culture supernatants were removed and the macrophage monolayers lysed with 1 ml of 0.1% (vol/vol) Triton X-100 containing 0.9% (wt/vol) NaCl. The supernatants were centrifuged at 500 *g* for 5 min to pellet any detached cells and these were combined with the Triton X-100 lysate. Additional macrophage monolayers were treated with Zapoglobin and the released nuclei counted on a Model ZH Coulter counter to quantify the number of adherent cells per well.

Enzyme Assays

Culture supernatants and cell lysates were analyzed for activities of the lysosomal enzymes acid phosphatase, hexosaminidase, and β-glucuronidase, and the cytoplasmic enzyme lactate dehydrogenase.

The lysosomal enzyme activities were determined in 96-well microtiter plates by mixing 50 μl of sample with 100 μl of pre-warmed buffered substrate. After incubation at 37°C, the reactions were stopped by the addition of 100 μl of 0.5 M borate buffer, pH 10.6. Hexosaminidase activity was detected using *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucosamine (2 mM) in 0.1 M citrate/phosphate buffer, pH 4.3, with an incubation time of 30 min; acid phosphatase was assayed with *p*-nitrophenyl phosphate (5 mM) in 0.1 M citric acid/citrate buffer, pH 4.3, and was incubated for 15 min; and β-glucuronidase activity was determined using *p*-nitrophenyl-β-D-glucuronic acid in 0.1 M sodium acetate/acetic acid buffer, pH 4.4, with an incubation time of 8 h. The *p*-nitrophenol released by the enzyme-dependent hydrolysis of the separate substrates was quantified spectrophotometrically using a Biotek Instruments Enzyme ImmunoAssay plate reader fitted with a 405-nm wavelength filter. Absorbances at 405 nm were converted into μmol substrate cleaved using the Beer-Lambert Law and the molar extinction coefficient for *p*-nitrophenol ($E = 18{,}700$). Units of specific activity are defined as μmol substrate cleaved/h per 10^6 cells.

Lactate dehydrogenase was assayed by measuring the oxidation of lactic acid to pyruvic acid in the presence of NAD^+ at pH 9.0. Pyruvic acid was quantified by coupling it with 2,4-dinitrophenylhydrazine (0.5 mg/ml in 1 M HCl) and reading the absorbance at 450 nm after alkalinization of the solution with 1 M NaOH (4).

Biosynthetic Labeling with [^{35}S]Methionine

The total protein synthesizing capacity of the cells was determined by biosynthetic labeling with [^{35}S]methionine.

Macrophage monolayers were incubated for various periods of time in the presence and absence of LPS (500 ng/ml). Immediately before labeling, the cells were rinsed twice with Hanks' balanced salt solution and then incubated with 200 μCi of [^{35}S]methionine dissolved in methionine-free RPMI 1640 at 37°C for 60 min. The supernatants were then removed, and the cell monolayers rinsed twice with ice-cold Hanks' balanced salt solution. The cells were lysed in an ice-cold detergent/inhibitor cocktail composed of 0.5% (vol/vol) Triton X-100, 0.25% (wt/vol) sodium deoxycholate, 10 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride dissolved in phosphate-buffered saline, pH 7.6.

The total amount of [^{35}S]methionine incorporated into cellular proteins during the 1-h pulse was determined by trichloroacetic acid (TCA) precipitation in filter paper discs. 10-μl aliquots of the cell lysates were spotted onto Whatman grade 3 filter paper discs (2.3-cm diameter). After drying, the discs were incubated in a solution of 10% (wt/vol) TCA containing 5 mM methionine for 10 min. The solution was then removed and replaced with a solution containing 5% (wt/vol) TCA and 5 mM methionine. The discs were then boiled for 10 min, before being rinsed successively with 5% (wt/vol) TCA and 5 mM methionine, absolute ethanol, ethanol/acetone (50/50), and absolute acetone. After drying, the discs were placed in scintillation vials to which were added 130 μl of distilled water and 700 μl of TS-2 tissue solubilizer. After shaking for 2 h at room temperature, 10 ml of scintillation fluid were added to each vial, before counting the vials for radioactivity. Counts per minutes were converted to disintegrations per minute by determining the amount of quench in each sample.

Immunoprecipitation of Hexosaminidase and C3

The amount of [^{35}S]methionine incorporated into pro-hexosaminidase and pro-C3 was determined by immunoprecipitation with specific antibodies, followed by analysis by SDS PAGE, localization of the radioactive bands by fluorography, and determination of the incorporation of [^{35}S]methionine into these two proteins by excision of the radioactive bands, digestion in hydrogen peroxide, and counting in a liquid scintillation spectrometer.

[^{35}S]Methionine-labeled macrophage lysates (obtained as described above) were initially pre-cleared by incubation at 4°C for 30 min with 200 μl of Immunoprecipitin. The Immunoprecipitin was then pelleted and discarded. SDS was then added to the lysate to a final concentration of 0.1% wt/vol, followed by 5 μl of anti-human C3 IgG. The mixtures were then incubated overnight at 4°C. The C3 containing immune complexes were isolated by incubation at 4°C for 30 min with 100 μl of Immunoprecipitin (which had previously been boiled in mercaptoethanol and SDS according to the manufacturers' recommendation). The immunoprecipitates were washed once in phosphate-buffered saline containing 1% (wt/vol) SDS, 1% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, and 5 mg/ml bovine serum albumin (BSA), and an additional five times in the washing buffer without BSA. Finally, the immunoprecipitates were boiled in Laemmli sample buffer, and subjected to SDS PAGE through a 7.5% acrylamide gel under reducing conditions using the buffer system of Laemmli (16). The amount of [^{35}S]methionine-labeled pro-hexosaminidase in the macrophage lysates was determined by immunoprecipitation with 2 μl of rabbit anti-human placental hexosaminidase A antiserum. This latter antiserum was prepared in our laboratory using highly purified human placental hexosaminidase A as immunogen. The hexosaminidase was purified using the method of Hasilik and Neufeld (12). The antiserum was found to precipitate >85% of the hexosaminidase catalytic activity contained in ~10^6 human monocyte–derived macrophages when used at a dilution of 1:2,000. Immunoprecipitation of [^{35}S]-labeled hexosaminidase was routinely conducted at a dilution of 1:500. Immunoprecipitation of macrophage mono-

layers that had been pulse labeled with 200 μCi of [^{35}S]methionine for 1 h, with this antiserum, followed by analysis of the labeled material by SDS PAGE through a 10% acrylamide gel under reducing conditions, and fluorography, revealed two components in the molecular weight range of 63,000–68,000, which were presumed to be the precursors of the α- and β-subunits of hexosaminidase. In pulse-chase experiments, these components disappeared and were replaced by two bands at ~53,000 and 30,000, which closely correspond to the published values for the molecular weights of the mature α- and β-subunits of hexosaminidase A (12). In addition, a single contaminant of ~185,000-mol-wt was detected in pulse-labeled macrophage monolayers. In pulse–chase experiments, this contaminant was found to chase to a two-chain product with reduced molecular weights of ~110,000 and 75,000. The identity of this component is believed to be that of complement component C3 since it could be completely removed by immunoprecipitation with anti-C3 IgG. Accordingly, [^{35}S]methionine-labeled macrophage monolayers were immunoprecipitated with anti-C3 IgG before immunoprecipitation of the labeled hexosaminidase. The anti-C3 IgG that was used in these studies was purchased from Atlantic Antibodies, and was found to be monospecific for C3. SDS PAGE was conducted through 10% acrylamide gels under reducing conditions. After fixation, the gels were immersed in EN^{3}HANCE, dried, and exposed at −70°C to Kodak XAR-5 film. The radioactive bands were then excised, digested for 18 h at 56°C in 15% (vol/vol) hydrogen peroxide, and then counted in a liquid scintillation counter after the addition of 10 ml "Scintiverse" scintillation cocktail. In addition, sections of dried gel, similar in area to the radioactive bands, were excised from each lane and digested in hydrogen peroxide to provide appropriate background counts. These were subsequently subtracted from the counts obtained from the radioactive bands. Counts per minute were corrected to disintegrations per minute by comparing the automatic quench control number with a standard quench curve constructed with [^{14}C]benzoic acid. This comparison is possible since the energy emission specta and intensities for [^{14}C] and [^{35}S] are nearly identical. Thus, at identical intensities, the mean particle energy for [^{14}C] is 0.050 MeV, while that of [^{35}S] is 0.0492 MeV (3).

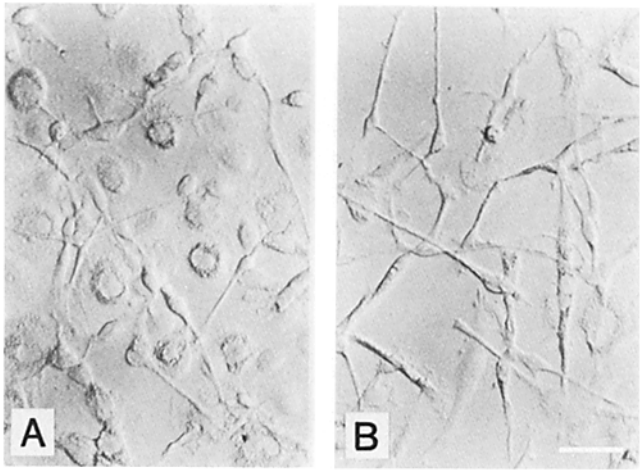

Figure 1. Changes in macrophage morphology after exposure of the cells to LPS (*E. coli* 0111:B4; 500 ng) for 72 h at 37°C. (*a*) Control cells; (*b*) LPS-treated cells. Hoffman interference optics. Bar, 12.5 μm.

Results

Morphology

Exposure of macrophages to LPS (0111:B4, 500 ng) for 72 h resulted in a profound change in the configuration of the cells. These morphological changes were characterized by bidirectional pseudopod extension associated with a narrowing of the nuclear transect and a tendency to form aggregates of four or more cells (Fig. 1). These changes in morphology, which initially became apparent after a 24–36-h exposure to LPS, gave the macrophages a markedly polarized appearance in comparison with unstimulated controls.

Concentration–Response Characteristics

The effect of LPS on the cellular and culture supernatant activities of three representative lysosomal enzymes, acid phosphatase, hexosaminidase, and β-glucuronidase, was investigated by exposing human macrophages to increasing concentrations of LPS (0111:B4; 5–500 ng/ml) for 72 h at 37°C. The results, illustrated in Fig. 2, show that the cellular-specific activities of all three lysosomal enzymes were reduced by LPS. Acid phosphatase showed the greatest decline in specific activity with a significant effect ($P < 0.05$) being seen with only 5 ng/ml of LPS; while the maximum reduction was seen at ~100 ng/ml of LPS. A significant reduction in specific activities of hexosaminidase and β-glucuronidase was achieved only with concentrations of LPS >50 ng/ml. The reduction in cellular and secreted specific activities was not associated with decrements in cell viability as evidenced by the failure to detect significant levels of the cytoplasmic enzyme lactate dehydrogenase above control values in culture supernatants (data not shown).

Effect of Different LPS Preparations

The effect of three different *E. coli* Westphal LPS preparations (0111:B4; 055:B5, and K235) on the reduction of cellular specific activities of acid phosphatase and hexosaminidase in

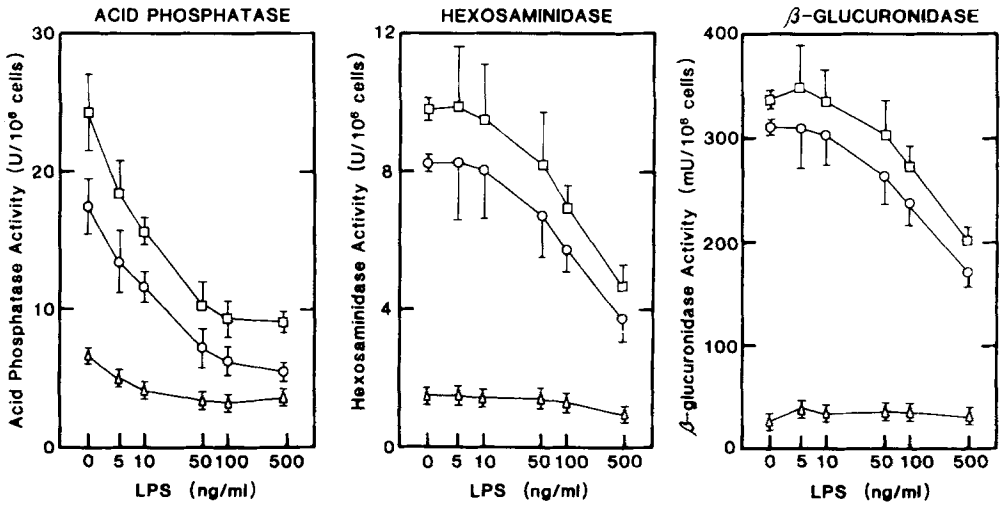

Figure 2. Concentration-dependent reductions in the cellular (○), secreted (△), and total (□) culture catalytic activities of acid phosphatase, hexosaminidase, and β-glucuronidase in macrophages exposed to LPS (*E. coli* 0111:B4; 5–500 ng/ml) for 72 h at 37°C. Cells were derived from a single donor.

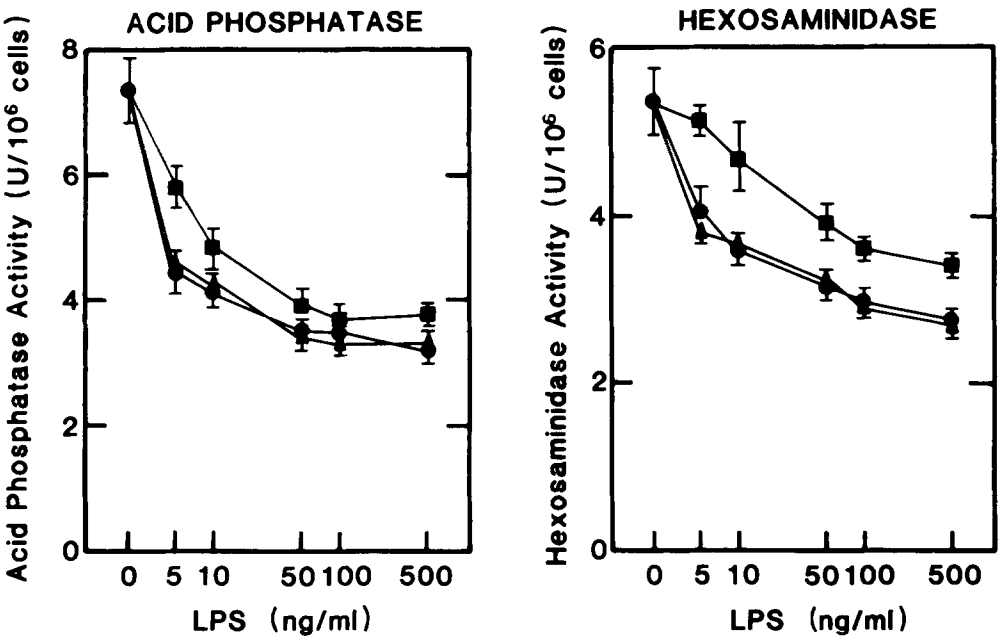

Figure 3. Concentration-dependent reductions in the cellular activities of acid phosphatase and hexosaminidase in response to three different *E. coli* LPS preparations. (●) 0111:B4; (■) 055:B5; (▲) K235. The macrophages were derived from a single donor and were exposed to the LPS preparations for 72 h at 37°C.

macrophages exposed to 5–500 ng of LPS for 72 h is illustrated in Fig. 3. It will be seen that K235 and 0111:B4 were equally effective in reducing the cellular activities of these two lysosomal enzymes. The LPS 055:B5 preparation reduced intracellular acid phosphatase and hexosaminidase activities to a similar degree as K235 and 0111:B4 at high concentrations (>100 ng/ml) but was somewhat less effective at lower concentrations (<10 ng/ml).

Effect of a Single LPS Preparation on Macrophages Derived from Different Individuals

The effect of multiple concentrations (5–500 ng/ml) of a single LPS preparation (0111:B4) on acid phosphatase activities of macrophages derived from different donors is shown in Fig. 4. The response to LPS was found to vary significantly from donor to donor. For example, after exposure to 5 ng/ml of LPS for 72 h, the acid phosphatase activity in macrophages derived from donor J. S. was suppressed by only 13%; while that of donor F. A. showed a 62% reduction in cellular specific activity under identical stimulation conditions. Successive cell preparations from the same four donors gave similar rank orders of sensitivity to LPS (data not shown) indicating that the observation of different responses in different donors was not due to daily or experimental variations, but probably, reflects a true variation of responses between individuals.

Effect of LPS in Lysosomal Enzyme Assays

To determine whether the observed decline in cellular and secreted lysosomal enzyme activities in response to LPS exposure was due to an inhibition of enzyme activity or interference of the catalytic activity assay by LPS, Triton X-100 lysates of human macrophages were incubated with concentrations of LPS varying between 10 and 1,000 ng/ml for 24 h before being assayed for catalytic activities of acid phosphatase and hexosaminidase. LPS was not found to inhibit the catalytic activities of the enzymes under study (data not shown).

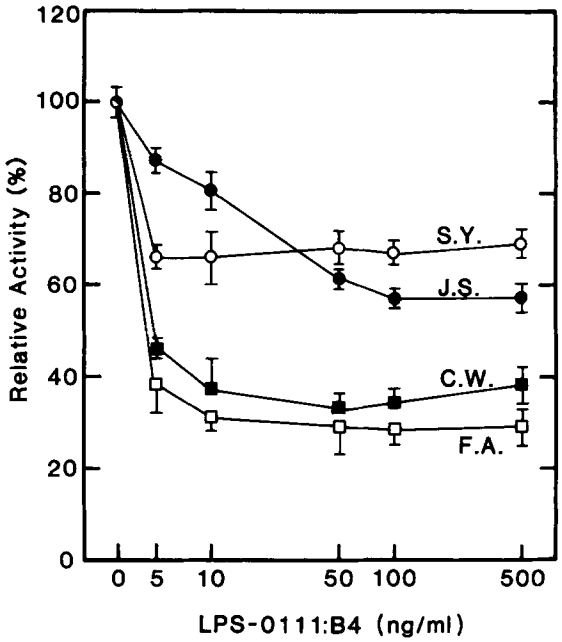

Figure 4. Effect of multiple concentrations of LPS (*E. coli* 0111:B4; 5–500 ng/ml) on the cellular catalytic activities of acid phosphatase in macrophages obtained from four different individuals. Cells were exposed to LPS for 72 h at 37°C. The specific activity of acid phosphatase in the absence of LPS has been normalized to 100% since the absolute baseline specific activities varied amongst the four individuals.

Effect of Indomethacin

Although LPS is known to directly activate or suppress certain macrophage functions, some of its properties (e.g., the induction of pro-coagulant activity) appear to be dependent on its ability to generate cyclooxygenase products. To investigate if the reduction of lysosomal enzyme activity was dependent on

prostanoid generation, human macrophages were pre-incubated with the cyclooxygenase inhibitor indomethacin, for 15 min, at a concentration (1 μM) which totally blocked PGE_2 production by these cells in response to LPS (data not shown). The cells were then exposed to LPS (0111:B4; 5–500 ng/ml) in the presence and absence of indomethacin (1 μM) for 72 h at 37°C. The results, shown in Fig. 5, indicate that inhibition of cyclooxygenase by indomethacin failed to affect the suppression of acid phosphatase activities by LPS, thus showing that this response to LPS is not dependent upon prostanoid generation. Similar results were obtained with hexosaminidase and β-glucuronidase (data not shown).

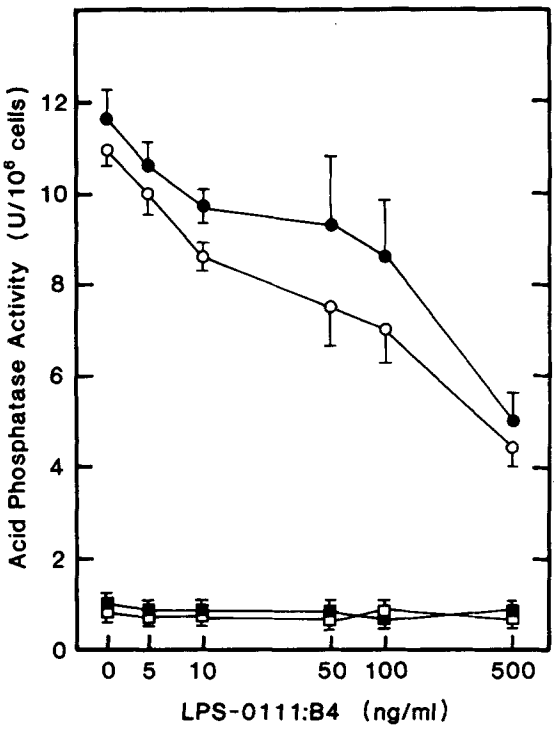

Figure 5. Influence of indomethacin (1 μM) on the decline in cellular and secreted acid phosphatase activities of human macrophages exposed to varying concentrations of LPS (0111:B4; 5–500 ng/ml) for 72 h. (○ and □) LPS alone; (● and ■) LPS + indomethacin (1 μM); (□ and ■) secreted activity; (○ and ●) cellular activity.

Time Course of LPS Action

The kinetic effect of LPS on human macrophage lysosomal enzyme activities was examined by exposing cells to a fixed concentration of LPS (0111:B4; 500 ng/ml). The incubations were terminated at 12-h intervals to a total of 60 h after the addition of LPS. The results of a representative experiment are illustrated in Fig. 6. It will be seen that in control cultures, the cellular catalytic activities of acid phosphatase, hexosaminidase, and β-glucuronidase continue to rise in a more or less linear fashion over the 60-h experimental period. In cultures with LPS, however, acid phosphatase cellular activities declined over the first 24 h of culture before beginning to increase 36 h after the addition of LPS. Hexosaminidase also showed a consistent decline in cellular-specific activity during the first 36 h of exposure to LPS, although the magnitude of the response was always less than that of acid phosphatase. By contrast, β-glucuronidase failed to show a decline in cellular specific activity in response to LPS, although the rate of increase in activity with time was markedly reduced compared with cells that had not been exposed to LPS.

Determination of the Minimum LPS Exposure Time

The minimum exposure period required for LPS to initiate the observed reduction in cellular lysosomal enzyme catalytic activities was established by exposing human monocyte-derived macrophages to an optimal concentration of LPS (500 ng/ml) for various time intervals (5–60 min) before washing the monolayers five times with Hanks' balanced salt solution and incubating them for 72 h in LPS-free Medium 199. The degree of reduction of lysosomal enzyme catalytic activities during the various pre-treatment periods was compared to the effect obtained by continuously exposing cells to LPS (500 ng/ml) for the entire 72-h challenge period. It will be seen from Fig. 7 that exposure of the cells to LPS for <15 min was sufficient to produce a reduction of lysosomal enzyme activities that was only marginally less than that seen when the

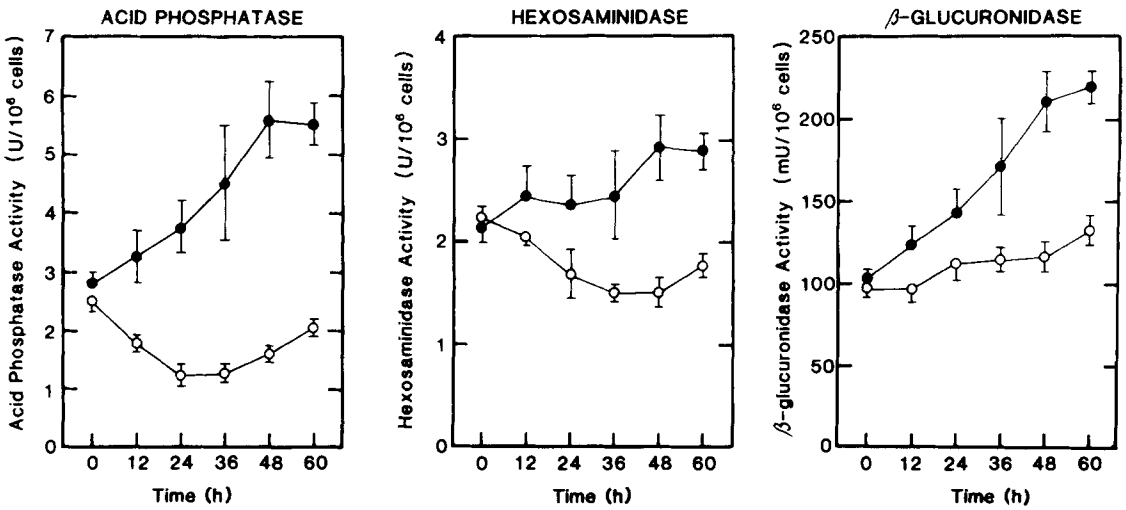

Figure 6. Time course of LPS-induced reduction of lysosomal enzyme activities in human macrophages. Cells were exposed to LPS (*E. coli* 0111:B4; 500 ng) at time 0. The incubations were terminated every 12 h thereafter up to 60 h. (●) Unstimulated cellular activity; (○) LPS-stimulated cellular activity.

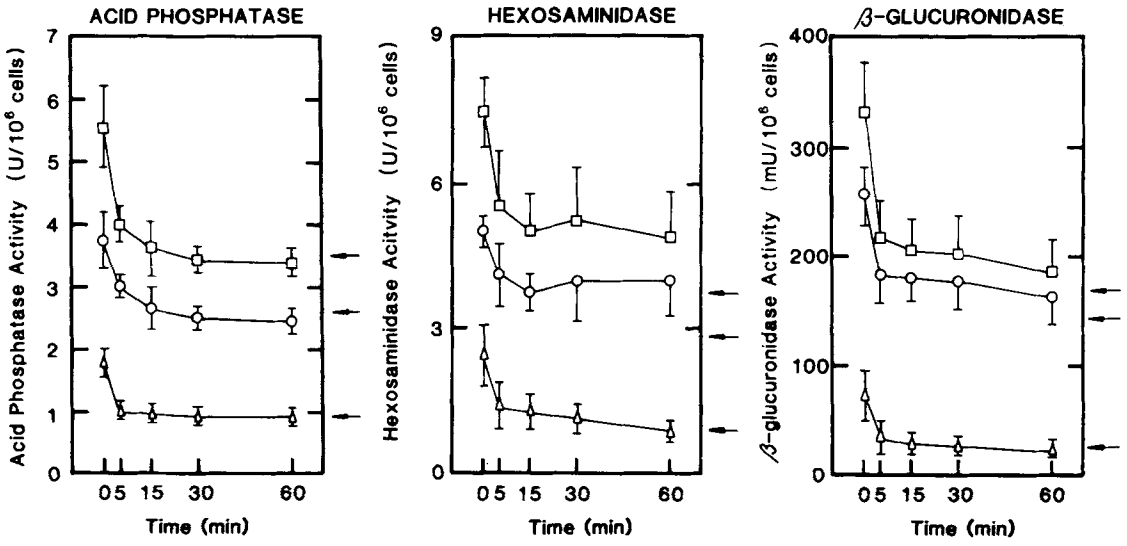

Figure 7. Determination of the minimum LPS exposure time. Human macrophages were exposed to a single dose of LPS (*E. coli* 0111:B4; 500 ng/ml) for 5–60 min at 37°C, then washed and cultured in the absence of LPS for 72 h. (△) Secreted activity; (○) cellular activity; (□) total activity. The arrows adjacent to the right ordinate of each graph represents the enzyme activities of each fraction in cells continuously exposed to LPS for 72 h.

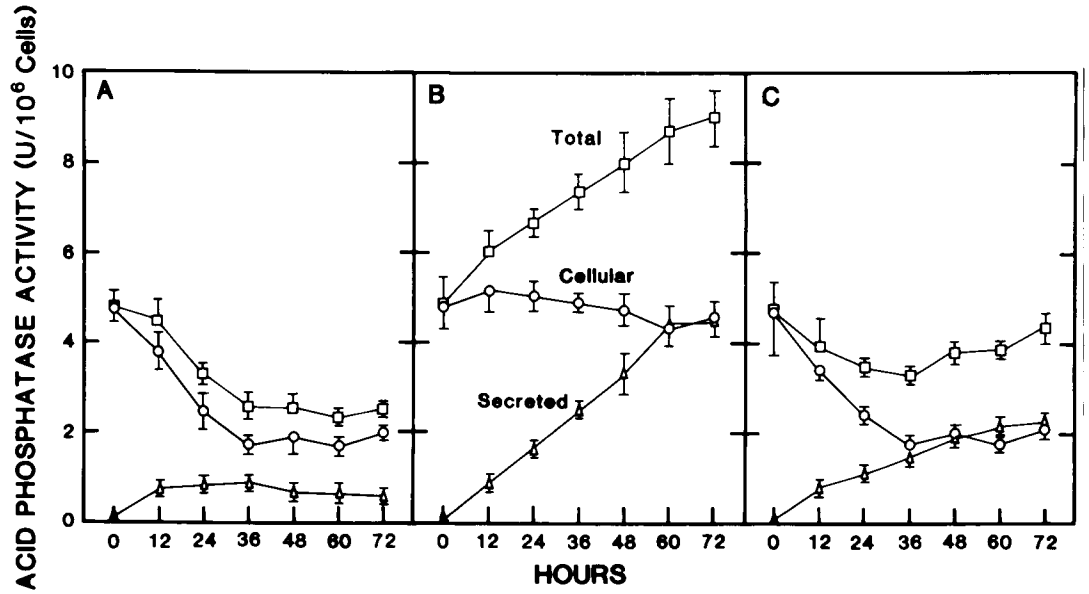

Figure 8. Effect of LPS on the down-regulation of acid phosphatase activities in human macrophages in the presence of methylamine (5 mM). (*A*) Cells exposed to LPS (*E. coli* 0111:B4; 500 ng). (*B*) Cells exposed to methylamine (5 mM). (*C*) Cells exposed to LPS (500 ng) and methylamine (5 mM). (△) Secreted activity; (○) cellular activity; (□) total activity. Incubations were terminated at 12-h intervals for a total of 72 h.

cells were exposed to LPS continuously for the entire 72-h incubation period. The spontaneous secretion of lysosomal enzymes into the culture medium was also suppressed in a similar fashion to the cellular specific activities.

Effect of Methylamine

To investigate whether the reduction of enzyme activity induced in human monocyte–derived macrophages by LPS was due to a decreased rate of production of catalytically active enzyme, lysosomal degradation was blocked by the addition, at the onset of incubation, of the lysosomotropic weak-base methylamine (5 mM), and the effect of LPS was re-examined. In the presence of methylamine, the cellular catalytic activity of acid phosphatase was insignificantly different from that of unstimulated cultures (Fig. 8*A*). However, exposure to methylamine was associated with an approximately fourfold increase in secretion of catalytically active enzyme to the exterior of the cells, presumably due to inhibition of mannose-6-phosphate receptor recycling (32). Thus, in the absence of lysosomal catabolism, the total culture specific activity of acid phosphatase was almost doubled after a 72-h exposure to methylamine.

When cells were exposed to LPS (0111:B4; 500 ng) the cells underwent the previously observed reduction of enzyme catalytic activity (Fig. 8*B*). In addition, when macrophages were exposed to LPS in the presence of methylamine, the cellular

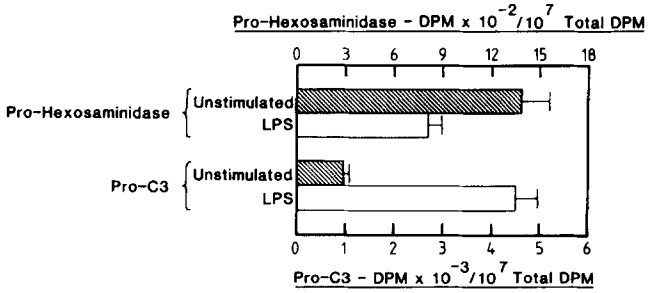

Figure 9. Effect of LPS (0111:134; 500 ng/ml) on the synthesis of pro-hexosaminidase and pro-C3. Macrophage monolayers were exposed to LPS for 24 h. The cells were then rinsed with Hanks' balanced salt solution, and pulsed with 200 μCi of [^{35}S]methionine for 1 h. Levels of intracellular pro-hexosaminidase and pro-C3 were determined by immunoprecipitation with specific antibodies. (▨) Unstimulated controls; (□) cells stimulated with LPS. Results represent the mean ± SEM for four experiments.

Table I. Effect of LPS (E. coli 0111:B4; 500 ng/ml) on the Incorporation of [^{35}S]Methionine into Human Macrophage Cellular Proteins

	0 h	3 h	6 h	12 h	24 h	48 h
Control cells*	9.50	11.46	10.78	15.91	11.47	13.58
LPS-stimulated cells*	11.50	9.14	12.49	15.57	15.27	14.38

Cells were exposed to LPS or to medium alone for up to 48 h, before pulsing with 200 μCi of [^{35}S]methionine, in the presence and absence of LPS, for 1 h. The amount of TCA-precipitable [^{35}S]methionine in the cell lysates was determined as described in the Materials and Methods section.

* Radioactivity is expressed as dpm × 10^{-6} per culture well. The amount of [^{35}S]methionine incorporated into protein varied between 25.8 and 33.7% of the total radioisotope added.

catalytic activities were reduced to the same degree as in the absence of methylamine (Fig. 8*C*). Interestingly, some enhanced secretion of catalytically active enzyme to the cells exterior was also seen, although it represented only about half of that seen in the absence of LPS. It thus appears that LPS suppresses the appearance of catalytically active lysosomal acid phosphatase rather than accelerating its degradation.

Effect of LPS on Total Protein Biosynthesis

To determine that the reduction of lysosomal enzyme activities in human macrophages exposed to LPS was not the result of a more generalized down-regulation of protein synthesis, we have examined the effect of LPS on total protein biosynthesis. Cells were exposed to LPS (0111:B4, 500 ng) for various time periods up to 48 h before being biosynthetically labeled by pulsing with 200 μCi of [^{35}S]methionine for 1 h. The amount of [^{35}S]methionine incorporated into protein was determined at each time point by TCA precipitation. As illustrated in Table 1, overall protein synthesis was not appreciably affected by LPS exposure.

Effect of LPS on the Biosynthesis of Hexosaminidase and C3

To determine whether the decline in lysosomal enzyme activities in response to LPS was due to a decline in the primary synthesis of these enzymes, macrophage monolayers (derived from previously determined high LPS-responding individuals) were exposed to LPS (0111:B4, 500 ng/ml) for 24 h. The cells were then rinsed twice in Hanks' balanced salt solution and incubated in RPMI-1640 containing 200 μCi of [^{35}S]methionine and 500 ng/ml LPS at 37°C for 1 h. The cells were then lysed and analyzed for [^{35}S]methionine-labeled pro-hexosaminidase. The effect of LPS on C3 synthesis was also determined as a positive control since other studies (33) have shown that the synthesis of this protein is markedly stimulated by LPS. The radioactivity associated with the precursors of both alpha and beta subunits of hexosaminidase (molecular weights of 67,000 and 63,000, respectively) and the precursor of C3 (pro-C3, molecular weight, 185,000) was determined by excision of the radioactive bands followed by digestion in hydrogen peroxide and counting in a liquid scintillation spectrometer.

As will be seen from Fig. 9, exposure of human macrophages to LPS for 24 h has resulted in a 42% reduction in the labeling of pro-hexosaminidase ($P = 0.02$). By contrast, in confirmation of previous work by Strunk et al. (33), the synthesis of pro-C3 was significantly stimulated by exposure of the cells to LPS. While this reduction in the primary synthesis of hexosaminidase protein will certainly account in large part for the reduction in the catalytic activity of this enzyme in response to LPS, it is not clear whether a similar situation exists for the other lysosomal enzymes identified in this study. However, it should be noted that the synthesis of β-glucuronidase by mouse bone marrow–derived macrophages is also strongly inhibited by LPS (Riches, D. W. H., and P. M. Henson, unpublished observation).

Discussion

It is becoming clear that bacterial LPS exerts multiple effects on mononuclear phagocytes. The data presented in this paper show that nanogram quantities of bacterial LPS can substantially reduce the intracellular levels of three representative lysosomal enzymes (namely, acid phosphatase, hexosaminidase, and β-glucuronidase) in cultured human monocyte–derived macrophages. At the same time, however, other studies have shown that the synthesis and secretion of a fibroblast growth factor (17) and complement component C3 (33) is markedly stimulated, indicating that this response was not the result of a generalized LPS-dependent inability of the cells to synthesize specific protein products. The reduction of enzyme activity was conveniently monitored by measuring the catalytic activities of the enzymes under study using artificial chromogenic substrates. This effect was, in confirmation of previously reported studies (2, 8), found not to be due to interference of the enzyme assays by LPS. Furthermore, the reduction of cellular lysosomal enzyme catalytic activities did not appear to be due to secretion of the enzymes to the exterior of the cells, since this response was not associated with a reciprocal and concurrent increase in culture supernatant enzyme activities. The reason for the differences in the magnitude of the reduction in catalytic activities is unclear, although it may be related to difference in the degradative rate of each enzyme. Difference in the rates of degradation of rat liver lysosomal enzymes have been reported by Tessitore et al. (35).

Exposure of the cells to LPS in the presence and absence of a concentration of indomethacin sufficient to block prostanoid biosynthesis indicated that the reduction of lysosomal enzyme levels was independent of prostanoid formation. A

similar lack of dependence on cyclooxygenase products has also recently been reported in the LPS-dependent synthesis and secretion of fibroblast growth factor(s) by human monocyte–derived macrophages (17), although the ability of LPS to induce pro-coagulant activity in macrophages is reported to require prostaglandin biosynthesis (31). The observed reduction of lysosomal enzyme activities in response to LPS was not associated with a decrease in cell viability as indicated by measurements of the release of the cytoplasmic enzyme lactate dehydrogenase. Furthermore, as we have shown, measurements of the total incorporation of radiolabeled methionine into cellular proteins was not appreciably stimulated or inhibited by the presence of LPS in the incubation medium, indicating that the decrease in lysosomal enzyme activities in macrophages exposed to LPS was not the result of a more generalized decline in the overall protein-synthesizing capacity of the cells. This indicates a general balance between those proteins whose synthesis is increased and those proteins whose synthesis is decreased, and thus implies that the synthesis of many proteins is decreased during exposure of macrophages to LPS. The phenomenon may bear analogy to a situation in virus-infected cells where the expression of viral and essential host products is conserved, while that of "non-essential" products is repressed (22).

To determine the effect of catabolism on the cellular activities of the lysosomal enzymes under study, macrophages were exposed to LPS either in the absence or presence of methylamine, a lysosomotropic weak-base which, following its accumulation in the lysosomes, elevates the intralysosomal pH from its basal value of 4.8 to pH ~6.5 (21). Under these conditions, the breakdown of material within the lysosomes, which we presume includes the lysosomal enzymes themselves, is strongly inhibited since the intralysosomal pH is well above the pH optimum of the lysosomal hydrolases (18, 30). In the presence of methylamine, the total culture activities of acid phosphatase were increased. A large proportion of this increase was associated with increased secretion of catalytically active enzyme to the exterior of the cells. Interestingly, these human cells did not show the extensive immediate secretion of lysosomal enzymes that is characteristically seen in mouse macrophages which have been exposed to weak-bases (26, 27). When human macrophages were exposed to LPS in the presence of methylamine, the catalytic activities of acid phosphatase and hexosaminidase were reduced to the level seen in the absence of methylamine indicating that when lysosomal catabolism was blocked, LPS could still clearly be shown to reduce the intracellular catalytic activities of the lysosomal enzymes. Thus, the reduction of lysosomal enzymes activities by LPS probably represents a down-regulation in the appearance of catalytically active enzyme, rather than an accelerated rate of enzyme catabolism. This observed reduction in catalytic enzyme activity is also consistent with the data of Hammarstrom and Unsgaard (11), who, microscopically, observed that the number of perinuclear granules, which they presumed were lysosomes, were diminished in number in human monocyte–derived macrophages that had been exposed to LPS, compared with unstimulated controls.

Definitive evidence that the reduction in lysosomal enzyme catalytic activity was due to a reduction in the rate of enzyme production was obtained by investigating the effect of LPS on hexosaminidase protein synthesis (by monocytes derived from previously determined high LPS responders) by biosynthetic labeling with [^{35}S]methionine and immunoprecipitation of the labeled enzyme with specific antibody. It was found that the synthesis of pro-hexosaminidase was reduced by 42% after a 24-h exposure to LPS. By contrast, the synthesis of C3 was stimulated 450% indicating full responsiveness to LPS. It is not clear whether the synthesis of β-glucuronidase and acid phosphatase are similarly regulated by LPS. However, other studies (Riches, D. W. H., and P. M. Henson, unpublished observations) have shown that the synthesis of β-glucuronidase by mouse bone marrow–derived macrophages is strongly inhibited by nanogram quantities of LPS.

A kinetic analysis of the effect of LPS on the reduction of lysosomal enzyme activities suggested that this response was manifested in the early hours after exposure to LPS. In the case of acid phosphatase, a reduction in catalytic activity was observed during the first 24 h of exposure to LPS. Unstimulated macrophages, however, generally continued to synthesize acid phosphatase as indicated by a net increase in its catalytic activity with time. Approximately 36 h after the addition of LPS, the cells appear to re-initiate the accumulation of catalytically active enzyme, even though they had been continuously exposed to LPS during this period. Furthermore, by determining the minimum time required for the interaction between LPS and human macrophages to elicit the reduction in enzyme levels, it was found that LPS need only be present for 15–30 min in order to bring about this effect. However, in the absence of specific information concerning the amount of LPS bound during the 15–30-min exposure, it is also possible that sufficient LPS to elicit the reduction of catalytic enzyme activity is bound during this brief period, and that the response is initiated later on.

The mechanism of reduction of lysosomal enzyme synthesis after exposure to LPS is not clear. LPS does down-regulate the synthesis of the polypeptide chains of hexosaminidase and if this is also true for acid phosphatase and β-glucuronidase, this may occur as a coordinate repression of the transcription of the genes for each enzyme, thereby resulting in a reduction in specific mRNA species and thus specific enzyme biosynthesis. Alternatively, LPS may induce the synthesis of an intracellular component, such as double-stranded RNA (36), that may block the transcription of lysosomal enzyme–specific mRNA. Answers to these questions however, could be obtained by measuring lysosomal enzyme mRNA levels by Northern blotting.

In broader terms, LPS has been found to regulate the expression and synthesis of a number of macrophage products. In addition to its effects on the products reported in this paper, LPS has also been found in our laboratory to stimulate the synthesis and secretion of fibroblast growth factor(s) by human monocyte–derived macrophages (17). Other studies have shown that in mouse macrophages, LPS can stimulate the synthesis of factor B of the complement system (34), but at the same time inhibit the synthesis of apolipoprotein E (38). Chen et al. (6) have also shown that exposure of mouse macrophages to LPS triggers a down-regulation in the expression of functional colony-stimulating factor receptors; while Pekala et al. (24) have provided strong evidence that LPS-stimulated mouse macrophages release a factor(s) that down-regulates the synthesis of specific protein products (e.g., acetyl CoA carboxylase) in adipocytes.

These changes in the protein synthetic phenotype of macrophages exposed to LPS may reflect a change in their functional status. One effect of LPS on macrophages from a variety of species is to stimulate their ability to nonspecifically lyse tumor cells (1, 5, 9). It would thus seem quite conceivable that to attain this and other new functional states, the macrophage must regulate the expression of the products it synthesizes. In the case of products whose expression is not required for the acquisition of the new functional state, this may be achieved by repressing the synthesis of the gene encoding a particular product.

As to the functional consequences of this response, previous studies have shown that the degradation of ingested Candida albicans is markedly reduced in human macrophages that have been exposed in vitro to LPS (11). Thus the response may have negative implications in host defense and macrophage scavenging roles.

This work was carried out in the F. L. Bryant, Jr. Research Laboratory for the Mechanisms of Lung Disease.

Dr. D. W. H. Riches gratefully acknowledges travel grants from the Wellcome Trust, U. K., and from Upjohn Pharmaceuticals (UK) Ldt.

This work was supported by grants GM 24834, AM 26609, and ACS IM-491 from the National Institutes of Health.

Received for publication 9 August 1985, and in revised form 8 January 1986.

References

1. Alexander, P., and R. Evans. 1971. Endotoxin and double-stranded RNA render macrophages cytotoxic. *Nature New Biol.* 232:76–78.

2. Allison, A. C., P. Davies, and R. C. Page. 1973. Effects of endotoxin on macrophages and other lymphoreticular cells. *J. Infect. Dis.* 128:5212–5220.

3. Brodsky, A. B. 1978. CRC Handbook of Radiation Measurement and Protection.

4. Caboud, P. G., and F. Wroblewski. 1958. Colorimetric measurement of lactic dehydrogenase activity of body fluids. *Am. J. Clin. Pathol.* 30:234–238.

5. Cameron, D. J., and W. H. Churchill. 1980. Cytotoxicity of human macrophages for tumor cells: enhancement by bacterial lipopolysaccharides (LPS). *J. Immunol.* 123:708–711.

6. Chen, B. D.-M., H. S. Lin, and S. Hsu. 1983. Lipopolysaccharide inhibits the binding of colony-stimulating factor (CSF-1) to murine peritoneal exudate macrophages. *J. Immunol.* 130:2256–2260.

7. Cooper, P. H., P. Mayer, and M. Baggiolini. 1984. Stimulation of phagocytosis in bone marrow-derived mouse macrophages by bacterial lipopolysaccharide: correlation with biochemical and functional parameters. *J. Immunol.* 133:913–922.

8. Di Natale, P., M. Stabile, L. Ransisrille, and R. Utili. 1981. Effect of bacterial endotoxin on lysosomal enzyme activities of normal and mucolipidosis III fibroblasts. *Biomedicine (Paris).* 35:46–49.

9. Doe, W. F., and P. M. Henson. 1978. Macrophage stimulation by bacterial lipopolysaccharides. I. Cytolytic effect on tumor target cells. *J. Exp. Med.* 148:544–556.

10. Glenn, K. C., and R. Ross. 1981. Human monocyte-derived growth factor for mesenchymal cells: activation of secretion by endotoxin and concanavalin A. *Cell.* 25:603–615.

11. Hammarstrom, J., and G. Unsgaard. 1979. In vitro influence of endotoxin on human mononuclear phagocyte structure and function. *Acta Pathol. Microbiol. Scand.* 87:381–389.

12. Hasilik, A., and E. F. Neufeld. 1980. Biosynthesis of lysosomal enzymes in fibroblasts. Synthesis as precursors of higher molecular weight. *J. Biol. Chem.* 255:4937–4945.

13. Haslett, C., L. A. Guthrie, M. M. Kopaniak, R. B. Johnston, and P. M. Henson. 1985. Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. *Am. J. Pathol.* 119:101–110.

14. Havell, E. A., and G. L. Spitalny. 1983. Endotoxin-induced interferon synthesis in macrophage cultures. *J. Reticuloendothelial Soc.* 33:369–374.

15. Kurland, J. I., and R. Bockman. 1978. Prostaglandin E production by human blood monocytes and mouse peritoneal macrophages. *J. Exp. Med.* 147:952–957.

16. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–682.

17. Leslie, C. C., R. A. Musson, and P. M. Henson. 1984. Production of growth factor activity for fibroblasts by human monocyte-derived macrophages. *J. Leukocyte Biol.* 36:143–160.

18. Lie, S. O., and B. Schofield. 1973. Inactivation of lysosomal function in normal cultured human fibroblasts by chloroquine. *Biochem. Pharmacol.* 22:3109–3118.

18a. McIntyre, F. C., G. H. Barlow, H. W. Sievert, R. A. Finlay, and A. L. Yoo. 1969. Studies on a lipopolysaccharide from E. Coli. Heterogeneity and mechanisms of reversible inactivation by sodium deoxycholate. *Biochemistry.* 8:4063–4066.

19. Murtaugh, M. P., W. P. Arend, and P. J. A. Davies. 1984. Induction of tissue transglutaminase in human peripheral blood monocytes. *J. Exp. Med.* 159:114–125.

20. Musson, R. A., and P. M. Henson. 1979. Humoral and formed elements of blood modulate the response of peripheral blood monocytes. I. Plasma and serum inhibit and platelets enhance monocyte adherence. *J. Immunol.* 122:2026–2031.

21. Ohkuma, S., and B. Poole. 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. *Proc. Natl. Acad. Sci. USA.* 75:3327–3331.

22. Oldstone, M. B. A., and M. J. Buchmeier. 1982. Restricted expression of viral glycoproteins in cells of persistently infected mice. *Nature (Lond.)* 300:360–362.

23. Pabst, M. J., and R. B. Johnston. 1980. Increased production of superoxide anion by macrophages exposed in vitro to muramyl dipeptide or lipopolysaccharide. *J. Exp. Med.* 151:101–114.

24. Pekala, P. H., M. Kawakami, C. W. Angus, M. D. Lane, and A. Cerami. 1983. Selective inhibition of synthesis of enzymes for *de novo* fatty acid biosynthesis by an endotoxin-induced mediator from exudate cells. *Proc. Natl. Acad. Sci. USA.* 80:2743–2747.

25. Riches, D. W. H., and Henson, P. M. 1986. Functional aspects of macrophage heterogeneity in lung inflammation. *Ann. NY Acad. Sci.* In Press.

26. Riches, D. W. H., and D. R. Stanworth. 1980. Primary amines induce selective release of lysosomal enzymes from mouse macrophages. *Biochem. J.* 188:933–936.

27. Riches, D. W. H., C. J. Morris, and D. R. Stanworth. 1981. Induction of selective acid hydrolase release from mouse macrophages during exposure to chloroquine and quinine. *Biochem. Pharmacol.* 30:629–634.

28. Rivers, R. P. A., W. E. Hathaway, and W. L. Weston. 1975. The endotoxin-induced coagulant activity of human monocytes. *British Journal of Haematology.* 30:311–319.

29. Schmidt, J. A., C. N. Oliver, J. L. Lepe-Zuniga, I. Green, and I. Gery. 1984. Silica-stimulated macrophages release fibroblast proliferation factors identical to interleukin 1. A potential role for interleukin 1 in the pathogenesis of silicosis. *J. Clin. Invest.* 73:1461–1472.

30. Seglen, P. O., B. Grinde, and A. E. Solheim. 1979. Inhibition of the lysosomal pathway of protein degradation in isolated rat hepatocytes by ammonia, methylamine, chloroquine and leupeptin. *Eur. J. Biochem.* 95:215–225.

31. Sitin, R. G., H. G. Kaltreider, and M. E. Goldyne. 1984. Prostaglandin E is required for the augmentation of procoagulant activity of LPS-stimulated rabbit alveolar macrophages. *J. Immunol.* 132:867–871.

32. Sly, W. S., and H. D. Fischer. 1982. The phosphomannosyl recognition system for intracellular and intercellular transport of lysosomal enzymes. *J. Cell. Biochem.* 18:67–85.

33. Strunk, R. C., A. S. Whitehead, and F. S. Cole. 1985. Pretranslational regulation of the synthesis of the third component of complement in human mononuclear phagocytes by the lipid A portion of lipopolysaccharide. *J. Clin. Invest.* 76:985–990.

34. Sundsmo, J. S., J. R. Chin, R. A. Papin, D. S. Fair, and Z. Werb. 1985. Factor B, the complement alternative pathway serine proteinase, is a major constitutive protein synthesized and secreted by resident and elicited mouse macrophages. *J. Exp. Med.* 161:306–322.

35. Tessitore, L., M. Curzio, G. Cecchini, M. F. Zuretti, and F. M. Baccino. 1979. Evidence for heterogeneous turnover of rat liver lysosome hydrolases. *Boll. Soc. Ital. Biol. Sper.* 55:60–64.

36. Torrence, P. F., and R. M. Friedman. 1979. Are double-stranded RNA-directed inhibition of protein synthesis in interferon-treated cells and interferon induction related phenomena? *J. Biol. Chem.* 254:1259–1263.

37. Wahl, L. M., S. M. Wahl, S. E. Mergenhagen, and G. R. Martin. 1974. Collagenase production by endotoxin-activated macrophages. *Proc. Natl. Acad. Sci. USA* 71:3598–3601.

38. Werb, Z., and J. R. Chin. 1983. Endotoxin suppresses expression of apolipoprotein E by mouse macrophages in vivo and in culture. *J. Biol. Chem.* 258:10642–10647.