Enhancement of O_2^- generation and tumoricidal activity of murine macrophages by a monosaccharide precursor of *Escherichia coli* lipid A

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The effects of a monosaccharide precursor of *Escherichia coli* lipid A (lipid X) on murine macrophages were studied. Lipid X is a diacylglucosamine 1-phosphate bearing β-hydroxymyristoyl groups at positions 2 and 3. Lipid X, as well as lipopolysaccharide and lipid A, enhanced O_2^- generation in mouse peritoneal macrophages and a macrophage-like cell line, J774.1, and further induced the tumor-cytotoxic activity of peritoneal macrophages. Elimination of a 1-phosphate or 3-*O*-β-hydroxymyristoyl group from lipid X completely abolished these effects, suggesting that both 1-phosphate and 3-*O*-β-hydroxymyristoyl groups are essential for the elevated O_2^- generation and induction of turmoricidal activity due to lipid X.

Lipid A precursor *Microphage activation* *O_2^- generation* *Tumor cytotoxicity*

1. INTRODUCTION

A number of studies have revealed that lipid A, the hydrophobic part of LPS in Gram-negative bacteria, is responsible for most of the pathophysiological and biological activities of endotoxins [1,2]. Recently, Nishijima and Raetz [3,4] isolated 2 new glycolipids (designated lipids X and Y) from certain *Escherichia coli* mutants. The covalent structures of lipids X and Y were determined [5] and found to resemble the reducing end of lipid A (fig.1). Furthermore, lipid X was shown to be a biosynthetic precursor of lipid A in *E. coli* [6,7].

Here we determined the effects of lipid X and its deacylated and dephosphorylated derivatives (fig.1) on macrophage activation, as judged from O_2^- generation and tumoricidal activity. It was found that lipid X, as well as LPS and lipid A, enhanced O_2^- production in macrophages and induced tumoricidal activity against EL4 lymphoma cells, but the deacylated and dephosphorylated derivatives of lipid X had lost these activities.

Abbreviations: FBS, fetal bovine serum; LPS, lipopolysaccharide; NBT, nitroblue tetrazolium; PMA, 4β-phorbol 12-myristate 13-acetate; SOD, superoxide dismutase; TNF, tumor necrosis factor

2. MATERIALS AND METHODS

NBT, cytochrome *c*, SOD and PMA were purchased from Sigma (St. Louis, MO). *E. coli* LPS 0111:B4 was from Difco (Detroit, MI), and lipid A, from *S. minnesota* R595, from List Biological Laboratories (Cambell, CA). Lipid X, and the deacylated and dephosphorylated derivatives of lipid X (fig.1) were isolated as described [3,4,8]. Other chemicals and reagents were obtained as in [8].

Maintenance of the J774.1 macrophage-like cell line and preparation of mouse peritoneal macrophages were performed as in [8]. For assaying O_2^- generation, 1×10^6 macrophages were cultured overnight in 1 ml Ham's F12 medium containing 10% (v/v) heat-inactivated FBS, 100

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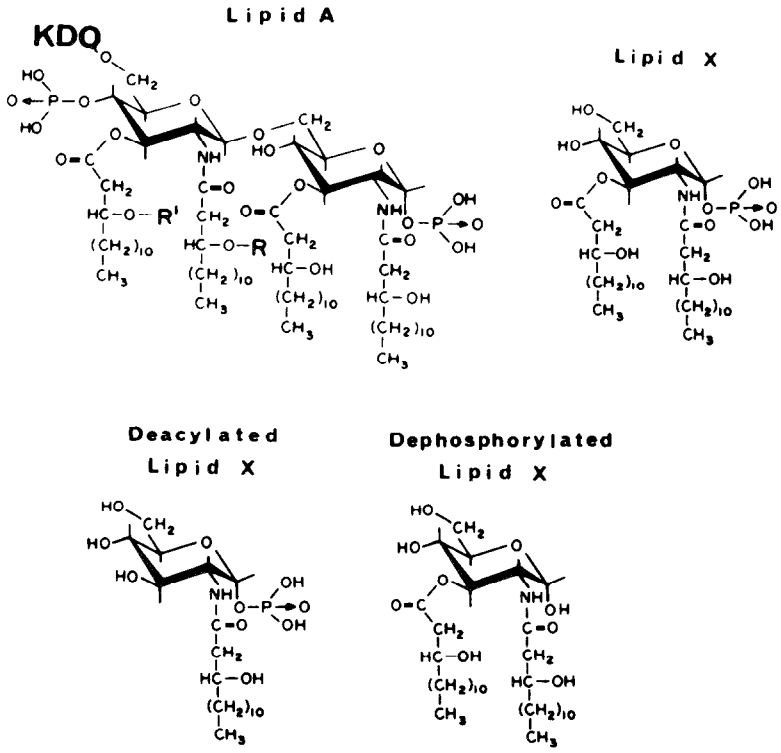

Fig.1. Structures of *E. coli* lipid A, lipid X and deacylated and dephosphorylated derivatives of the latter. KDO at 6′-position of lipid A denotes ketodeoxyocturosonate.

units/ml penicillin and 100 μg/ml streptomycin in a 35 mm diameter plastic petri dish at 37°C in 5% CO_2/air, and then activated with the reagents at 37°C for 24 h. NBT reduction was assayed as described [9], in the presence of 5 μg/ml PMA and 0.05% NBT. The cells were incubated at 37°C for 90 min in 5% CO_2/air, and then after removal of the reaction buffer, air-dried, fixed with methanol, stained with 0.1% safranin O, and finally observed under a light microscope in random fields. The cells stained with insoluble blue formazane were counted.

O_2^- generation was also determined from SOD-sensitive reduction of cytochrome *c*, according to Johnston et al. [10]. After incubation at 37°C for 90 min, the reaction mixture was transferred to the cuvettes of a double-beam spectrophotometer (Shimadzu model UV-240) and the difference in absorbance at 550 nm was determined. O_2^- generation was calculated on the basis that a change in absorbance of 1.0 corresponds to 47.4 nmol O_2^-, and the results were expressed as specific activities. Cell proteins were determined as described [11] with bovine serum albumin as a standard.

Assaying of macrophage-mediated cytotoxicity against EL4 leukemic cells was performed with peptone-induced peritoneal macrophages from ICR mice as in [8].

3. RESULTS

The effect of lipid X on NBT reduction was dependent on its concentration, an effect being evident at and over 0.1 μg/ml (fig.2). Blue-black insoluble precipitates of formazane were frequently observed in the cells stimulated with PMA, but not in the negative control without PMA (not shown). J774.1 cells were not able to reduce NBT, but about 25% of the cells became formazane-positive on treatment with 10 μg/ml of lipid X. Lipid A was as effective as lipid X for NBT reduction, but LPS was more effective, especially at low doses. In

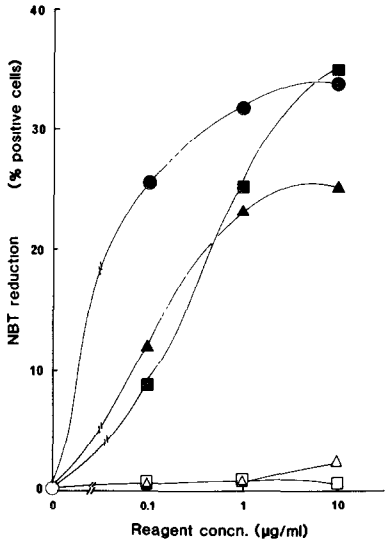

Fig.2. Effects of LPS (●), lipid A (▲), lipid X (■), and deacylated (△) and dephosphorylated (□) lipid X on NBT reduction by J774.1 cells. Approx. 300 cells in 4 random fields were examined. The point on the ordinate (○) is for the negative control.

contrast, the deacylated and dephosphorylated derivatives of lipid X did not make the macrophages reduce NBT (fig.2).

O_2^- generation was more quantitatively estimated from the reduction of cytochrome *c* (fig.3). Lipid X activated both J774.1 macrophages (fig.3a) and mouse peritoneal macrophages (fig.3b), and enhanced the production of O_2^- significantly, although the extents were lower than those with LPS. Neither deacylated nor dephosphorylated lipid X was effective for O_2^- generation even at 10 μg/ml. Lipid A was as effective as lipid X (not shown). The results in figs 2 and 3 show that lipid X, as well as LPS and lipid A, enhances O_2^- production in mouse peritoneal macrophages and J774.1 macrophages, but that removal of a 1-phosphate or 3-*O*-acyl group from lipid X completely abolishes these activities.

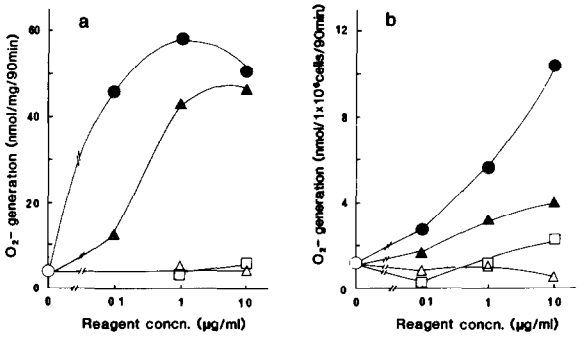

Fig.3. Effects of LPS (●), lipid X (■), and deacylated (△) and dephosphorylated (□) derivatives of the latter on O_2^- generation by J774.1 cells (a) and the resident macrophages from ICR mouse peritoneum (b). The points on the ordinates (○) are for the negative controls. The results are the means for 2 representative experiments.

We next examined the effect of lipid X on the tumoricidal activity of the macrophages. As shown in table 1, lipid X caused peptone-induced mouse

Table 1

Effects of LPS, lipid A, lipid X and deacylated and dephosphorylated derivatives of the latter on tumor cell killing by peptone-induced mouse peritoneal macrophages

Reagent dose (μg/ml)	Expt 1			Expt 2			
	LPS	Lipid A	Lipid X	LPS	Lipid X	Deacyl X	Dephospho X
0	0.0 ± 0.0	–	–	−4.8 ± 0.7	–	–	–
0.1	8.4 ± 3.0	1.8 ± 0.8	N.T.	N.T.	N.T.	N.T.	N.T.
1	9.9 ± 1.7	2.2 ± 12.0	2.6 ± 1.0	12.9 ± 5.0	−4.1 ± 2.4	−2.0 ± 2.0	0.4 ± 1.3
10	29.1 ± 4.5	8.2 ± 1.8	9.4 ± 2.2	15.2 ± 1.2	27.5 ± 1.8	−4.8 ± 1.9	−0.6 ± 0.4
50	N.T.	20.9 ± 2.0	26.9 ± 2.3	21.5 ± 1.5	17.6 ± 2.9	−2.8 ± 2.0	−0.8 ± 0.8
100	N.T.	26.9 ± 4.8	29.8 ± 1.9	23.6 ± 4.5	14.0 ± 3.6	−2.5 ± 2.2	−2.0 ± 0.8

Experimental details were as given in [8], and cytotoxicity is expressed as % lysis, defined as [(experimental release) − (spontaneous release)]/[(maximal release) − (spontaneous release)] × 100. Results are means ± SD for triplicate samples for representative experiments. N.T., not tested

peritoneal macrophages to kill ^{51}Cr-labeled EL4 leukemic cells just as LPS and lipid A did, although the effective dose of lipid X was considerably higher than that of LPS. Again, neither the deacylated nor dephosphorylated derivative of lipid X was effective in this system.

4. DISCUSSION

Lipid X is a biosynthetic precursor of lipid A in *E. coli* [6,7]. Judging from previous results with macrophages [8] and the present data, and also with B-lymphocytes [12], lipid X retains the biological activities of lipid A but the deacylated and dephosphorylated derivatives of lipid X have no effects, showing that the 1-phosphate and 3-*O*-β-hydroxymyristoyl groups are essential for manifestation of the biological activities of lipid X.

O_2^- generation and tumor-cytotoxic reactions are important criteria for the activation of macrophages [2,10]. Furthermore, O_2^- is one of the active molecules that attack tumor cells in macrophage-mediated cytotoxic reactions [13]. This study showed that lipid X enhanced the O_2^- generation in mouse peritoneal macrophages and a macrophage-like cell line, J774.1 (figs 2 and 3), and induced cell-mediated cytotoxicity against EL4 leukemic cells in vitro (table 1). In addition, lipid X induced marked production of a TNF by J774.1 cells in the culture medium, and this factor effectively killed L929 tumor cells in vitro (submitted). These results in vitro suggest that lipid X may be useful in cancer immunotherapy in vivo through its induction of the production of O_2^-, TNF and so on by macrophages. Recently, Takayama et al. [14] reported that lipid X was far less toxic, as judged from its pyrogenicity and the results of a lethal dose test with chicken embryos. These results also seem to show the advantages of the therapeutic application of lipid X as an immunopotentiator with low toxicity.

ACKNOWLEDGEMENTS

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