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Evidence that Lipopolysaccharide and Pertussis Toxin Bind to Different Domains on the Same p73 Receptor on Murine Splenocytes

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In previous studies, we used a photoactivable, radioiodinated lipopolysaccharide (LPS) derivative to define and characterize a specific bacterial endotoxic LPS-binding protein (p73) on mammalian lymphoreticular cells, including B and T lymphocytes and macrophages. More recently, using the same methodology, we characterized a specific interaction of LPS with the S2 subunit of *Bordetella pertussis* pertussis toxin (PT) in the fluid phase (M.-G. Lei and D. C. Morrison, J. Biol. Chem., 268:1488–1493, 1993). Furthermore, we showed that lysozyme (LZM) but not polymyxin B can compete with PT for binding to LPS in the fluid phase, a result suggesting that these two molecules compete for the same binding site on LPS. In this report, we demonstrate that the binding of PT to murine splenocytes (cell-bound PT) reduces the ability of the LPS photo-cross-linking probe to bind to the p73 receptor. The reduction can also be demonstrated with the PT B oligomer, a result indicating that the observed reduction of LPS binding to the p73 receptor by PT is A-protomer (S1-subunit) independent. More importantly, our studies document that cell-bound PT can be radiolabelled by the LPS probe, coincident with the observed reduction in p73 photoaffinity labelling. The preferential interaction of LPS with the PT S2 subunit in the fluid phase was, however, not observed with cell-bound PT. The reduction in radiolabelling of the p73 receptor by the LPS probe and in radiolabelling of cell-bound PT was shown to be concentration dependent. The data presented here document, however, that LZM does not reduce the ability of the LPS probe to bind to the p73 receptor on mouse splenocytes, nor does the presence of LZM bound to LPS influence the observed reduction in photoaffinity labelling of p73 by the LPS probe or radiolabelling of cell-bound PT by the LPS probe. Collectively, these results support the concept that the ability of LPS to interact with PT in the fluid phase is not responsible for the ability of cell-bound PT to influence the binding of the LPS probe to the p73 receptor. Thus, it is suggested that PT and LPS bind to different sites on the p73 molecule and that this same p73 protein may recognize both LPS and PT.

In 1985, Wollenweber and Morrison (30) reported the synthesis of a photoactivable, radioiodinated lipopolysaccharide (LPS) derivative (^{125}I-ASD-LPS). Since that time, many investigators have used their technique and have used the resulting LPS derivatives as probes to identify specific LPS-binding proteins on various mammalian cells (5, 9, 10, 15, 23). The technique is based on the ability of the radiolabelled (*p*-azidosalicylamido)-1,3′-dithiopropionate (ASD) group to chemically cross-link and iodinate molecules that are topologically in close proximity to the bound LPS on the cell surface. We have identified a major predominant membrane protein, the 73-kDa (p73) LPS receptor, on murine splenocytes (12). This protein was detected on splenic B and T lymphocytes and macrophages and various murine cell lines (12, 13, 15). Proteins similar to the p73 LPS receptor were also detected on lymphoreticular cells of many mammalian species (24) and on human peripheral blood lymphocytes, monocytes, neutrophils, and platelets but not on erythrocytes (8). The protein has specificity for the 2-keto-3-deoxyoctulosonic acid-lipid A determinant of the LPS macromolecule (13, 15). A monoclonal antibody, MAb5D3, raised against the murine p73 LPS receptor (2) was found to activate murine bone marrow culture-derived macrophages for tumor cell killing (3) and resident peritoneal macrophages for nitric oxide production (7). The antibody also shows a capacity to protect mice against the lethal effect of LPS (18).

Dziarski (5) has used identical photoaffinity labelling procedures and has recently reported that LPS and soluble peptidoglycan (sPGN) bind to the same protein on murine B lymphocytes; this protein has a relative molecular mass of 70 kDa. This LPS- and sPGN-binding protein was confirmed to be similar to the p73 LPS receptor reported by our laboratory (5). Although we have shown that a peptidoglycan and various peptidoglycan-containing polysaccharides isolated from many bacterial strains cannot inhibit LPS probe binding to the p73 receptor (15), the data of Dziarski (5) suggest that the LPS receptor can serve as a receptor for other ligands as well.

Pertussis toxin (PT), an exotoxin produced by virulent *Bordetella pertussis*, has an A-B type structure typical of many bacterial toxins (25). PT expresses many biological activities (19, 26). It is a hexamer, composed of five different subunits. The S1 subunit, an A protomer with ADP-ribosyltransferase activity, has the capacity to modify GTP-binding regulatory proteins (G proteins). The remainder of the subunits form the B oligomer, which has the capacity to bind to target cells via specific receptors (1, 25, 29). Very recently, our laboratory used PT to investigate the potential role of G proteins in LPS-induced signal transduction and the relationship of G proteins to the p73 LPS receptor (31).

Recently, we found that the LPS probe also binds specifically to the S2 subunit of both intact PT and the PT B

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oligomer (14). Of importance, Ohno and Morrison reported that hen egg white lysozyme (LZM) can interact specifically with LPS (20–22). When the capacity of LZM to compete with the PT S2 subunit for LPS binding was tested, it was found that the binding of LPS to the S2 subunit of PT could be competitively inhibited by LZM. Somewhat surprisingly, analysis of the N-terminal amino acid sequence showed a high degree of sequence similarity between the S2 subunit of PT and LZM (14).

In unrelated studies, Clark and Armstrong (4) have identified a PT receptor on lymphocytes that has a relative molecular mass of 70 kDa; this receptor is similar in molecular mass to the 73-kDa LPS receptor that we have identified. This observation has prompted us to investigate whether a potential relationship of the p73 LPS receptor with the 70-kDa PT receptor exists. In this report, we demonstrate that both intact PT and the PT B oligomer reduce the ability of the LPS probe to photoaffinity label the p73 LPS receptor on murine splenocytes, coincident with an LPS-specific radiolabelling of cell-bound PT. To exclude the possibility that the observed reduction of LPS probe binding to the p73 LPS receptor by PT was due to a direct interaction of LPS and PT, we investigated the effect of LZM on the binding of the LPS probe to the p73 LPS receptor and the radiolabelling of cell-bound PT. We found that, although LZM binds LPS with a high affinity, it manifests no capacity to reduce LPS probe binding to the p73 LPS receptor, nor does it in any way alter the ability of the LPS probe to radiolabel cell-bound PT. Therefore, we hypothesize that LPS and PT may bind to either topologically closely related or identical sites on lymphoreticular cells.

(Some of this work was presented at the Second International Endotoxin Society Meeting in Vienna, Austria, 17 to 20 August 1992.)

MATERIALS AND METHODS

Reagents. LPS from *Escherichia coli* O111:B4 was extracted and purified by the phenol-water procedure of Westphal and Luderitz (28) as previously described (17). PT and the PT B oligomer were purchased from List Biological Laboratories (Campbell, Calif.). Hen egg white LZM (grade I) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Sulfosuccinimidyl-2-(*p*-azidosalicylamido)-1,3′-dithiopropionate (SASD) was purchased from Pierce Chemical Co. (Rockford, Ill.).

Animals. Mice of the C3HeB/FeJ strain were purchased from Jackson Laboratory (Bar Harbor, Maine) and maintained in the animal facility at the University of Kansas Medical Center until used for experiments. Mice used were between 2 and 3 months of age and were of either sex.

Splenocytes. Mouse spleen cells were prepared as described elsewhere (6). Erythrocytes were lysed by the hypotonic shock procedure as described by Mishell and Shiigi (16). After lysis of erythrocytes, spleen cells were washed three times with cold medium (RPMI 1640) by centrifugation at 300 × *g*.

Photoaffinity labelling. Photoactivable, iodinated *E. coli* O111:B4 LPS (^{125}I-ASD-LPS; approximate specific activity, 2 μCi/μg) was prepared by radioiodination of the SASD-conjugated LPS exactly as described previously (30).

Photoaffinity labelling was carried out as described previously (15). For studying the effect of PT on LPS binding to the p73 LPS receptor (unless otherwise indicated), 2.5 μg of PT and 1 μg of ^{125}I-ASD-LPS were incubated with approximately 2 × 10^6 splenocytes in a final total volume of 40 μl at 0°C for 45 to 60 min. After incubation, the reaction mixture was irradiated with short-wavelength UV light (4-W maximum emission at 254 nm) for 10 min to effect photo-cross-linking of LPS to the target molecules. Spleen cells were washed three times with cold medium by centrifugation to remove unbound LPS probe, reduced with 2-mercaptoethanol, lysed by use of sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (15).

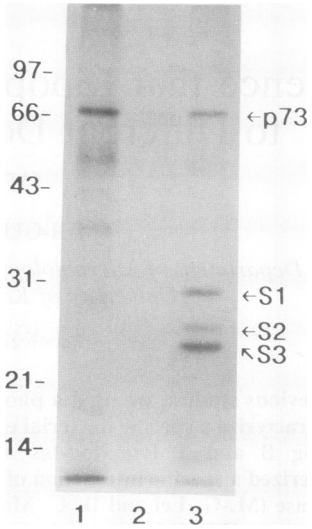

FIG. 1. Reduction of ^{125}I-ASD-LPS binding to the p73 receptor on murine splenocytes by PT and LPS. Lanes: 1, control without inhibitor; 2, with a 50-fold excess of underivatized *E. coli* O111:B4 LPS; 3, with 2.5 μg of PT. Subunits of PT were identified by their apparent molecular masses (in kilodaltons) (25).

SDS-PAGE. One-dimensional 11% SDS-PAGE was performed as described by Laemmli (11). Molecular weight markers were purchased from Bio-Rad Laboratories (Richmond, Calif.). Gels were stained, destained, dried, and autoradiographed to detect LPS-binding molecules as described previously (15).

RESULTS

Since ^{125}I-ASD-LPS was used to identify the p73 LPS receptor, our initial strategy for investigating the potential relationship of the LPS receptor with the PT receptor was to determine whether PT would reduce the binding of the LPS probe to the p73 receptor on murine splenocytes. For these experiments, mouse splenocytes were incubated at 0°C for 45 min with ^{125}I-ASD-LPS and the effect of either excess LPS or excess PT on binding to p73 was determined as outlined in Materials and Methods. Samples of solubilized cell lysates were analyzed by SDS-PAGE and autoradiography. The results are shown in Fig. 1. Photoaffinity labelling of p73 by ^{125}I-ASD-LPS alone is shown in lane 1. Lane 2 shows, as anticipated, the inhibition of LPS probe binding to the receptor by an excess of underivatized LPS. In the presence of PT, photoaffinity labelling of p73 by the LPS probe was reduced (lane 3), compared with that in the absence of PT (lane 1). Since the photo-cross-linking procedure allows the radioiodination of molecules in close proximity to the bound LPS, these results suggest that PT and LPS may bind to topologically closely related or identical receptor proteins.

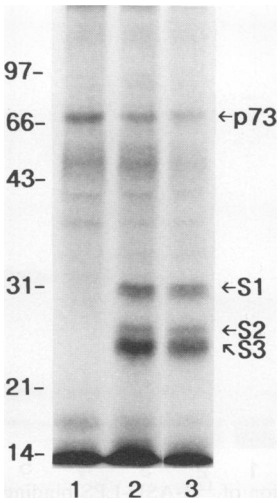

FIG. 2. PT reduction of binding of ^{125}I-ASD-LPS to the p73 receptor on murine splenocytes. Shown is evidence that the interaction of PT with LPS occurs at the cell surface. Lanes: 1, control without inhibitor; 2, with 2.5 μg of PT; 3, with 2.5 μg of PT and washing of the mixture of PT and cells before the LPS probe was added to the mixture. Numbers at the left are in kilodaltons.

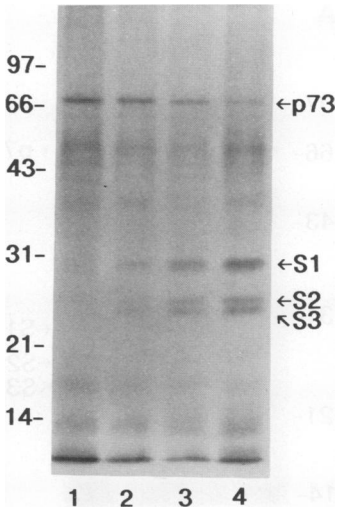

FIG. 3. Concentration dependence of PT-mediated reduction of ^{125}I-ASD-LPS binding to the p73 receptor on murine splenocytes. Lanes: 1, control without inhibitor; 2 to 4, with 0.125, 0.5, and 2.0 μg of PT, respectively. ^{125}I-ASD-LPS was added to the cells subsequent to the addition of PT without washing of the cells. Numbers at the left are in kilodaltons.

Interestingly, however, the results shown in lane 3 of Fig. 1 also indicate the photoaffinity labelling of PT subunits by the LPS probe. This result could have been due to the interaction of PT with the LPS probe as reported previously (14). Since the splenocytes were washed with cold medium by centrifugation before being analyzed by SDS-PAGE and autoradiography, the results might suggest that these radiolabelled PT molecules are cell bound. For proving that the interaction of the LPS probe with PT in the presence of splenocytes occurs on the cell surface, splenocytes were treated with PT, washed with cold medium by centrifugation, and then incubated with the photo-cross-linking LPS probe. The results shown in Fig. 2 establish that the pattern of photoaffinity labelling of PT subunits by the LPS probe in the washed PT-splenocyte mixture is similar to that obtained without washing (lane 3 versus lane 2). Taken together, the results provide evidence that both the reduction of LPS binding to the p73 LPS receptor on splenocytes by PT and the interaction of the LPS probe with PT occur at the cell surface.

For examination of whether the above-described results were concentration dependent, additional experiments were carried out with various amounts of PT. Figure 3 shows that as the concentration of PT is increased, there is a corresponding decrease in LPS probe binding to the p73 receptor on splenocytes (lanes 2 to 4) and a coincident increase in radiolabelling of the PT subunits. These data suggest that the observed reduction of LPS probe binding to the p73 receptor may be directly related to the binding of PT to the cell surface.

Recently, we reported a specific interaction of LPS with the S2 subunit of PT in the fluid phase (14) that was reflected by a preferential radiolabelling of the S2 subunit in the fluid-phase PT-LPS photo-cross-linking interactions. However, the preferential binding of the LPS probe to the S2 subunit was not observed with cell-bound PT (Fig. 1 to 3). We next examined, therefore, whether mixing PT and ^{125}I-ASD-LPS first would affect the binding of PT to the cell surface and/or the binding of ^{125}I-ASD-LPS to the p73 receptor and cell-bound PT. The results of one such experiment are shown in Fig. 4A. Mixing PT and the LPS probe prior to addition of the mixture to cells had no detectable effect on the observed reduction of ^{125}I-ASD-LPS binding to the p73 receptor by PT, in contrast to the results of a similar experiment in which splenocytes were incubated with PT prior to the addition of ^{125}I-ASD-LPS (lane 2 versus lane 3). The results also show that mixing PT and ^{125}I-ASD-LPS had no apparent significant effect on the binding of PT to the cell surface and the accompanying radiolabelling by the LPS probe. These results suggest that the specific interaction of the S2 subunit with LPS in the fluid phase (lane 4) (14) may not directly contribute to the observed reduction by PT of the binding of the LPS probe to the p73 receptor on splenocytes.

For quantitative comparison of the differences in the interaction of the LPS probe with cell-bound PT versus fluid-phase PT, the autoradiographs shown in Fig. 4A, lanes 2 and 4, were scanned with a laser densitometer. The relative intensities of radiolabelling of the S1, S2, and S3 subunits of PT by ^{125}I-ASD-LPS are shown in Fig. 4B. These results show clearly that the interaction of the LPS probe with fluid-phase PT was preferential for the S2 subunit; however, this preference was not observed with cell-bound PT. Thus, the relative binding of LPS to the S2 subunit of cell-bound PT is much reduced.

The intact PT molecule contains the A protomer (the S1 subunit, which has ADP-ribosyltransferase activity) and the B oligomer. We therefore used the purified B oligomer of PT to test the role of the S1 subunit in the observed reduction of ^{125}I-ASD-LPS binding to the p73 receptor by PT. The results of this experiment are shown in Fig. 5. With equal microgram amounts of intact PT (lane 2) and PT B oligomer (lane 3), it appears that the PT B oligomer may be more effective in reducing the binding of ^{125}I-ASD-LPS to the p73 receptor. However, the B oligomer molar concentration is about 25% higher than that of intact PT. Therefore, these data suggest that the PT B oligomer has a capacity to reduce the binding of the LPS probe to the p73 receptor similar to that of intact

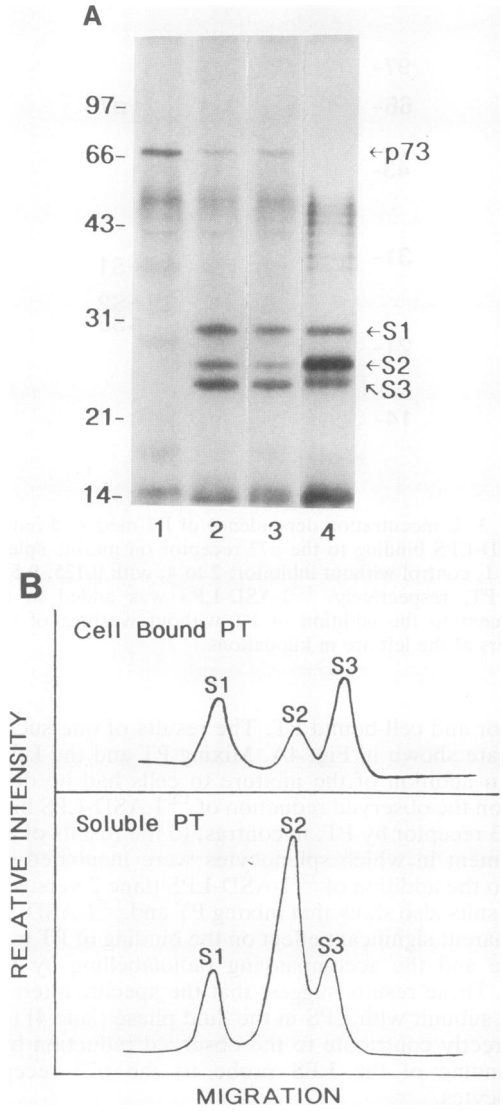

FIG. 4. (A) Reduction of ^{125}I-ASD-LPS binding to the p73 receptor on murine splenocytes by either premixing PT and the LPS probe or premixing PT and splenocytes. Lanes: 1, control without PT; 2, PT and ^{125}I-ASD-LPS were mixed first and then added to splenocytes; 3, PT was mixed with splenocytes first, and then the LPS probe was added to the mixture; 4, photo-cross-linking of ^{125}I-ASD-LPS to PT in the fluid phase. Numbers at the left are in kilodaltons. (B) SDS-PAGE analysis of the interaction of ^{125}I-ASD-LPS with cell-bound PT versus soluble PT. The autoradiographs of the SDS-PAGE analysis (panel A, lanes 2 and 4) were scanned with a laser densitometer.

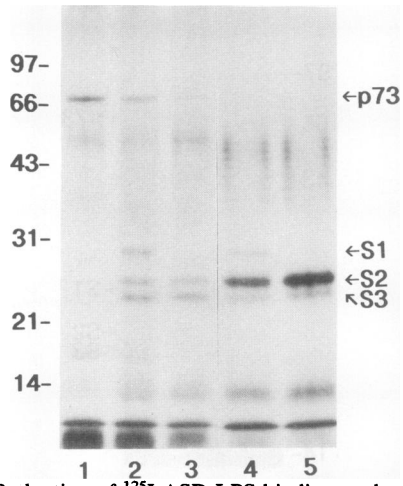

FIG. 5. Reduction of ^{125}I-ASD-LPS binding to the p73 receptor on murine splenocytes by either intact PT or the PT B oligomer. Lanes: 1, control without inhibitor; 2, with 2.5 μg of intact PT; 3, with 2.5 μg of the PT B oligomer; 4 and 5, photo-cross-linking of ^{125}I-ASD-LPS to intact PT and the PT B oligomer in the fluid phase, respectively. Numbers at the left are in kilodaltons.

PT. Since the B oligomer is devoid of the S1 subunit, the radiolabelling of the S1 subunit by the LPS probe was not observed with the cell-bound B oligomer (lane 3). These results indicate that the observed reduction in LPS probe binding to the p73 receptor by PT is S1 subunit independent.

The above-described results demonstrate that, in the presence of PT, the ability of ^{125}I-ASD-LPS to bind to the p73 receptor is reduced, and the reduction in p73 receptor binding is accompanied by the binding of ^{125}I-ASD-LPS to cell-bound PT (but not preferentially to the S2 subunit). Ohno and Morrison (20–22) previously demonstrated the specific interaction of LZM and LPS, and we recently reported that LZM can effectively compete with the S2 subunit of PT for LPS binding (14). These observations have allowed us to carry out experiments to determine whether ^{125}I-ASD-LPS radiolabelling of cell-bound PT and the reduction in p73 radiolabelling could result from a direct binding of ^{125}I-ASD-LPS to cell-bound PT (i.e., does the presence of cell-bound PT provide an alternative binding site for LPS, which results in the reduction in LPS binding to p73?). For these experiments, splenocytes were treated with PT at 0°C for 20 min. In parallel, ^{125}I-ASD-LPS was incubated with LZM to block the PT binding sites on LPS. Then the ^{125}I-ASD-LPS–LZM complexes were added to the PT-treated splenocytes. The final mixture was incubated at 0°C for 45 min, irradiated with UV for 10 min, and washed with cold medium by centrifugation to remove non-cell-bound constituents, and the radiolabelled solubilized cell lysates were then assessed by standard techniques.

The results of this experiment are shown in Fig. 6 and illustrate a number of important points. First, as demonstrated earlier, when PT was added to splenocytes simultaneously with the LPS probe, PT significantly reduced the binding of the LPS probe to the p73 receptor, compared with the results of the control experiment carried out with the LPS probe alone. This reduction in the radiolabelling of p73 was coincident with the radiolabelling of PT subunits (lane 1 versus lane 4). Second, and in contrast to the results observed with PT, the binding of LZM to the LPS probe did not influence the capacity of the LPS probe to bind to the p73 receptor (compare lanes 2 and 4), even though LZM was now associated with the splenocytes through its LPS binding site. The third and most important finding, however, was that when the LPS probe was incubated with LZM and then the mixture was added to PT-treated splenocytes (lane 3), the binding of the LPS probe to the p73 receptor was reduced to an extent equivalent to that observed without LZM treatment (lane 1); the subunits of cell-bound PT were radiolabelled to similar extents in the absence and presence of LZM (lane 1 versus lane 3), and LZM was radiolabelled to similar extents in the absence and presence of PT (lane 2

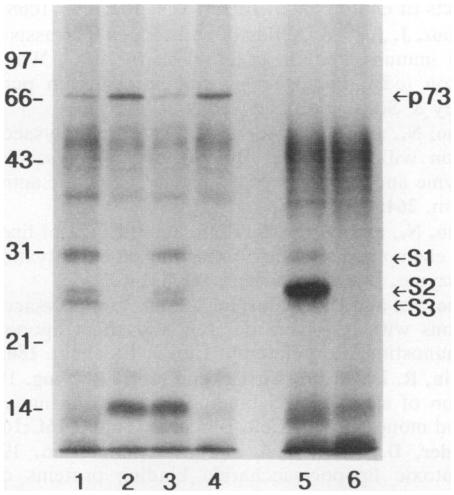

FIG. 6. Effects of LZM and PT on the binding of ^{125}I-ASD-LPS to the p73 receptor on murine splenocytes. Lanes: 1, with 2.5 μg of PT; 2, with 5 μg of LZM; 3, LZM (5 μg) incubated with ^{125}I-ASD-LPS at room temperature for 20 min and then added to the splenocyte-PT (2.5 μg) mixture, which had been incubated at 0°C for 20 min; 4, control without any inhibitors; 5, photo-cross-linking of ^{125}I-ASD-LPS to PT in the fluid phase; 6, photo-cross-linking of ^{125}I-ASD-LPS to itself in the absence of a ligand, showing the typical ladder pattern of *E. coli* O111:B4 LPS in SDS-PAGE. Numbers at the left are in kilodaltons.

versus lane 3). Since the interaction of LZM with LPS is of a high affinity (20) and the photoaffinity-labelled splenocytes were washed three times with cold medium by centrifugation before being analyzed by SDS-PAGE, these data allow the conclusion that LZM molecules (the 14-kDa bands in lanes 2 and 3) must be associated with cell-bound LPS. Collectively, the results of Fig. 6 support the hypothesis that LPS does not bind to cell-bound PT via the LZM-PT S2 subunit binding site but rather that PT binds to the cell surface at sites topologically closely related to or (more likely) identical to the p73 LPS receptor.

DISCUSSION

The above-described results demonstrate that PT can effectively reduce the photo-cross-linking of ^{125}I-ASD-LPS to the p73 LPS receptor and that cell-bound PT can be radiolabelled by the LPS probe. However, in contrast to our earlier findings (14), the preferential binding of ^{125}I-ASD-LPS to the S2 subunit of PT was not observed with cell-bound PT, suggesting that different mechanisms may be involved in the interaction of LPS with fluid-phase PT and cell-bound PT.

In addition to the p73 LPS receptor, there are some minor LPS-binding proteins that could also be eliminated by the addition of underivatized LPS (Fig. 1). Recently, we characterized one of these minor LPS-binding proteins, a 38-kDa protein, which shows specificity for the inner core 2-keto-3-deoxyoctulosonic acid determinants of the LPS molecule (14a). The characteristics of the other minor LPS-binding proteins are still under investigation in our laboratory.

Since our previous data suggested that LZM and PT bind to the same site on the LPS macromolecule (14) and our preliminary studies have indicated that the binding affinity of LPS for LZM is relatively higher than that for PT (unpublished observations), the results showing that LZM does not reduce the binding of LPS to the p73 receptor (Fig. 6) would suggest that the specific interaction of the LPS probe with PT either in the fluid phase or cell bound is not likely to be responsible for the reduction in the binding of LPS to the p73 receptor. The fact that the S2 subunit of cell-bound PT appeared not to bind preferentially to LPS would further support this conclusion. Although we cannot exclude the possibility that cell-bound PT may undergo a conformational change to generate a new binding site for LPS, there is no apparent specificity for this new binding site on the PT molecule.

While the S1 subunit of cell-bound intact PT was radiolabelled with a relative intensity approximately equal to those of the S2 and S3 subunits, the S2 and S3 subunits were radiolabelled equally well when the B oligomer of PT (which lacks the S1 subunit) was used in the study (Fig. 5). Thus, the S1 subunit would appear not to be essential for cell-bound PT–LPS interactions.

The most likely interpretation of these data is that PT binds specifically to the p73 receptor at a site that does not interfere with LPS binding and that p73-bound PT simply provides alternative acceptor sites for the cross-linking group of ^{125}I-ASD-LPS; thus, p73-bound PT is radiolabelled after UV irradiation. PT has been established to bind to mammalian cells via carbohydrate residues (1, 27, 29), and we earlier provided suggestive evidence that p73 is a glycoprotein (2). Since it is not likely that the interaction of LPS lipid A with the p73 receptor is dictated by carbohydrate residues on p73, it is not unreasonable to hypothesize that PT and LPS interact with different domains of p73.

Since LPS and sPGN can compete for binding to the 70- and/or 73-kDa receptor (5) and Dziarski has suggested that the binding may be specific for the $(GlcNAc)_2$ moiety of lipid A and the $(GlcNAc\text{-}MurNAc)_n$ backbone of sPGN (5), it would appear that both LPS and sPGN may bind to the same or similar domains on p73 (although the possibility of steric hindrance has yet to be excluded). Collectively, therefore, these data provide support for the concept that the p73 protein may serve as a binding receptor for LPS, sPGN, and PT (and maybe for other ligands as well). Why this p73 protein is singled out by nature for binding to such a diversity of potentially important bacterial virulence factors as LPS, sPGN and PT remains an intriguing question for future research.

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