

Synthesis of [^{3}H]-Labeled Bioactive Lipid A Analogs and Their Use for Detection of Lipid A-Binding Proteins on Murine Macrophages[1]

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Both endotoxic and antagonistic [^{3}H]-labeled 2-(phosphonooxy)ethyl (PE) analogs of lipid A were synthesized with high purity and high specific radioactivity. Lipid A-binding proteins were detected by using the endotoxic analog of hexaacyl *Escherichia coli*-type designated [^{3}H] PE-506. The plasma membrane fractions from peritoneal macrophages derived from LPS-responder C3H/HeN mice and LPS-hyporesponder C3H/HeJ mice were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were then incubated with the [^{3}H] PE-506. Several [^{3}H] PE-506 binding proteins were detected in both C3H/HeN and C3H/HeJ macrophages. Unlabeled hexaacyl lipid A inhibited the interaction between [^{3}H] PE-506 and these proteins. The result suggests that there exist multiple binding sites for lipid A on macrophages. LPS-induced change in the profile of the cell surface lipid A binding proteins was observed in C3H/HeN macrophages, but not in C3H/HeJ macrophages, by preincubation of macrophages with LPS.

Lipopolysaccharide (LPS), a cell surface glycoconjugate characteristic of Gram-negative bacteria, has been well-known as an endotoxin. LPS stimulates immunocompetent cells such as macrophages, monocytes, and endothelial cells to produce mediators such as cytokines, prostagrandins, platelet activating factor, oxygen free radicals, and NO. These mediators activate immune system and also cause clinical manifestations of bacterial infections such as fever, inflammation, hypotension, and, in severe cases, lethal shock.[2,3] LPS consists of a glycolipid component termed lipid A covalently bound to a polysaccharide part. In our previous study, we have clearly demonstrated by means of the total synthesis of *Escherichia coli* lipid A **1** that lipid A is the chemical entity responsible for the biological activities of LPS (a synthetic **1** is termed compound 506) (Fig. 1).[4,5] Lipid A samples of various bacterial origins were shown to be structurally closely related and to consist of: 1) β (1→6) disaccharide of two D-glucosamines, 2) phosphono groups at the reducing end and the 4-position of the non-reducing sugar, and 3) long-chain acyl groups bound at 2, 2′, 3, and 3′ positions. It has been shown that both phosphono groups at the 1- and 4′-positions and acyl groups are crucial for the biological activities of lipid A.[6–11] For example, a biosynthetic precursor of LPS **2** (a synthetic **2** is termed compound 406) which has four 3-hydroxytetradecanoyl groups shows antagonistic activity in humans (Fig. 1).

The recognition mechanism of endotoxin by host animals has been extensively studied and a multiple receptor system, including CD14, CD55, and Toll-like receptor 4, has been identified. The membrane-bound CD14 (mCD14), which is a glycosylphosphatidylinositol (GPI)-linked protein and is anchored on myeloid lineage cells,[12] lacks transmembrane and intracellular signaling domains. CD14, though it binds LPS, probably works as a LPS shuttle for presenting LPS to a transmembrane receptor, or LPS/CD14 complexes interact with a transmembrane receptor responsible for signal transduction. Recently, the Toll protein family was first identified in *Drosophila* as proteins characterized by leucine-rich repeats and involved in responses to products of microbial pathogens.[13] Two independent groups then reported that Toll-like receptor 2 (TLR2) in humans conferred LPS responsiveness.[14,15] TLR2 has an intracellular signaling domain with sequence homology to the interleukin-1 (IL-1) receptor. Both studies demonstrated that LPS induced activation of the transcription factor, nuclear factor κB (NF-κB), via the IL-1 receptor-like signaling cascade. Poltorak et al. found that LPS hyporesponsive C3H/HeJ mice had a mutation on the Toll-like receptor-4 gene (*Tlr4*), where a highly conserved proline is replaced with histidine at position 712.[16] Another LPS-resistant strain C57BL/10ScCr mice exhibited a deletion of *Tlr4*. Golenbock et al. showed that LPS stimulates NF-κB-mediated gene expression in cells transfected with *Tlr4*.[17] As mentioned above, compound 406 (**2**) shows antagonistic activity in humans but displays endotoxic activity in rodents. *Rhodobacter sphaeroides* lipid A (RSLA) also shows similar biological activity. Golenbock et al. demonstrated that the species-specific responses to **2** and RSLA are mediated by TLR4 but not by TLR2 and therefore TLR4 is the LPS receptor.[18] Other studies also demonstrated TLR4 is responsible for LPS signaling.[19,20] Miyake et al. reported that small protein MD-2 is associated with the extracellular domain of TLR4 and that TLR4-MD-2 complex signals the presence of LPS.[21] On the other hand, TLR2 was shown to be the receptor for peptidoglycan,[22–24] lipoproteins,[25] and li-

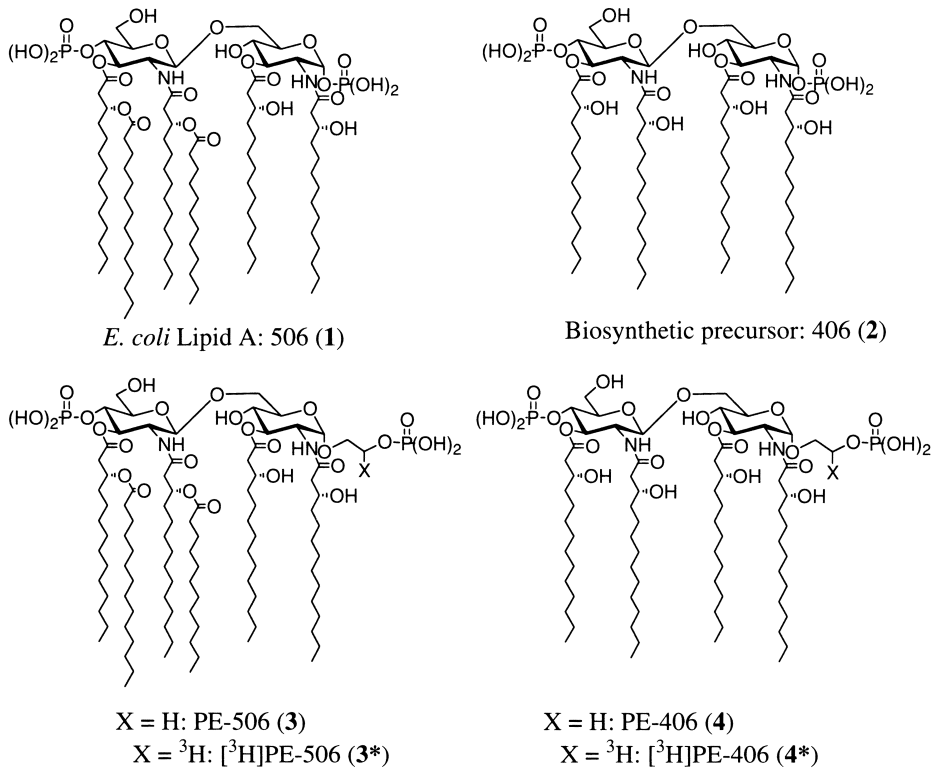


Fig. 1. Structure of lipid A and PE-analogs.

poteichoic acids.[23]

Various issues, however, remain to be solved: e.g., whether LPS directly binds TLR4, MD-2, or TLR4-MD2, or whether other proteins such as CD14 confer LPS binding function on the TLRs. Kirikae et al. reported that CD14-negative ST2 cells responded to LPS in serum-free medium.[26,27] This result suggests that LPS-signaling can be transmitted without CD14 but can not rule out the existence of other LPS binding molecules. Direct binding experiments of the membrane proteins with radiolabeled LPS or lipid A have been expected in order to investigate these issues. Preparation of homogeneous radiolabeled LPS or lipid A, however, has been difficult from natural sources. Since biological studies using heterogeneous samples may afford ambiguous results, chemical synthesis of homogeneous radiolabeled endotoxin equivalents has been strongly required. The large number of synthetic steps and the chemical instability of the glycosyl phosphate moiety, however, rendered the synthesis of radiolabeled compounds particularly difficult. 2-(Phosphonooxy)ethyl analogs [hexaacyl *E. coli* type: PE-506 (**3**) and tetraacyl biosynthetic precursor type: PE-406 (**4**)] of lipid As proved to be chemically stable and to exhibit activities indistinguishable from those of *E. coli* type lipid A **1** (506) and biosynthetic precursor type lipid A **2** (406), respectively (Fig. 1).[8–10] In addition, the ethylene glycol moieties of the PE analogs are suitable for labeling with tritium. In the present study, we synthesized two [^{3}H]-labeled bioactive lipid A analogs [[^{3}H] PE-506 (**3***) and [^{3}H] PE-406 (**4***)] with high chemical purity and high specific radioactivity. Several lipid A binding proteins were clearly detected on macrophages of both LPS-responder C3H/HeN and LPS-hyporesponder C3H/HeJ mice by using [^{3}H]-labeled bioactive lipid A analogs, suggesting the existence of a possible multiple recognition system of lipid A and LPS by macrophages.

Experimental

Materials and Measurements for Synthesis of [^{3}H]-Labeled Lipid A Analogs [*E. coli* Type: [^{3}H] PE-506 (3*), Biosynthetic Precursor Type: [^{3}H] PE-406 (4*)]. Silica-gel column chromatography was carried out using Kieselgel 60 (E. Merck. 0.040–0.063 mm) at medium-pressure (2–4 kg cm^{-2}). Sephadex® LH-20 was purchased from Pharmacia Biotech, Sweden. Anhydrous CH_2Cl_2 and $CHCl_3$ were prepared by distillation from calcium hydride. Anhydrous tetrahydrofuran (THF) and benzene were purchased from Kanto Chemicals, Tokyo. NaB^3H_4 (specific activity 222 GBq mmol^{-1}) was purchased from Amersham LIFE SCIENCE, Japan. Autoradiography was carried out by the use of Fuji imaging plate BAS-TR2040S (Fuji Photo Film Co., Tokyo, Japan) with Bio-imaging Analyzer BAS-1500 MAC (Fuji).

Formylmethyl 4-*O*-Benzyl-6-*O*-[6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3-yl)-2-[(*R*)-3-(dodecanoyloxy)tetradecanoylamino]-3-*O*-[(*R*)-3-(tetradecanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-3-*O*-[(*R*)-3-(benzyloxy)tetradecanoyl]-2-[(*R*)-3-(benzyloxy)tetradecanoylamino]-2-deoxy-α-D-glucopyranoside (6). To a vigorously stirred solution of **5**[10] (450 mg, 202 μmol) in THF/*t*-butyl alcohol/water (10:10:1, 12 mL) at rt were added 4-methylmorpholine *N*-oxide (NMO) (94 mg, 0.80 mmol) and OsO_4 in water (2.5%, 400 μL, 40 μmol). After 6 h, saturated aqueous $Na_2S_2O_3$ (50 mL) was added, and the mixture was extracted with EtOAc (50 mL). The EtOAc layer was washed successively with saturated aqueous $Na_2S_2O_3$ (50 mL × 2) and brine (20 mL), dried over Na_2SO_4, and concentrated in vacuo to give a crude diol (458 mg). The crude diol thus obtained was dissolved in anhydrous benzene (10 mL).

To this solution was added lead(IV) acetate (Pb $(OAc)_4$) (90% purity, 119 mg, 242 μmol). After 30 min, the mixture was filtered through a silica-gel column (3 g) using EtOAc as an eluent. After removal of the solvent in vacuo, the residue was purified by silica-gel flash chromatography (20 g, toluene/EtOAc = 5:1) to give **6** as a colorless syrup (377 mg, 84%). FAB-MS (positive) *m/z* 2244 $[(M + Na)^+]$; ^1H NMR (500 MHz, $CDCl_3$) δ 9.37 (s, 1 H, CHO).

2-Hydroxy-[2-$^3H_1$]ethyl 4-*O*-Benzyl-6-*O*-[6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3-yl)-2-[(*R*)-3-(dodecanoyloxy)tetradecanoylamino]-3-*O*-[(*R*)-3-(tetradecanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-3-*O*-[(*R*)-3-(benzyloxy)tetradecanoyl]-2-[(*R*)-3-(benzyloxy)tetradecanoylamino]-2-deoxy-α-D-glucopyranoside (7*). To a solution of **6** (150 mg, 62.4 μmol) in 2-propanol/methanol/CH_2Cl_2 (5:1:1, 3.5 mL) at 0 °C was added NaB^3H_4 (590 μL, 26.4 μmol/mL, 240 GBq $mmol^{-1}$). After being stirred for 30 min, the reaction was stopped with saturated aqueous NH_4Cl, and the mixture was extracted with EtOAc. The extract was dried over Na_2SO_4 and concentrated in vacuo to give colorless syrup in a quantitative yield (144 mg). The product was subjected to the next reaction without purification.

2-(1,5-Dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3-yloxy)-[2-$^3H_1$]ethyl 4-*O*-Benzyl-6-*O*-[6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3-yl)-2-[(*R*)-3-(dodecanoyloxy)tetradecanoylamino]-3-*O*-[(*R*)-3-(tetradecanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-3-*O*-[(R)-3-(benzyloxy)tetradecanoyl]-2-[(*R*)-3-(benzyloxy)tetradecanoylamino]-2-deoxy-α-D-glucopyranoside (8*). To a solution of **7*** (144 mg, 59.9 μmol) in CH_2Cl_2 (14 mL) at 0 °C were added *N*,*N*-diethyl-1,5-dihydro-3*H*-2,4,3-benzodioxaphosphepin-3-amine (89 mg, 0.37 mmol) and 1*H*-tetrazole (25 mg, 0.32 mmol). The mixture was stirred at rt for 30 min and then cooled to −78 °C. *m*CPBA (70%, 81 mg, 0.37 mmol) was added to the reaction mixture, and stirring was continued for another 45 min. The excess reagent was quenched with saturated aqueous $Na_2S_2O_3$, and the mixture was extracted with $CHCl_3$. The $CHCl_3$ solution was washed successively with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica-gel column chromatography [3.0 g, toluene/EtOAc/hexafluoro-2-propanol (HFIP) = 100:50:1] to give a colorless syrup (134 mg, 87%). The product was chromatographically identified with unlabeled **8** obtained by the same procedure using cold $NaBH_4$. Data for unlabeled **8**: $[\alpha]_D^{25}$ = +17.8 (*c* 1.00, $CHCl_3$); FAB-MS (positive) *m/z* 2428 $[(M + Na)^+]$. Found: C, 69.96; H, 9.21; N, 1.14%. Calcd for $C_{140}H_{218}N_2O_{26}P_2$: C, 69.85; H, 9.13; N, 1.16%.

2-(Phosphonooxy)[2-$^3H_1$]ethyl 2-Deoxy-6-*O*-[2-deoxy-2-[(*R*)-3-(dodecanoyloxy)tetradecanoylamino]-4-*O*-phosphono-3-*O*-[(*R*)-3-(tetradecanoyloxy)tetradecanoyl]-β-D-glucopyranosyl]-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[(*R*)-3-hydroxytetradecanoylamino]-α-D-glucopyranoside ([^{3}H] PE-506: 3*). To a solution of **8*** (57 mg, 22 μmol) in THF (8 mL) was added Pd-black (130 mg). The mixture was stirred under 7 kg cm^{-2} of hydrogen at rt for 2 h. The reaction mixture was then neutralized with triethylamine. After removal of the Pd catalyst by filtration, the solvent was evaporated in vacuo. The residue was purified by liquid–liquid partition column chromatography (20 g of Sephadex® LH-20, $CHCl_3$/methanol/water/2-propanol = 9:9:9:1), wherein the organic layer was the stationary phase and the aqueous layer was the mobile phase, to give a white powder (25 mg, 841 MBq, 62 GBq $mmol^{-1}$, 63%). The product was chromatographically identified with unlabeled **3** obtained in the same manner and fully characterized. Data for unlabeled **3**: FAB-MS (negative) *m/z* 1840 $[(M - H)^-]$; ^1H NMR (600 MHz, $CD_3OD/CDCl_3$ = 1:1) δ 5.23 (m, 1 H), 5.19 (t, *J* = 8.2 Hz, 1 H), 5.17 (m, 1 H), 5.14 (t, *J* = 8.2 Hz, 1 H), 4.82 (d, *J* = 3.0 Hz, 1 H), 4.56 (d, *J* = 7.4 Hz, 1 H), 4.23 (q, *J* = 8.0 Hz, 1 H), 4.18 (dd, *J* = 8.9, 3.0 Hz, 1 H), 4.08–3.93 (m, 5 H), 3.92–3.82 (m, 4 H), 3.80 (dd, *J* = 10.3, 4.5 Hz, 1 H), 3.74 (d, *J* = 10.6 Hz, 1 H), 3.63 (m, 1 H), 3.56 (t, *J* = 8.1 Hz, 1 H), 3.37 (m, 1 H), 2.72 (dd, *J* = 14.0, 6.6 Hz, 1 H), 2.64 (dd, *J* = 14.0, 4.5 Hz, 1 H), 2.52–2.46 (m, 2 H), 2.44–2.36 (m, 2 H), 2.36–2.27 (m, 6 H), 1.66–1.39 (m, 12 H), 1.38–1.20 (m, 108 H), 0.89 (t, *J* = 5.6 Hz, 18 H).

Formylmethyl 4-*O*-Benzyl-6-*O*-[6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3-yl)-2-[(*R*)-3-(benzyloxy)tetradecanoylamino]-3-*O*-[(*R*)-3-(benzyloxy)tetradecanoyl]-β-D-glucopyranosyl]-3-*O*-[(*R*)-3-(benzyloxy)tetradecanoyl]-2-[(*R*)-3-(benzyloxy)tetradecanoylamino]-2-deoxy-α-D-glucopyranoside (10). To a vigorously stirred solution of **9**[28] (230 mg, 0.15 mmol) in THF/*t*-butyl alcohol/water (10:10:1, 9 mL) at rt were added NMO (40 mg, 0.45 mmol and OsO_4 in water (2.5%, 230 μL, 0.02 mmol). After 6 h, saturated aqueous $Na_2S_2O_3$ (50 mL) was added, and the mixture was extracted with EtOAc (50 mL). The EtOAc layer was successively washed with saturated aqueous $Na_2S_2O_3$ (50 mL × 2) and brine (20 mL), dried over Na_2SO_4, and concentrated in vacuo to give a crude diol (228 mg), which was dissolved in anhydrous benzene (6 mL). To this solution was added lead(IV) acetate as described above (90% purity, 68 mg, 0.14 mmol). After 30 min, the mixture was filtered through a silica-gel column (3 g) using EtOAc as an eluent. After removal of the solvent in vacuo, the residue was purified by silica-gel flash chromatography (6 g, toluene/EtOAc = 4:1) to give **10** as a colorless syrup (190 mg, 83%). FAB-MS (positive) *m/z* 2032 $[(M + Na)^+]$; ^1H NMR (500 MHz, $CDCl_3$) δ 9.30 (s, 1H, CHO).

2-Hydroxy-[2-$^3H_1$]ethyl 4-*O*-Benzyl-6-*O*-[6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3-yl)-2-[(*R*)-3-(benzyloxy)tetradecanoylamino]-3-*O*-[(*R*)-3-(benzyloxy)tetradecanoyl]-β-D-glucopyranosyl]-3-*O*-[(*R*)-3-(benzyloxy)tetradecanoyl]-2-[(*R*)-3-(benzyloxy)tetradecanoylamino]-2-deoxy-α-D-glucopyranoside (11*). To a solution of **10** (100 mg, 49.8 μmol) in 2-propanol/methanol (5:1, 1.46 mL) at 0 °C was added NaB^3H_4 (474 μL, 26.3 μmol/mL, 240 GBq $mmol^{-1}$). After being stirred for 30 min, the excess reagent was quenched with 2 M HCl and brine, and the mixture was extracted with $CHCl_3$. The extract was dried over Na_2SO_4 and concentrated in vacuo to give colorless syrup in a quantitative yield (100 mg).

2-(1,5-Dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3-yloxy)-[2-$^3H_1$]ethyl 4-*O*-Benzyl-6-*O*-[6-*O*-benzyl-2-deoxy-4-*O*-(1,5-dihydro-3-oxo-3λ^5-3*H*-2,4,3-benzodioxaphosphepin-3-yl)-2-[(*R*)-3-(benzyloxy)tetradecanoylamino]-3-*O*-[(*R*)-3-(benzyloxy)tetradecanoyl]-β-D-glucopyranosyl]-3-*O*-[(*R*)-3-(benzyloxy)tetradecanoyl]-2-[(*R*)-3-(benzyloxy)tetradecanoylamino]-2-deoxy-α-D-glucopyranoside (12*). To a solution of **11*** (100 mg, 49.8 μmol) in CH_2Cl_2 (14 mL) at 0 °C were added *N*,*N*-diethyl-1,5-dihydro-3*H*-2,4,3-benzodioxaphosphepin-3-amine (70 mg, 0.29 mmol) and 1*H*-tetrazole (20 mg, 0.25 mmol). The mixture was stirred at rt for 30 min and then cooled to −20 °C. *m*CPBA (70%, 65 mg, 0.29 mmol) was added, and stirring was continued for another 40 min. After addition of a saturated aqueous $NaHCO_3$ solution, the mixture was extracted with $CHCl_3$. The $CHCl_3$ solution was washed successively with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated in vac-

uo. The residue was purified by silica-gel column chromatography (2.3 g, toluene/EtOAc/HFIP = 100:50:1) to give **12*** as a colorless syrup (63.9 mg, 59%). The product was chromatographically identified with unlabeled **12** obtained in the same manner and fully characterized. Data for unlabeled **12**: $[\alpha]_D^{25} = +16.9$ (*c* 1.00, $CHCl_3$); FAB-MS (positive) *m/z* 2428 [$(M + Na)^+$]. Found: C, 69.98; H, 8.40; N, 1.33%. Calcd for $C_{128}H_{182}N_2O_{24}P_2$: C, 70.05; H, 8.36; N, 1.28%.

2-(Phosphonooxy)[2-$^3H_1$]ethyl 2-Deoxy-6-*O*-[2-deoxy-2-[(*R*)-3-hydroxytetradecanoylamino]-4-*O*-phosphono-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-β-D-glucopyranosyl]-3-*O*-[(*R*)-3-hydroxytetradecanoyl]-2-[(*R*)-3-hydroxytetradecanoylamino]-α-D-glucopyranoside ([^{3}H] PE-406: 4*). To a solution of **12*** (17 mg, 7.8 μmol) in THF (6 mL) was added Pd-black (120 mg). The mixture was stirred under 7 kg cm^{-2} of hydrogen at rt for 2 h. The reaction mixture was then neutralized with triethylamine. After removal of the Pd catalyst by filtration, the solvent was evaporated in vacuo. The residue was purified by liquid–liquid partition column chromatography (20 g of Sephadex® LH-20, $CHCl_3$/methanol/water/2-propanol = 8:8:9:1), wherein the organic layer served as the stationary phase and the aqueous layer was the mobile phase, to give a white powder (7.5 mg, 331 MBq, 64 GBq $mmol^{-1}$, 66%) after lyophilization. The product was chromatographically identified with unlabeled **4** obtained in the same manner and fully characterized. Data for unlabeled **4**: FAB-MS (negative) *m/z* 1448 [$(M - H)^-$]. ^{1}H NMR (500 MHz, SDS-d_{25}–D_2O) δ 5.18 (dd, *J* = 9.4, 7.5 Hz, 1H), 5.17 (dd, *J* = 9.8, 9.6 Hz, 1 H), 4.87 (d, *J* = 3.8 Hz, 1 H), 4.67 (d, *J* = 7.0 Hz, 1 H), 4.12 (dd, *J* = 9.8, 3.8 Hz, 1 H), 4.11 (dd, *J* = 15.5, 1.6 Hz, 1 H), 4.11 (q, *J* = 7.5 Hz, 1 H), 4.02 (dd, *J* = 9.4, 7.0 Hz, 1 H), 3.99 (m, 1H), 3.96 (m, 1H), 3.91 (m, 3 H), 3.87 (ddd, *J* = 9.6, 1.6, 2.9 Hz, 1 H), 3.82 (dd, *J* = 9.8, 8.6 Hz, 1 H), 3.82 (dd, *J* = 15.5, 2.9 Hz, 1 H), 3.82 (m, 1H), 3.81 (m, 3H), 3.71 (m, 1H), 3.59 (m, 1H), 2.56–2.23 (m, 8 H), 1.54–1.11 (m, 80 H), 0.86 (m, 12 H).

Lipid A and Its Analog. *E. coli* lipid A (506: **1**) and its phosphonooxyethyl analog (PE-506: **3**) were synthesized as described in our previous paper.[10] Synthesis of biosynthetic precursor type lipid A (406: **2**) was also described.[29] PE-406 (**4**) was synthesized as described in the previous paper.[10,28]

LPS. The Re-chemotype LPS (Re-LPS) from *Salmonella minnesota* R595 was kindly provided by Prof. K. Hisatsune, Josai University, Sakado, Japan. The *S*-form LPS from *S. minnesota* wild-type (S-LPS) was purchased from Sigma Chemical Co., St. Louis, Mo.

Animals. LPS-responsive C3H/HeN mice and LPS-hyporesponsive C3H/HeJ mice aged 6 to 7 weeks were obtained from Nippon Clea Co., Tokyo, Japan and maintained under standard conditions. The mice were used at 8 to 10 weeks of age.

Antiserum against ST2 Cell Membrane Antigen. Purified plasma membrane fraction was prepared from ST2 cells, derived from bone marrow stroma cells of BALB/c mice, according to the literature.[30] Antiserum against ST2 cell membrane antigen was obtained by immunizing rabbits with the purified fraction.

Macrophage Isolation and Culture. Murine peritoneal macrophages were isolated by peritoneal lavage 4 days after i.p. injection of 1.5 mL of 3% Brewer thioglycolate broth (Difco Laboratories, Detroit, Mich.). The cells were washed with serum-free RPMI 1640 medium (ICN Biomedicals, Costa Mesa, Calf.) containing 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin; plated at the concentration of 2 × 107 per dish in 100 mm culture dishes; cultured for 2 h at 37 °C with 5% CO_2; washed with serum-free RPMI 1640 medium to remove non-adherent cells; and then were incubated in the presence or absence of various doses of LPS for 24 h in 10 mL of RPMI 1640 medium containing 2% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, Lenexa, Kans). After the cultivation, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and used for detection of PE-506- or PE-406-binding proteins.

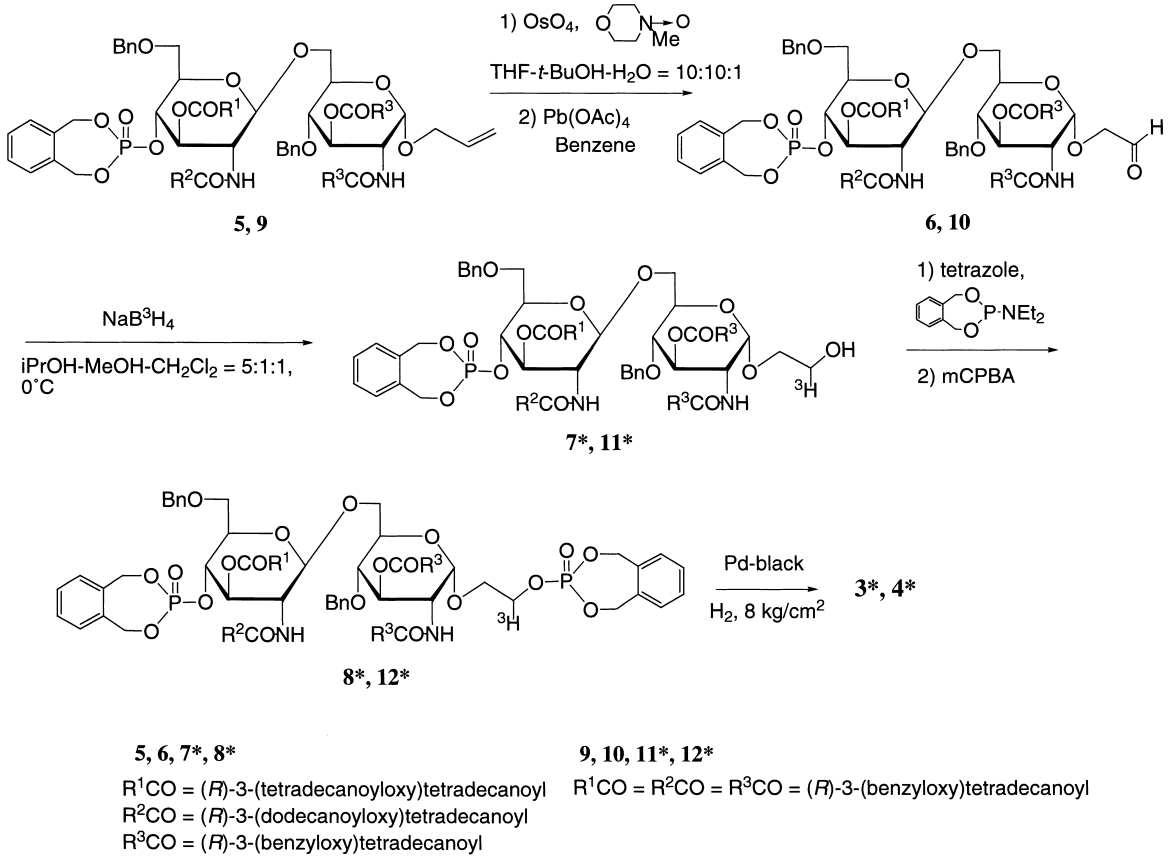
Detection of [^{3}H] PE-506- or [^{3}H] PE-406-Binding Proteins in Macrophages. The peritoneal macrophages were lysed with ice-cold PBS containing 0.5% sodium deoxycholate, 10% (v/v) Nonidet P-40, 10% sodium dodecyl sulfate, 0.5 M EDTA and 100 U/mL of aprotinin (Bayer Co., Leverkusen, Germany) and immunoprecipitated with protein A agarose (Life Technologies, Inc., Gaithersburg, Md.) and rabbit antiserum to ST2 cell membrane antibody. The precipitant was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% acrylamide gels with 3% stacking gels. Resolved proteins were blotted onto nitrocellulose membrane (0.2 mm, Bio-Rad Laboratories, Calif.). Blots were blocked by soaking for 1 h in PBS containing 0.1% Tween 20, then incubated for additional 1 h with 25 ng/mL of [^{3}H] PE-506 or [^{3}H] PE-406 in the presence or absence of 10 μg/mL of competitors, washed four times with 0.1% Tween 20, and dried. Fuji imaging plates (Fuji Photo Film Co.) exposed to the blots for 2-3 days were visualized with a Bio-image Analyzer BAS200 (Fuji).

Results

Synthesis of [^{3}H]-Labeled Bioactive Analogs of Lipid A. Previously, we reported the synthesis of [^{3}H] PE-506 (**3***) with ca. 4 GBq $mmol^{-1}$ of specific radioactivity ($^3H/^1H = 0.4\%$).[31] The total yield of the synthesis, however, was too low owing to several unsatisfactory synthetic steps and the specific radioactivity of the final product was not high enough for actual biological studies. We, therefore, improved the procedure and newly synthesized [^{3}H]-labeled PE analogs **3*** and **4*** corresponding to *E. coli* lipid A and the biosynthetic precursor, respectively, with high chemical and radiochemical purity and high specific radioactivity.

In our recent work, two unlabeled PE analogs, **3** and **4**, were successfully synthesized by an improved method in the yields over 20% for 12 steps starting from allyl 4,6-*O*-benzylidene-2-deoxy-2-(trichloroethoxycarbonylamino)-D-glucopyranoside.[10,28] In those syntheses, the α-glycosidically bound ethylene glycol unit was formed via oxidative cleavage of the glycosidic allyl group, followed by $NaBH_4$ reduction of the aldehydes formed. This synthetic route is directly applicable to the preparation of the corresponding [^{3}H]-labeled PE analogs (**3*** and **4***) by using NaB^3H_4 in place of $NaBH_4$.

The synthetic route for [^{3}H]-labeled analog **3*** of *E. coli* lipid A is shown in Scheme 1. The allyl group of **5** was dihydroxylated by using a catalytic amount of osmium(VIII) oxide (OsO_4) and 4-methylmorpholine *N*-oxide (NMO). Oxidative cleavage of the resulting diol with lead(IV) acetate ($Pb(OAc)_4$) afforded aldehyde **6** in 84% yield.[10] Aldehyde **6** was reduced by the use of NaB^3H_4 to give the [^{3}H]-labeled alcohol **7*** in a quantitative yield. Phosphitylation of **7*** and subsequent oxidation of the phosphite with *m*CPBA gave the bisphosphate **8***. We recently found that the benzyl protecting groups on the 3-hydroxyacyl moieties were so susceptible to oxidation by even molecular oxygen that a considerable portion of the benzyl group was oxidized by *m*CPBA into benzoyl groups. In-



Scheme 1.

deed, oxidation of the corresponding benzylic positions of the [^{3}H]-labeled compounds was more evidently observed in the present study because of their radioactivity: even during the storage and *m*CPBA treatment, the amount of benzoyl compounds apparently increased. Removal of the benzoylated compounds was difficult by silica-gel column chromatography with various conventional solvent systems. Removal of the phosphoramidate by-product formed by oxidation of the excess reagent was also difficult. This problem in purification was solved by the addition of a small amount of hexafluoro-2-propanol (HFIP) to the eluent for silica-gel column chromatography. Purification by silica-gel column chromatography by using toluene/EtOAc/HFIP (100:50:1) as an eluent thus afforded highly purified **8*** in 87% yield. Finally, one-step hydrogenolytic cleavage of all the benzyl-type protecting groups was carried out with Pd-black under 7 kg cm^{-2} of hydrogen atmosphere to give [^{3}H] PE-506 (**3***). The final product was purified by liquid-liquid partition column chromatography on Sephadex® LH-20 gel to give **3*** in 63% yield with 62 GBq mmol^{-1} of specific radioactivity. The radiochemical purity of the final product **3*** was estimated to be higher than 98% by autoradiography of TLC using an imaging plate (Fig. 2).

Synthesis of [^{3}H] PE-406 (**4***) was carried out in a similar manner starting from the corresponding allyl glycoside **9** (Scheme 1). Tritium-labeled precursor-type analog **4*** was thus obtained with 64 GBq mmol^{-1} of specific radioactivity and a radiochemical purity higher than 98% (Fig. 3).

Detection of [^{3}H] PE-506-Binding Proteins in Murine Macrophages. We have demonstrated previously that a murine marrow-derived stromal ST2 cell line responds to LPS and paclitaxel (Taxol), an LPS-agonist, in IL-6 production.[26] Although ST2 cells do not express mRNA for CD14, an LPS-binding protein, the cells produce IL-6 and express IL-6 mRNA in response to LPS in a serum-free condition (i.e., without soluble CD14). Taxol also induces IL-6 mRNA expression in ST2 cells. *Rhodobacter sphaeroides* lipid A (RSLA) inhibited both LPS- and Taxol-induced IL-6 mRNA expression. These results suggest that LPS, RSLA, and Taxol are recognized by the same receptor or receptor complex on ST2 cells, and that the receptor functions without CD14. To detect the receptor(s), therefore, we prepared an antiserum that was made from rabbits immunized with a purified plasma membrane fraction from ST2 cells, and tried to detect the molecule(s), using the antiserum, which might be expressed in murine macrophages in response to LPS. The peritoneal exudate macrophages of LPS-responsive C3H/HeN and LPS-hyporesponsive C3H/HeJ mice were lysed and immunoprecipitated with rabbit anti-ST2 cell membrane antibody. After separation by SDS-PAGE, the precipitant was electroblotted onto nitrocellulose membranes. The blot was incubated with [^{3}H] PE-506 (**3***) in the presence or absence of competitors and visualized with an image analyzer. In the absence of competitors, [^{3}H] PE-506 (**3***) bound predominantly to a protein (50 kDa) corresponding in molecular mass to the heavy chain of immunoglobulin derived from the rabbit antiserum (Fig. 4). Other [^{3}H] PE-506-binding proteins were detected, including 80 kDa

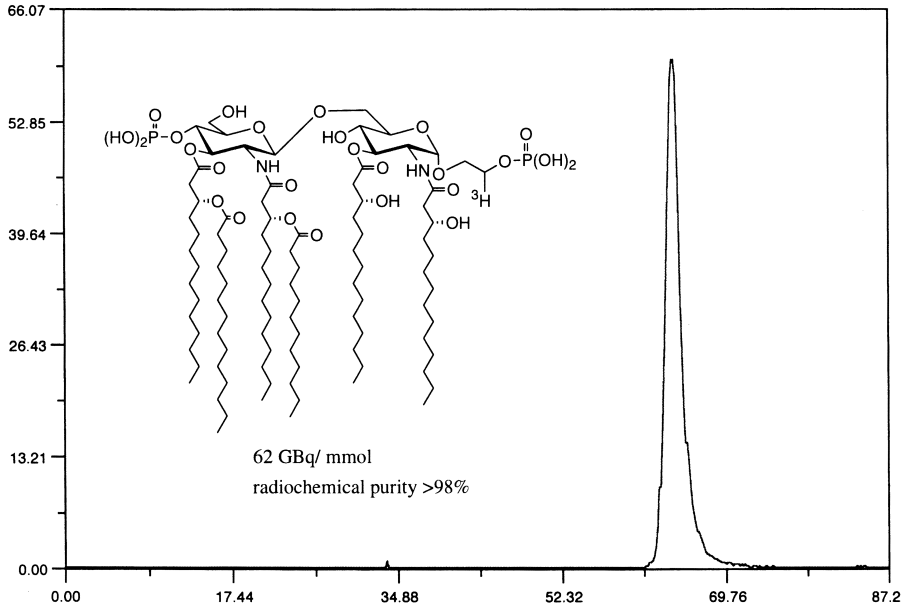


Fig. 2. Autoradiography of silica-gel TLC ($CHCl_3$–MeOH–H_2O–Et_3N = 60:40:10:0.2) of [^{3}H] PE-506.

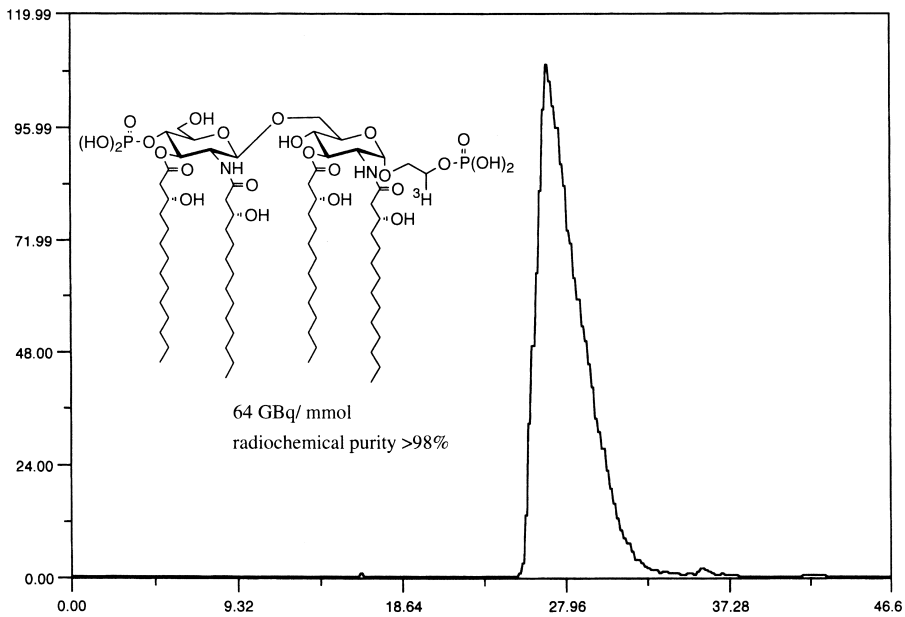


Fig. 3. Autoradiography of silica-gel TLC ($CHCl_3$–MeOH–H_2O–Et_3N = 70:50:10:0.2) of [^{3}H] PE-406.

protein (p80), a doublet of 66 kDa and 65 kDa proteins (p66 and p65), and a doublet of 60 kDa proteins (p60, p60′). In addition, no difference in the pattern of [^{3}H] PE-506-binding proteins was observed between C3H/HeN and C3H/HeJ macrophages. To identify the bands involved in the specific recognition process of lipid A, various competitors were examined for their ability to inhibit [^{3}H] PE-506 binding. Excess of cold PE-506 (**3**) and *E. coli* lipid A (506: **1**) inhibited effectively the binding of [^{3}H] PE-506 to the heavy chain of immunoglobulin, p80, p66, p65, one of the proteins with 60 kDa. This protein p60 had a slightly smaller molecular weight than that of the other p60′ and a weak but fine signal of p60′ was still detected in the presence of PE-506. By contrast, excess of compound 406 (**2**) did not inhibit the binding of [^{3}H] PE-506 to all of the above-mentioned binding proteins including p60′. S-form LPS and Re-chemotype LPS inhibited the binding to p80, p60, and p60′, but not the binding to the heavy chain of immunoglobulin and a doublet of p66 and p65. LPS preparations inhibited the binding to p60 and p60′ more efficiently than PE-506 and 506, but inhibition by LPS was less efficient for the binding to p80. No significant difference in the inhibition pattern was observed between C3H/HeN and C3H/HeJ macrophages. It should be considered that the competitors must be equilibrated between the monomeric form and the aggregated form at 10 μg/mL (The reported critical aggregation concentrations are *S. minnesota* Re 595: 10 μg/mL (4 μM), Lipid A from *S.*

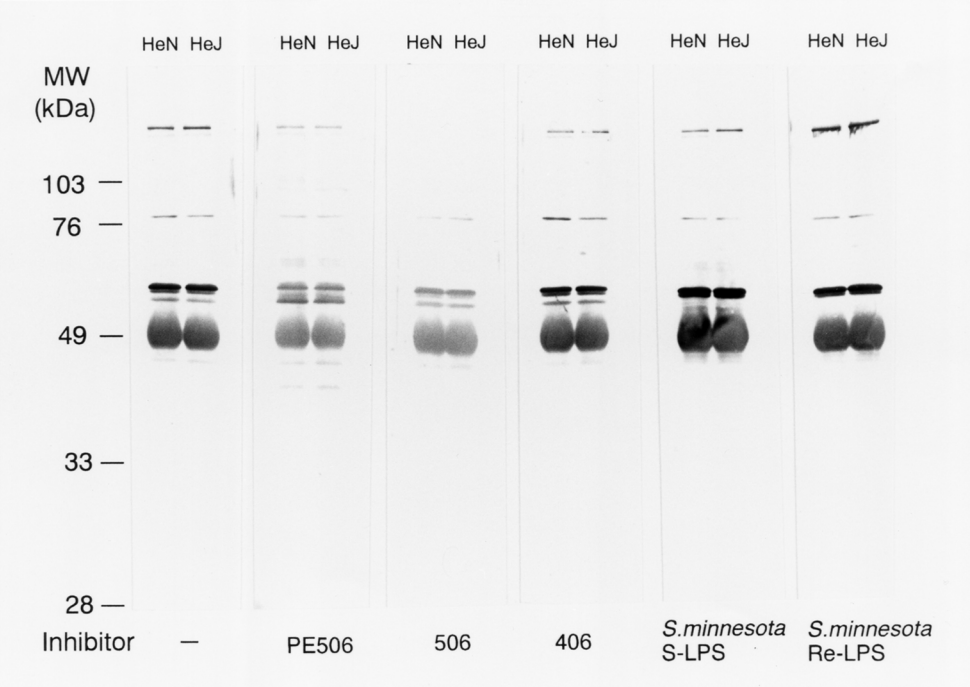


Fig. 4. Detection of [^{3}H] PE-506-binding proteins in murine macrophages.

minnesota Re 595: 10 μg/mL (5 μM), *S. minnesota* wildtype: 11 μg/mL).[32] The aggregates of competitors, in particular PE-506 and 506, can incorporate [^{3}H] PE-506 present in the medium. Some of the proteins probably bind to the aggregates containing radiolabeled compounds. This is the plausible reason why some protein bands did not completely disappear even in the presence of large excesses of competitors.

The above results suggest that p80, p66, p65, p60, and p60′ are specific lipid A-binding proteins. But they have different structural requirements for the binding of lipid A and LPS. The p80, p66, and p65 preferentially bind to lipid A. The p80, but neither p66 nor p65, is also able to bind to LPS. The p60 preferentially binds to LPS.

Detection of [^{3}H] PE-406-Binding Proteins in Murine Macrophages. [^{3}H] PE-406 (**4***) were tested to detect specific lipid A-binding proteins in murine macrophage in a manner similar to the above binding experiment with **3***. In the absence of competitors, [^{3}H] PE-406 (**4***) bound many minor proteins in addition to [^{3}H] PE-506 binding proteins, p80, p66, p65, and p60. No significant difference in the inhibition pattern was observed between C3H/HeN and C3H/HeJ macrophages, either. Excess of cold PE-406 (**4**) and 406 (**2**) did not inhibit the binding. Cold 506 seemed to slightly inhibit the binding. S-form LPS and Re-chemotype LPS also partly inhibited the binding to p60. The reason why binding of [^{3}H] PE-406 to these proteins was not clearly inhibited by competitors is probably the same as that described for [^{3}H] PE-506. In addition, some of the observed binding of [^{3}H] PE-406 might be not specific. Although the critical aggregation concentration of 406 was unknown, we recently found that both 406 and PE-406 form a similar supramolecular structure in SDS micelles.[28]

Effects of LPS Treatment on Expression of [^{3}H] PE-506-Binding Proteins in Murine Macrophages. We then investigated the effect of LPS-induced cell activation to the expression of the [^{3}H] PE-506-binding proteins on both C3H/HeN and C3H/HeJ macrophages. Macrophages were incubated for 24 h with various concentrations of *S. minnesota* S-LPS. [^{3}H] PE-506-binding proteins on the treated macrophages were then detected in the same manner as above. As can be clearly seen in Fig. 5, p80 of C3H/HeN decreased in a dose-dependent manner and completely disappeared by the treatment with 10 ng/mL of *S. minnesota* S-LPS. In addition, three new high molecular weight proteins (> 100 kDa) were detected by the treatment with LPS in a dose-dependent manner on macrophages of C3H/HeN. No changes in cell surface [^{3}H] PE-506-

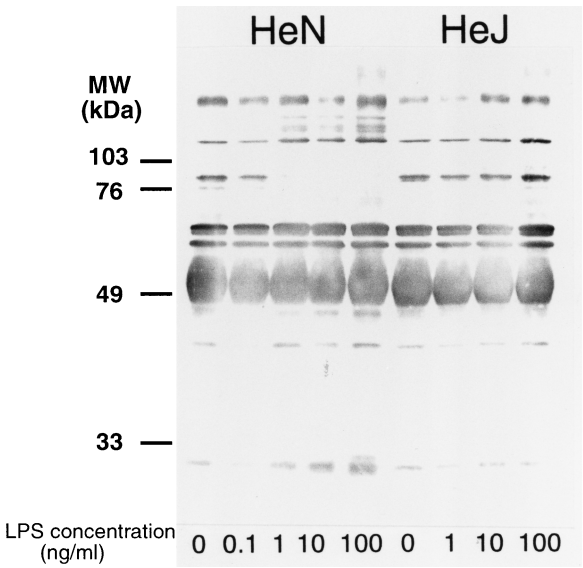


Fig. 5. Effects of LPS pretreatment on the profile of [^{3}H] PE-506-binding proteins in murine macrophages.

binding proteins on C3H/HeJ macrophage were observed by the pretreatment with LPS.

Discussions

The above results show that there exist multiple binding sites for lipid A and LPS on macrophages. The different inhibition patterns by *E. coli* lipid A and LPS might explain the differences in their biological action. Since *S. minnesota* S-LPS and *S. minnesota* R-LPS inhibited the binding of [^{3}H] PE-506 to p80, p60, and p60′, these proteins are LPS-binding proteins. The fact that no significant difference in the binding was observed between C3H/HeN and C3H/HeJ macrophages indicates that LPS and lipid A may be recognized by the receptor complex of both C3H/HeN and C3H/HeJ macrophages in the same manner, at least at the initial stage. We, however, found a difference in the pattern of cell surface LPS binding proteins between C3H/HeN and C3H/HeJ macrophages after stimulation with LPS. The band of p80 of C3H/HeN disappeared and three new proteins were detected over 100 kDa by the pretreatment with LPS. There are three possible explanations for the change of the pattern of the cell surface lipid A binding proteins. The first possibility is that p80 was just down-regulated by the contact of the cells with LPS and the three new proteins were newly expressed. Recently, Akira et al. reported the down-regulation of TLR4 on mouse macrophages by treatment of LPS.[33] The second possibility is that p80 formed stable complexes with LPS and/or other proteins to afford three new proteins of over 100 kDa. The other explanation is based on the following observations. Wright reported LPS internalization from the plasma membrane to an intracellular site but LPS-hyporesponsive mice exhibit a defect in LPS internalization.[34,35] Ulmer et al. also reported LPS internalization, which does not correspond to its activation kinetics in monocytes.[36] Internalization of p80 associated with LPS might, therefore, have occurred and three lipid A binding proteins might have been newly expressed.

The above binding studies on the nitrocellulose membrane were carried out under artificial conditions in the absence of serum proteins in order to observe direct binding of cell-surface proteins with lipid A analogs. Some of the above bindings, therefore, might be artificial. The important point is, however, that LPS-stimulated alteration in the profile of the cell surface lipid A binding proteins was observed by using the tritium-labeled bioactive lipid A analog.

Ulmer has reported the 80 kDa LPS-binding protein (LMP80), which is present on human monocytes and endothelial cells, and recently identified it as decay-accelerating factor (DAF, CD55).[37] They proved that CD55 is involved in LPS-signaling but did not observe direct binding of LPS or lipid A to TLRs by their detection methods, using monoclonal antibodies against lipid A and LPS. Our research now focuses on what these [^{3}H] PE-506-binding proteins are and whether these proteins include TLRs and CD55.

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