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Endotoxic-Lipopolysaccharide-Specific Binding Proteins on Lymphoid Cells of Various Animal Species: Association with Endotoxin Susceptibility

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Endotoxic lipopolysaccharide (LPS), a common structural component of all gram-negative bacteria, is well recognized for its capacity to interact with and perturb immunologically relevant cells. Using a radioiodinated, photoactivatable LPS probe, we have recently identified an 80-kilodalton LPS-specific binding protein on murine B lymphocytes. We now have extended these studies to determine if other mammalian species, as well as representative endotoxin-resistant species (frog and chicken), have a similar LPS-binding protein. We have identified what appears to be a relatively conserved 80-kilodalton LPS-binding protein on mononuclear cells of all mammalian species tested. However, both frog and chicken leukocytes failed to show the presence of a similar LPS-binding protein. It is possible that the presence of specific LPS-binding proteins may be important for endotoxin sensitivity of most mammalian species.

Bacterial lipopolysaccharides (LPS) are common constituents of the cell wall of gram-negative bacteria. These molecules are potent microbial toxins which contribute significantly to the pathophysiological manifestations of infections with gram-negative bacteria, including hypotension, intravascular coagulation, multisystem organ failure, shock, and, in the most severe situations, death (reviewed in references 21 and 22). Thus, despite major advances in intensive antibiotic therapy to treat infections, morbidity and mortality caused by endotoxin remain as serious problems in both human and veterinary medicine (2). In addition to the severe deleterious consequences of endotoxin, however, these molecules have been shown to be potent stimulants of the host immune system (reviewed in reference 20). In many species, LPS serves as a mitogen to stimulate B-lymphocyte proliferation and differentiation, as well as initiating the activation of mononuclear phagocytes. Multiple effects of LPS on immunologically active cells result in enhancement of immune responses to unrelated antigens, constituting the adjuvant properties of this immunostimulant.

Research carried out during the last several decades has provided considerable information on molecular components of LPS responsible for its potent biological activities. In this respect, it is now well recognized that the lipid A component of LPS, consisting of a β(1-6)-linked diglucosamine backbone with both amide- and ester-linked long-chain fatty acids as well as charged residues such as pyrophosphate, phosphorylethanolamine, and 4-amino arabinose, is important for both the toxic and immunostimulatory activities of LPS. Indeed, the recent elegant studies of Shiba et al. (25) and Rietschel et al. (E. T. Rietschel, L. Brade, U. Schade, U. Serydel, U. Zahringer, S. Kusumoto, and H. Brade, *in* U. Schwartz and M. Richmond, ed., *Surface Structures of Microorganisms and Their Interaction with the Mammalian Host*, in press), which demonstrated the biological activity of synthetic lipid A prepared by total organic synthesis, has provided convincing proof for the important role of lipid A in endotoxin responses.

Despite these advances in lipid A biochemistry, the mechanism by which LPS, and more specifically lipid A, interacts with cells of immunologic interest to initiate, e.g., B-lymphocyte or macrophage activation has not been determined. Many investigators have postulated the existence of specific LPS receptors on mammalian cells (reviewed in reference 9); however, the capacity of LPS to bind nonspecifically to mammalian cells has confounded many efforts to identify such receptors (17, 19). In an attempt to circumvent this problem, we have recently synthesized a photoreactive, radioiodinated LPS derivative which maintains all of the biological activity of native LPS (32). We have shown that this derivative of LPS may be used to detect specific binding to both heavy and light chains of monoclonal antibody directed against LPS as well as to human serum albumin (32).

In very recent studies we have used this photoreactive LPS to detect specific LPS-binding sites on murine lymphocytes and macrophages (11, 12). The results of these studies have shown the presence on both B and T lymphocytes, as well as on splenic macrophages, of a specific LPS-binding protein with a molecular mass of 80 kilodaltons (kDa) and a pI of approximately 6.5. This LPS-binding protein shows specificity for lipid A, and binding can be inhibited by both homologous and heterologous underivatized LPS as well as by purified lipid A. We have demonstrated that this 80-kDa LPS-binding protein is expressed on the cell membrane of lymphoid cells and have postulated that this protein might represent a specific receptor for LPS.

In this study, we have investigated whether similar LPS-binding proteins exist on the surface of peripheral blood mononuclear cells from other vertebrate species. The results presented here suggest that an 80-kDa LPS-binding protein may be a common constituent of many mammalian species. However, the LPS-binding protein does not appear to be expressed on lymphoid cells isolated from either chickens or frogs. This latter observation is particularly interesting in light of the reported insensitivity of these species to the toxic effects of endotoxic LPS (3, 5, 10).

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MATERIALS AND METHODS

LPS. LPS from *Escherichia coli* O111:B4 was extracted and purified by a modification of the phenol-water procedure of Westphal and Luderitz (29) as previously described (18) and was used for all of the studies reported here.

Animals. Mice of the C3HeB/FeJ inbred strain were purchased from Jackson Laboratories, Bar Harbor, Maine. New Zealand White rabbits were purchased from White Hare Rabbitry, Stark City, Mo. Outbred mongrel dogs were purchased from Brinks Kennel, Paola, Kans. White leghorn chickens 3 to 4 months of age were purchased from Colonial Poultry, Pleasant Hill, Mo. All of these animals were maintained in the animal facility at the University of Kansas Medical Center. Goats, horses, pigs, cows, and sheep were maintained at the University of Missouri School of Veterinary Medicine. Bullfrogs were taken from a farm pond near Kearney, Mo.

Mononuclear cells. Normal mouse spleen cells were prepared as described in detail elsewhere (11). Frog spleen cells were isolated by Ficoll-Hypaque centrifugation as described previously (28). Peripheral blood was collected from all other species studied, including normal human volunteers. Blood was obtained aseptically and placed in anticoagulant (1 liter of H_2O, 13.65 g of citric acid, 25 g of sodium citrate, and 20 g of dextrose) at a ratio of 1:6 (vol/vol). Peripheral blood mononuclear cells were purified by centrifugation through Ficoll-Hypaque gradients. The actual densities either were adjusted to conditions reported in the literature to give optimal yields of peripheral blood mononuclear cells or were determined empirically in our laboratory. The separated mononuclear cell fraction was concentrated by centrifugation, and the cells were washed three times by centrifugation with RPMI 1640 medium containing glutamine, penicillin, and streptomycin. All cells were maintained at 4°C until used for experiments.

Photoaffinity labeling. Sulfosuccinimidyl-2-(*p*-azidosalicylamino)-1,3′-dithiopropionate (SASD) was obtained from Pierce Chemical Co., Rockford, Ill. Photoactivatable radiolabeled *E. coli* O111:B4 LPS (^{125}I-ASD-LPS) was prepared as described previously (32). For standard reactions (unless otherwise indicated), approximately 10×10^6 cells in 100 μl of medium (RPMI 1640 containing 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml) were incubated with 5 μg of ^{125}I-ASD-LPS (approximate specific activity, 2 μCi/μg) at 37°C for 30 min. After incubation, the mixture of the cells and LPS was irradiated with short-wavelength UV light (4-Watts maximum emission at 254 nm) for 10 min to induce covalent cross-linking of LPS to target cells. Treated cells were then washed three times, reduced with 2-mercaptoethanol, and lysed by using the lysis buffer described by O'Farrell (23) for two-dimensional gel electrophoresis.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed as described by O'Farrell (23). Ampholytes (2D-Pharmalyte; Pharmacia, Uppsala, Sweden) of pH 3 to 10 were used for isoelectric focusing in the first dimension. Samples were applied at the acidic end of the gel. The second dimension was a sodium dodecyl sulfate–11% polyacrylamide gel. Gels were stained with 0.2% Coomassie blue R250–50% methanol–12% acetic acid and destained with 20% ethanol–10% acetic acid and then dried and autoradiographed to detect LPS-binding sites. The autoradiography was carried out with Kodak X-Omat XK-1 film, using a Dupont cassette with two Dupont Cronex Lightning-Plus intensifying screens, for 1 to 3 days.

RESULTS

We have previously shown the existence of specific LPS-binding proteins on the surface of murine lymphocytes and macrophages (11, 12). To assess the generality of these findings, peripheral blood mononuclear cells were isolated from the following species: human, rabbit, dog, cow, pig, sheep, horse, goat, and chicken. Mouse and frog spleen cells were prepared and utilized as a source of lymphoreticular cells for these two species. All cells were photo-cross-linked with the ^{125}I-ASD-LPS probe, washed three times, reduced with 2-mercaptoethanol, and analyzed by two-dimensional electrophoresis and autoradiography.

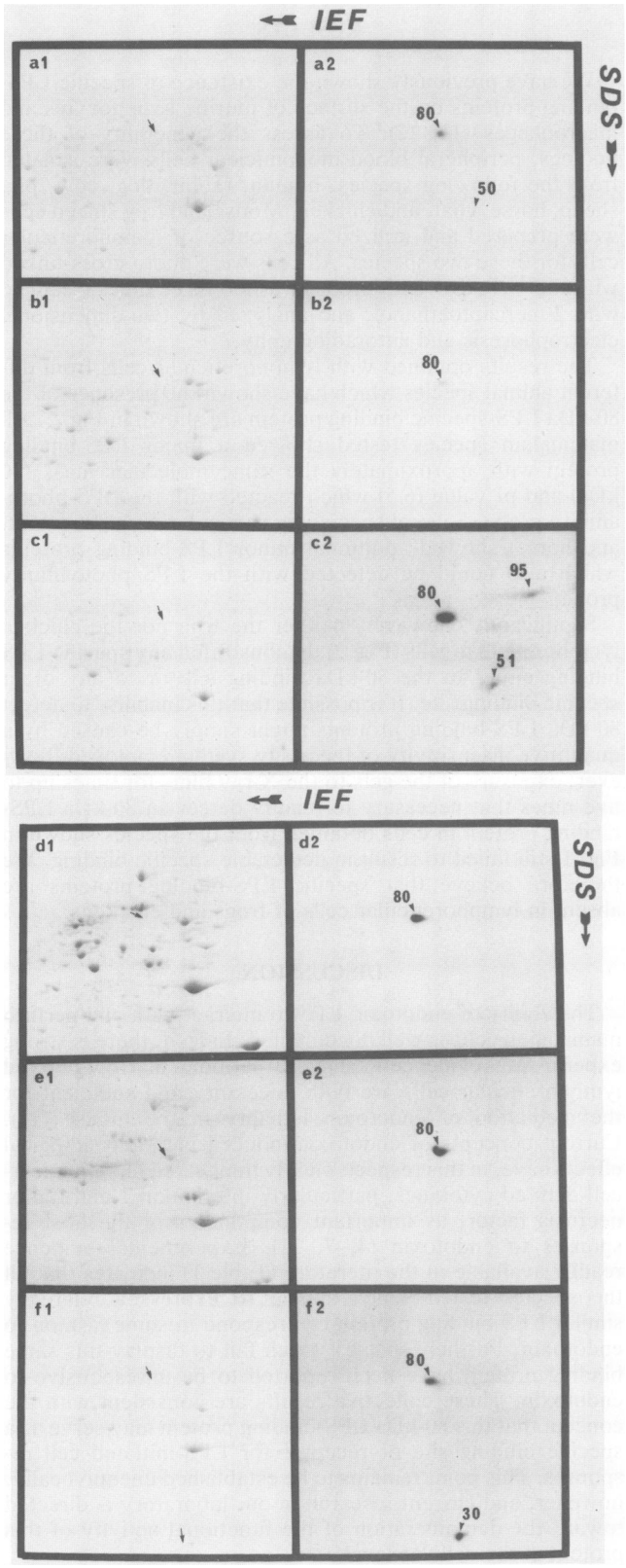
The results obtained with lymphoreticular cells from different animal species which have shown the presence of the 80-kDa LPS-specific binding protein are shown in Fig. 1. All mammalian species tested showed a major LPS-binding protein with approximately the same molecular mass (80 kDa) and pI value (6.5) which reacted with the LPS photoaffinity probe. Several species (in particular, human, rabbit, and horse) showed additional minor LPS-binding proteins which also could be detected with the LPS photoaffinity probe.

Significantly, however, neither the frog nor the chicken lymphoreticular cells (Fig. 2) demonstrated any specific LPS binding either to the 80-kDa binding site or to any other specific binding site. It is possible that this inability to detect 80-kDa LPS-binding proteins might simply be caused by a quantitive insensitivity of the assay system employed; however, exposure of the gels to the X-ray films for times up to five times that necessary to readily detect an 80-kDa LPS-binding protein in cells obtained from the species shown in Fig. 1 still failed to result in detectable specific binding. We therefore believe that specific LPS-binding proteins are absent in lymphoreticular cells of frogs and chickens.

DISCUSSION

The ability of endotoxic LPS to interact with and perturb mammalian cells is well documented. Indeed, there is strong experimental evidence to suggest that bone marrow-derived lymphoreticular cells are both necessary and sufficient for the mediation of endotoxin lethality in the mouse (14). Current concepts of endotoxin-induced pathophysiological effects have, in this respect, clearly implicated mononuclear-cell-derived cytokines, particularly interleukin 1 and tumor necrosis factor, as important components of the host responses to endotoxin (4, 7, 21). Experimental evidence readily available in the literature (Table 1) indicates that all the species tested which appear to express a relatively similar LPS-binding protein can respond in some fashion to endotoxin. Further, species which fail to display this same binding protein have been reported to be unresponsive to endotoxin. These collective results are consistent with the concept that this 80-kDa LPS-binding protein may serve as a specific binding site or receptor for LPS-initiated cell responses. This point remains to be established unequivocally, however, and current research in our laboratory is directed toward the demonstration of the functional activity of this protein at the cellular level.

For the majority of the species reported here, we have investigated only the binding to unfractionated peripheral blood mononuclear cells or splenocytes, containing both T and B lymphocytes and mononuclear phagocytes. However, in some of our previously reported studies, we have documented the presence of the 80-kDa LPS-binding protein on



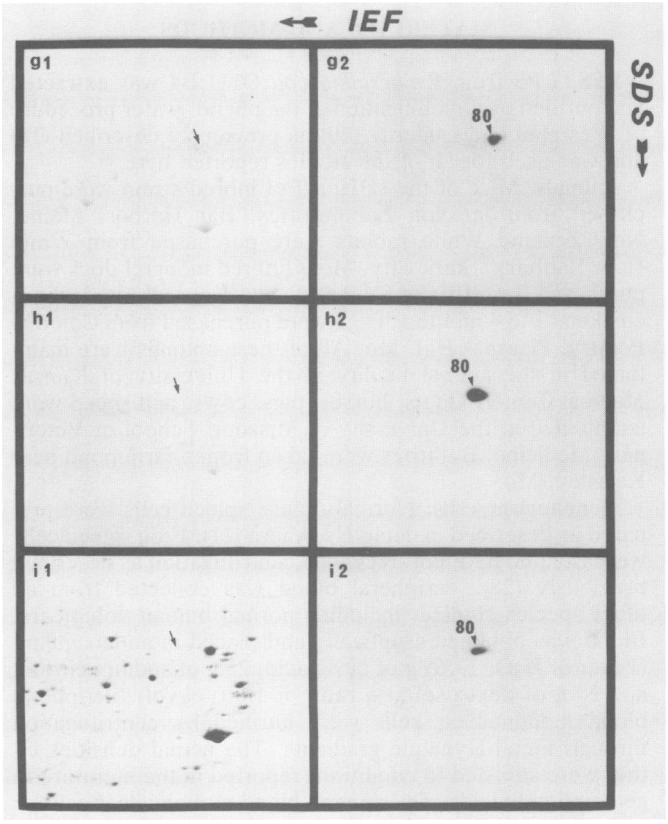


FIG. 1. Two-dimensional gel electrophoresis of photo-cross-linked, iodinated, and reduced lymphoid cells from various animal species sensitive to endotoxic LPS. Panels a1 through i1 show the Coomassie blue stain of the protein pattern; panels a2 through i2 show the corresponding autoradiographs of the gels. (a) Human peripheral blood mononuclear cells; (b) mouse spleen cells; (c) rabbit peripheral blood mononuclear cells; (d) pig peripheral blood mononuclear cells; (e) cow peripheral blood mononuclear cells; (f) horse peripheral blood mononuclear cells; (g) dog peripheral blood mononuclear cells; (h) sheep peripheral blood mononuclear cells; (i) goat peripheral blood mononuclear cells. The arrows in panels a1 through i1 indicate the positions of the major proteins specifically reacting with the LPS photoaffinity probe. IEF, Isoelectric focusing; SDS, sodium dodecyl sulfate.

purified populations of murine splenic B lymphocytes, thymocytes, and splenic macrophages. In addition, we have shown that this protein is expressed on murine B and T cell lines as well as on some murine macrophage cell lines. Finally, we have recently demonstrated (unpublished observations) the presence of the 80-kDa LPS-binding protein on purified human B lymphocytes (kindly provided by Ken Grabstein, Immunex, Seattle, Wash.). Therefore, our available data would be consistent with the existence of this LPS-binding protein on all major subpopulations of lymphoreticular cells.

If this LPS-binding protein does in fact participate in LPS-mediated cell activation, it is of considerable interest that LPS receptors with similar molecular weights and pI values can be detected on many mammalian cells of lymphoreticular origin. One explanation for this observation might be the potential importance to the host in recognizing the presence of LPS-bearing, gram-negative bacteria. Lewis Thomas (27) has suggested that effective recognition of LPS by host tissues might be a requisite event in the successful host response to bacterial infection. In this respect, however, it would be surprising to find that both frogs and chickens lack any specific mechanisms for LPS recognition, since both of these species exist in an environment of

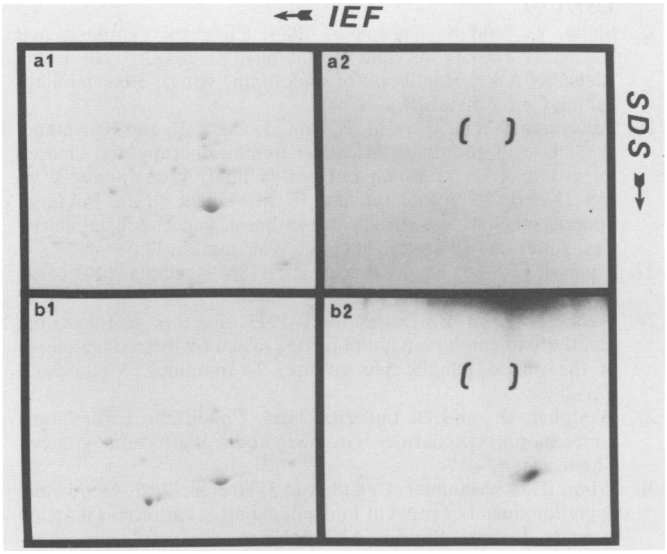


FIG. 2. Two-dimensional gel electrophoresis of photocross-linked, iodinated, and reduced lymphoid cells of chickens and frogs. Panels a1 and b1 show the Coomassie blue stain of the protein pattern; panels a2 and b2 show the corresponding autoradiographs of the gels. (a) Chicken peripheral blood mononuclear cells; (b) frog spleen cells. IEF, Isoelectric focusing; SDS, sodium dodecyl sulfate.

gram-negative organisms. Concerning the latter species, it is of potential interest that the 10-day-old chicken embryo is highly sensitive to the lethal effects of endotoxin but that its resistance is increased almost 5 orders of magnitude by day 15 (8). It would be of interest to investigate whether this 80-kDa LPS-binding protein might be temporally expressed as a differentiation antigen during embryogenesis.

A second possibility for expression of a specific LPS-binding protein might derive from its requirement for an unrelated cellular function, equally vital to the cell but independent of LPS recognition per se. Such a function would, of course, also ensure the conservation of the LPS-binding protein on lymphoreticular cells. Bacterial components such as LPS might then be viewed as opportunistically utilizing the putative receptor protein as a means of either weakening or stimulating the host defenses. In this latter respect, the capacity of bacterial exotoxins to utilize native membrane constituents of susceptible target cells such as gangliosides in order to gain entry to the intracellular milieu is now well recognized. The LPS-binding protein must first be characterized before these possibilities can be differentiated.

TABLE 1. Relationship of 80-kDa LPS-binding protein on lymphoid cells to species-specific endotoxin-initiated host responses

Species	Endotoxin sensitivity	Representative references	Presence of 80-kDa LPS-binding protein
Human	Yes	15, 26, 31	Yes
Sheep	Yes	6, 13	Yes
Horse	Yes	16, 26	Yes
Rabbit	Yes	3, 26	Yes
Pig	Yes	6, 24	Yes
Cow	Yes	3, 26	Yes
Goat	Yes	1, 30	Yes
Dog	Yes	6, 26	Yes
Mouse	Yes	3, 4, 14	Yes
Chicken	No	9, 10	No
Frog	No	5, 28	No

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