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Enhanced Lysosomal Enzyme Activity in Mouse Cells Treated with Bacterial Endotoxin *in Vitro*[1] (39814)

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The possibility that lysosomes might be the target for the effects of gram-negative bacterial endotoxin was suggested by Martini (1), who observed increased free cathepsin activity in homogenates of rat liver and rat muscle after injection of endotoxin (bacterial lipopolysaccharide; LPS) although the total activity was relatively unchanged. Similarly, Weissmann and Thomas (2) found that LPS administered to rabbits caused a significant release of the lysosomal enzymes cathepsin and β-glucuronidase within 30 min. However, LPS administered to rabbits rendered resistant to LPS by pretreatment with LPS did not cause enhanced release of cathepsin and β-glucuronidase activities. Moreover, lysosomes isolated from LPS-treated animals are more fragile than those isolated from untreated animals (3, 4). Furthermore, LPS can stimulate the release of lysosomal hydrolases from polymorphonuclear leukocytes *in vitro* (5).

In this study, the β-glucuronidase activity of mouse cells treated with LPS *in vitro* has been measured. Evidence is presented that LPS enhances acid phosphatase and β-glucuronidase activities of adherent spleen cells from LPS-sensitive mice but not of those from LPS-refractory mice.

Materials and methods. Adult male ICR mice weighing 19 to 23 g and adult male C3H/HeDub mice weighing 18 to 22 g were obtained from Flow Research Animals, Inc., Dublin, Virginia. Adult male C3H/HeJ mice weighing 18 to 22 g were obtained from Jackson Laboratory, Bar Harbor, Maine. The animals were acclimated to their new environment for 1 week prior to experimentation and were allowed free access to food (Purina Lab Chow, Purina Ralston Corp., St. Louis, Mo.) and water. The mice were maintained on a 12-hr diurnal light schedule.

The bacterial endotoxin preparation, except where indicated, was *Escherichia coli* 0127:B8 LPS (Westphal preparation) obtained from Difco Laboratories, Detroit, Michigan. Inorganic chemicals (certified A.C.S. grade) were purchased from Fisher Scientific Co., Fair Lawn, New Jersey. Organic chemicals and biochemicals were purchased from Sigma Chemical Co., St. Louis, Missouri.

To obtain spleen cells, mice were killed by cervical dislocation and the spleens were removed aseptically and placed in Hanks' balanced salt solution (BSS; Flow Laboratories, Rockville, Md.). Spleens were forced through a syringe with no needle, homogenized with a loosely fitting pestle, and filtered through sterile cotton gauze prior to subculture. Cells from one spleen were incubated in a T-25 flask containing modified Eagle's medium (Flow), BSS or BSS lacking glucose, without serum, for 4 hr at 37°, with or without 10 μg of LPS/ml. Peritoneal exudate cells were collected after injecting 5 ml of BSS ip and gently massaging the mouse. These cells were incubated in modified Eagle's medium lacking serum, with or without 10 μg of LPS/ml, for 4 hr at 37°.

The mouse cells in maintenance culture (three flasks for spleen cultures and six flasks for peritoneal exudate cells) were collected and suspended in 10 m*M* phosphate buffer containing 0.15 *M* KCl, pH 7.0, at a concentration of 100 mg wet weight of cells/ml. The cells were disrupted by homogenization with a motor-driven Teflon pestle in a fitted glass tube (1300 rpm). The homogenate was centrifuged at 500*g* for 5 min; this is referred to as the cell extract. The extract

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was centrifuged at 20,000*g* for 20 min; the clear supernatant fraction and the sediment, called the granular fraction, were assayed for enzyme activities as indicated (6).

β-Glucuronidase activity (EC 3.2.1.31) was measured by the method of Talalay *et al.* (7). Activity was expressed as micrograms of phenolphthalein formed per minute per milligram of protein. Acid phosphatase activity (EC 3.1.3.2) was assayed according to the method of Valentine and Beck (8). Activity was expressed as micrograms of phosphate formed per minute per milligram of protein. Catalase activity (EC 1.11.1.6) was measured by the method of Beers and Sizer (9). The activity was expressed in k min^{-1} values (first-order rate constant). The Lowry modification of the Folin procedure was used to estimate protein concentrations (10). Bovine serum albumin was used as the standard.

Results. The specific activity of β-glucuronidase was greater in the granular fraction than in the supernatant fraction or the cell extract of the whole spleen, adherent spleen cells, or peritoneal exudate cells. The specific activity in the granular fractions from whole spleen, adherent spleen cells, or peritoneal exudate cells was greater for samples from LPS-treated cultures than for corresponding untreated cultures (Table I). Although nonadherent cells contained appreciable β-glucuronidase activity, the specific activity was the same in the cell extract, supernatant fraction, and the granular fraction. Moreover, the specific activities of fractions from LPS-treated cultures were essentially the same as those from untreated cultures (Table I). Subsequent studies were restricted to adherent spleen cells.

The LPS-stimulated elevation in β-glucuronidase and acid phosphatase activities was obtained with adherent spleen cells incubated in modified Eagle's medium and Hanks' balanced salt solution, with or without glucose. None of these maintenance media contained serum. Catalase activities were not different in fractions from LPS-treated and untreated cultures of adherent spleen cells (Table II). In general, the greatest specific activities for both acid phosphatase and β-glucuronidase were in the granular fractions.

The specific activities of β-glucuronidase in the granular fraction of adherent spleen cells from ICR and C3H/HeDub mice were higher for LPS-treated cultures than for their untreated counterparts. However, the specific activities of adherent spleen cells from ICR mice rendered tolerant or resistant to LPS by prior treatment with LPS, or from C3H/HeJ mice, were essentially the same for LPS-treated cultures and their untreated counterparts (Table III). The specific activities of the fractions from untreated cultures of adherent spleen cells were essentially the same for all three mouse strains and for the LPS-tolerant ICR mouse (Table III).

Discussion. The concentration of LPS that elicits a marked rise in lysosomal enzyme activities in primary cell cultures (10 μg/ml) is comparable to the LPS dose that kills 50% of treated mice (15 μg of LPS/g of mouse weight). Furthermore, LPS causes a substantial elevation of lysosomal enzyme activities *in vitro* within 4 hr, which corresponds to the time that animals display loss of thermal regulation and pactamycin-treated mice die after administration of LPS iv (3). Moreover, this enhanced lysosomal enzyme activity develops in the absence of serum. Our results, along with those of Bradley and Howe (11), demonstrate that LPS can affect cellular metabolic processes directly and that all of the cellular effects of LPS cannot be attributed to immunologic reactions, as claimed by many workers (12–14).

The present investigations do not differentiate between activation of the lysosomal enzymes and new enzyme synthesis. Exogenously supplied glucose and amino acids are not required for development of enhanced hydrolase activities. A parallel may be drawn with the release of endogenous pyrogen by polymorphonuclear leukocytes or macrophages treated with LPS *in vitro*. It has not been definitively established whether endogenous pyrogen is synthesized *de novo* or is activated from a less active precursor (15).

Spleen cells from both endotoxin-sensitive strains of mice responded to LPS *in vitro*, but spleen cells from both the genetically LPS-refractory C3H/HeJ mouse (16)

TABLE I. β-GLUCURONIDASE ACTIVITIES IN MOUSE CELLS TREATED WITH BACTERIAL ENDOTOXIN (LPS) *in Vitro*.[a]

Source of cells	LPS (10 μg/ml)	β-Glucuronidase activity		
		Cell extract	Supernatant	Granular
Whole spleen	−	5.7 ± 0.7	4.9 ± 0.3	8.9 ± 5.4
Whole spleen	+	182 ± 12%*	198 ± 15%*	174 ± 13%*
Spleen, nonadherent	−	6.7 ± 2.0	6.6 ± 0.5	5.1 ± 1.1
Spleen, nonadherent	+	112 ± 29%	103 ± 40%	124 ± 16%
Spleen, adherent	−	1.5 ± 0.2	1.9 ± 0.1	5.5 ± 1.8
Spleen, adherent	+	133 ± 5%*	105 ± 11%	182 ± 3%*
Peritoneal exudate	−	2.3 ± 1.7	2.5 ± 1.8	3.9 ± 3.2
Peritoneal exudate	+	87 ± 16%	88 ± 7%	185 ± 9%*

[a] Spleens were dispersed in Hanks' balanced salt solution (BSS) by gentle homogenization. The spleen cells were incubated in modified Eagle's medium (MEM) lacking serum for 4 hr at 37°, with or without bacterial endotoxin (LPS). Next the nonadherent cells were separated from the adherent cells. Peritoneal exudate cells were collected after injecting 5 ml of BSS ip and gently massaging the mouse. These cells were incubated in MEM lacking serum, with or without LPS, for 4 hr at 37°. Cells were harvested, extracts were prepared, and β-glucuronidase was assayed by the procedures described in *Materials and methods*. The values for preparations without LPS are the means ± standard errors of the specific activities determined in three independent experiments. The values for preparations treated with LPS are the means ± standard errors of the relative enzyme activities, expressed as the percentage of the corresponding sample without LPS, computed for three independent experiments.

* Significantly different from control without LPS at $P < 0.05$.

TABLE II. ENHANCED ACTIVITIES OF ACID PHOSPHATASE AND β-GLUCURONIDASE IN ADHERENT MOUSE SPLEEN CELLS TREATED WITH BACTERIAL ENDOTOXIN IN DIVERSE MEDIA.[a]

Incubation medium	Cell fraction	LPS (10 μg/ml)	Enzyme activity		
			Acid phosphatase	β-Glucuronidase	Catalase
MEM	Extract	−	55 ± 14	1.2 ± 0.4	0.16 ± 0.04
MEM	Extract	+	224 ± 23%*	225 ± 23%*	113 ± 12%
MEM	Supernatant	−	29 ± 7	1.6 ± 0.3	0.10 ± 0.02
MEM	Supernatant	+	79 ± 16%	169 ± 37%	120 ± 12%
MEM	Granular	−	191 ± 9	2.6 ± 1.0	0.20 ± 0.04
MEM	Granular	+	134 ± 5%*	185 ± 14%*	85 ± 9%
BSS	Extract	−	95 ± 33	1.4 ± 0.5	0.16 ± 0.05
BSS	Extract	+	280 ± 32%*	300± 34%*	113 ± 15%
BSS	Supernatant	−	185 ± 55	2.5 ± 1.2	0.11 ± 0.01
BSS	Supernatant	+	136 ± 21%	116 ± 29%	91 ± 5%
BSS	Granular	−	165 ± 15	3.5 ± 2.4	0.18 ± 0.02
BSS	Granular	+	230 ± 21%*	231 ± 19%*	117 ± 11%
SS	Extract	−	234 ± 21	1.6 ± 0.4	0.14 ± 0.05
SS	Extract	+	136 ± 14%	188 ± 17%*	129 ± 25%
SS	Supernatant	−	299 ± 69	2.0 ± 0.4	0.09 ± 0.05
SS	Supernatant	+	112 ± 9%	120 ± 12%	100 ± 27%
SS	Granular	−	354 ± 37	2.4 ± 0.6	0.18 ± 0.03
SS	Granular	+	219 ± 19%*	200 ± 15%*	94 ± 9%

[a] Spleens were dispersed in Hanks' balanced salt solution (BSS) by gentle homogenization. The spleen cells were incubated in modified Eagle's medium (MEM) supplemented with 5% fetal calf serum for 24 hr at 37°. The nonadherent cells were decanted off and the adherent cells were incubated for 4 hr at 37° in the indicated medium, with or without bacterial endotoxin (LPS). The adherent cells were released with EDTA and trypsin, harvested, and disrupted. Cell extracts or fractions were prepared and assayed for enzyme activity as described in *Materials and methods*. SS denotes BSS lacking glucose. The values for preparation without LPS are the means ± standard errors of the enzyme activities determined in three independent experiments. The values for preparations treated with LPS are the means ± standard errors in the relative enzyme activities, expressed as the percentage of the corresponding sample without LPS.

* Significantly different from control without LPS at $P < 0.05$.

and the ICR mice rendered resistant by pretreatment with LPS failed to produce enhanced hydrolase activity after exposure to LPS *in vitro*. Nonresponsiveness to LPS may reflect the absence of a cellular or organelle population vulnerable to LPS rather

TABLE III. ENHANCED β-GLUCURONIDASE ACTIVITY IN ADHERENT SPLEEN CELLS FROM DIVERSE MICE TREATED WITH BACTERIAL ENDOTOXIN *in Vitro*.[a]

Source of cells	LPS (10 μg/ml)	β-Glucuronidase activity		
		Extract	Supernatant	Granular
Normal ICR	−	2.1 ± 0.8	2.7 ± 0.6	3.4 ± 0.4
Normal ICR	+	100 ± 7%	122 ± 9%	179 ± 14%*
Tolerant ICR	−	1.4 ± 0.5	1.7 ± 0.4	5.6 ± 0.5
Tolerant ICR	+	107 ± 5%	118 ± 11%	88 ± 9%
Normal C3H/HeDub	−	3.5 ± 0.6	2.2 ± 0.3	4.1 ± 0.5
Normal C3H/HeDub	+	114 ± 11%	145 ± 5%*	188 ± 12%*
Normal C3H/HeJ	−	2.3 ± 0.4	1.5 ± 0.6	3.8 ± 0.4
Normal C3H/HeJ	+	96 ± 9%	87 ± 6%	84 ± 8%

[a] Adherent spleen cells from different strains of normal mice and from endotoxin-tolerant mice were prepared by the methods described in Table II. ICR mice were rendered tolerant to endotoxin by treatment with 1 mg/kg of *E. coli* O26:B6 (boivin) lipopolysaccharide on Day 1, 2 mg/kg on Days 2 and 3, and 4 mg/kg on Day 4. Spleens were removed on Day 5. All spleen cultures were incubated for 4 hr at 37° in MEM, with or without LPS. The values for the preparations without LPS are the means ± standard errors of the enzyme activity determined in three independent experiments. The values for preparations treated with LPS are the means ± standard errors of the relative enzyme activities, expressed as the percentage of the corresponding sample without LPS.

* Significantly different from control without LPS at $P < 0.05$.

than the presence or production of protective agents. It should be noted that C3H/HeJ mice have normal numbers of B cells and respond normally to other B-cell mitogens such as polyinosinic acid (17). Lymphoid cells from C3H/HeJ mice, however, are unable to give a mitogenic response with LPS. Furthermore, partially purified T cells or macrophages do not inhibit the mitogenic response to LPS of spleen cells from LPS-susceptible mice (18). These observations support the proposition that LPS unresponsiveness reflects a defect or depletion in B cells (17, 18).

It is reasonable to propose that enhanced lysosomal enzyme activity may be the primary mechanism of action of LPS *in vivo*. Lysosomal hydrolases, for example, may generate products that are responsible for activation of the complement system, activation of the coagulation pathway, and fever, the characteristic responses of animals to LPS (12).

Summary. The specific activity of β-glucuronidase in the granular fraction from whole spleen, adherent spleen cells, or peritoneal exudate cells of ICR mice was greater for samples from cell cultures treated with 10 μg of endotoxin/ml *in vitro* than that for the corresponding untreated cultures. The endotoxin-stimulated elevation in β-glucuronidase and acid phosphatase activities was obtained with adherent spleen cells incubated in modified Eagle's medium and Hanks' balanced salt solution, with or without glucose. The specific activities of β-glucuronidase in adherent spleen cells from endotoxin-tolerant ICR mice or genetically resistant C3H/HeJ mice were essentially the same for endotoxin-treated cultures and their untreated counterparts.

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