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A Lipooligosaccharide-Binding Site on HepG2 Cells Similar to the Gonococcal Opacity-Associated Surface Protein Opa

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The lacto-*N*-neotetraose-containing lipooligosaccharide (LOS) present on the surface of most *Neisseria gonorrhoeae* organisms may serve many important functions in gonococcal pathogenesis. This surface glycolipid contains the cross-reactive epitope to human paragloboside and can be sialylated by gonococci grown in the presence of CMP-*N*-acetylneuraminic acid. Another possible role for this glycolipid could be to mimic human asialocarbohydrates and act as a ligand for asialoglycoprotein receptors contained on numerous human cells. The most noted of this large family of receptors is that expressed on the surface of hepatic cells. In a model cell system, using the hepatoma tissue culture cell line HepG2, we wanted to investigate if the presence of this asialoglycoprotein receptor influenced the adherence and/or invasion of gonococci expressing the lacto-*N*-neotetraose structure. Piliated variants of the gonococcal wild-type strain 1291 and its isogeneic LOS mutant 1291E were used in adherence-invasion assays. This gonococcal strain is somewhat unusual in that it expresses large amounts of predominantly one species of LOS, thus reducing the complexity of interpreting the data. The data from these assays suggested that the Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc carbohydrate structure on the wild-type LOS affected the adherence-invasion of gonococci into the HepG2 cells. In studies to determine whether the major hepatic asialoglycoprotein receptor was involved in these interactions, we found that the HepG2 cells contained two receptors which bound gonococcal LOS. One of these was the asialoglycoprotein receptor, and the data concerning this receptor will be reported elsewhere. The data on the second receptor are reported here. Purified, ^{125}I-labeled gonococcal LOS was used to identify specific high-affinity LOS-binding sites. These binding experiments revealed one major binding site corresponding to a protein with a molecular mass of 70 kDa (p70). Several lines of evidence in this study suggested that the oligosaccharide region of LOS played an important role in LOS binding to the p70 of HepG2 cells. In addition, we show that this human LOS receptor has some similarities to the gonococcal Opa proteins.

Many of the glycolipid structures contained in gonococcal LOS and the biosynthesis of these compounds are only now being elucidated. One of these moieties which has received a great deal of attention is that containing Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc, present on its nonreducing terminus (7, 21, 46). This lacto-*N*-neotetraose structure is identical to the nonreducing terminus of oligosaccharides found on many human glycoproteins and glycolipids (33, 34). The possibility has been suggested that gonococci may use these structures to mimic human cell surface antigens and evade the immune system. This hypothesis is supported by the observation that upon exposure to an external source of CMP-NANA, gonococci could sialylate the galactose residue of this terminal oligosaccharide and mimic human I and i antigens (36, 40). Other studies have demonstrated that the sialylation of this LOS component increased the resistance of the gonococcus to complement-dependent bactericidal activity by antibodies directed at the outer membrane (40, 64).

These studies have focused considerable attention on the role of LOS sialylation in pathogenesis. A number of questions related to sialylation of the gonococcus and its role in pathogenesis remain to be answered. Could asialo-LOS and the smaller LOS species seen on SDS-PAGE gels also have an important function for the gonococcus during infection? Is there sufficient CMP-NANA in genital secretions to support LOS sialylation when the organism is in the extracellular environment? Can and do naturally occurring neuraminidases in the genital tract remove the sialic acid from the LOS? If sialylation is crucial to survival during infection, why has the gonococcus failed to evolve mechanisms to produce its own CMP-NANA, similar to the meningococcus (35)? Does this suggest that it is important at some point in human infection for gonococcal LOS to remain unsialylated? Studies have demonstrated that while the majority of the LOS of the intracellular organisms is sialylated during urethral infection, approximately 10% of the Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc residues remain unsialylated (2).

The conversion from the sialo-[NANA(α2-3)Gal(β1-4)GlcNAc] to the asialo-[Gal(β1-4)GlcNAc] form by the loss of the terminal NANA occurs as human glycoproteins and glycolipids near the end of their functional life (see references 51 and 62 for reviews). These "aged" compounds are bound by and removed from the circulation by asialoglycoprotein receptors. The most thoroughly studied asialo-receptor of this rather large family of galactose-binding proteins is that which is found on hepatocytes (51, 55). However, similar and/or identical galactose-binding proteins have also been found on human macrophages (18) and sperm (17) and recently on male urogenital cells (unpublished data).

The ultimate goal of these investigations was to determine if these asialoglycoprotein receptors could be recruited in the adherence and internalization of gonococci into nonprofessional phagocytic cells. For these studies, we elected to utilize the hepatoma cell line HepG2 as a model because most techniques and assay methods to study these galactose-binding

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proteins had been previously well established using these tissue culture cells. In addition, gonococci of strain 1291 were used because they produce predominantly one species of LOS (10), which has been well characterized for both the parent, which contains the Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc carbohydrate structure on its LOS (10, 21), and the isogeneic mutant 1291E, which is truncated at the branched heptose (21). Using piliated Opa$^-$ gonococci from both the parent and the isogeneic 1291E mutant in gentamicin-selective adherence and internalization assays, we found that gonococci containing the Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc oligosaccharide were found in vastly greater abundance on the HepG2 cells after gentamicin selection than the 1291E Opa$^-$ gonococci. Comparisons were also made with bacteria expressing Opa proteins. These data suggest that these terminal oligosaccharides could function as ligands for mammalian cell receptors. One of these receptors was demonstrated to be the major hepatic asialoglycoprotein receptor studied by numerous other investigators. The data pertaining to this receptor have been published separately (46a). In the present study, using purified radiolabeled LOS isolated from WT 1291, we characterized a second carbohydrate receptor on HepG2 cells which (i) has a higher affinity for gonococcal LOS than the major asialoglycoprotein receptor; (ii) can bind to LPS from other gram-negative bacteria; (iii) is a protein with a molecular mass of 70 kDa, similar to that of the LPS-binding proteins described by Lei et al. (26–28); and (iv) has some functional and antigenic similarities to the gonococcal Opa proteins.

MATERIALS AND METHODS

Abbreviations. LPS, lipopolysaccharide; LOS, lipooligosaccharide; PBS, phosphate-buffered saline; PGN, peptidoglycan; KDO, 3-deoxy-D-manno-2-octulosonic acid; Opa, opaque; Gal, galactose; GlcNAc, *N*-acetylglucosamine; SASD, sulfosuccinimidyl-2-(*p*-azidosalicylaminol)-1,3′-dithiopropionate; ASD-LOS, SASD-derivatized LOS; ASD-LPS, SASD-derivatized LPS; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CMP-NANA, cytidine monophosphate N-acetylneuraminic acid; WT, wild type; MAb, monoclonal antibody; FBS, fetal bovine serum.

Materials. Chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. LPS from WT *Salmonella minnesota* and *Escherichia coli* O111:B4 and R60 were purchased from List Biological Laboratories, Inc. (Campbell, Calif.). Synthetic lipid A (*E. coli* biphosphate type) was purchased from ICN Biomedicals Inc. (Cleveland, Ohio).

Cells. All experiments were carried out with the human hepatoma cell line HepG2 (23). HepG2 cells were plated in either 6- or 24-well tissue culture plates (Falcon) from confluent cultures in minimal essential medium (GIBCO-BRL, Grand Island, N.Y.) containing 10% FBS (Hyclone, Logan, Utah) and 1 mM sodium pyruvate. Experiments were carried out on cultures near confluency, 5 to 6 days after plating.

Adherence-invasion of HepG2 cells by gonococci of WT strain 1291 and the isogeneic LOS mutant 1291E. The adherence-invasion assays were similar to that described by Shaw and Falkow (52). Briefly, HepG2 cells were seeded in 24-well microtiter plates at 10^5 cells per well and allowed to grow for 24 h. Gonococci from WT strain 1291 and its isogeneic LOS mutant, 1291E (10), were grown on solid typing medium made up as follows: Protease Peptone #3 (15 g) (Difco), K_2HPO_4 (4 g), KH_2PO_4 (1 g), and NaCl (5 g) are dissolved in 250 ml of distilled water and filtered through a 10,000-molecular-weight-cutoff filter; the flow-through volume is adjusted to 1 liter by the addition of distilled water, and 10 g of Bacto-Agar is added; after the mixture has been autoclaved and cooled to 45°C, 10 ml of a solution identical to IsoVitaleX (BBL, Baltimore, Md.) is added and mixed and the mixture is poured into 100-mm-diameter dishes and allowed to cool overnight. Piliated organisms of the various phenotypes (piliated opaque and piliated transparent) were identified and grown separately. Gonococci were grown overnight, harvested and suspended in minimal essential medium buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.3) containing 2 mM $CaCl_2$. The gonococci were diluted, and an aliquot was removed for CFU determination. Approximately 10^5 to 10^6 gonococci were placed in each well containing the HepG2 cells. The mixture was incubated for 6 h, the wells were washed thoroughly, and gentamicin (50 μg/ml) was added. The cells were incubated for an additional 90 min and washed, and the infected cells were removed from the microtiter plate surface with 5 mM EDTA in PBS. Samples of each culture were plated in 10-fold dilutions and, after a 24-h growth period, counted. The results are given as a percentage of invasion, i.e., (the CFU after gentamicin selection divided by the total CFU prior to infection) × 100.

Isolation and purification of LOS. LOS was isolated from nonpiliated transparent gonococcal strain 1291 and its isogeneic LOS mutant 1291E. The bacteria were grown to mid-log phase in a complex liquid medium similar to gonococcal medium base (Difco) supplemented with IsoVitaleX. The LOS was extracted by either the hot phenol-water method described by Westphal and Jann (63) or the PCP method of Galanos et al. (14). LOS preparations were then purified as previously described (45).

Photoaffinity labeling of HepG2 cells. SASD was purchased from Pierce Chemical Co. (Rockford, Ill.). Photoactivatable, iodinated *Neisseria gonorrhoeae* 1291 LOS (^{125}I-ASD-LOS) and *E. coli* O111:B4 LPS were synthesized according to Wollenweber and Morrison (65). For standard reactions (unless otherwise indicated) 10^5 cells in a 24-well plate were washed three times with PBS and then incubated with 2 μg of ^{125}I-ASD-LOS (approximate specific activity of 1 μCi/mg) in a total volume of 200 μl for 1 h. Incubation temperatures varied with experiments and are indicated in the figure legends. After incubation, the cell cultures were washed three times with PBS to remove unbound ^{125}I-ASD-LOS, irradiated with short-wavelength UV light, and then lysed with SDS sample buffer containing 2-mercaptoethanol. The reduced cell lysates were collected, radioactivity was counted in a Packard auto-gamma scintillation spectrometer for 1 min, and total protein was determined by the Pierce BCA protein assay as recommended by the manufacturer. Samples were adjusted to equivalent protein concentration, boiled for 5 min, and analyzed by SDS-PAGE. Quantitative determination of the effect of additives and/or inhibitors was calculated by the following equation: $[1 - (\text{cpm/mg}_{\text{with additive}} \div \text{cpm/mg}_{\text{without additive}})] \times 100 = \%$ inhibition.

Binding of monoclonal immunoglobulin 4B12 to HepG2 cells. In all experiments using MAbs, the hybridoma cells were grown in serum-free medium (Optimum; GIBCO-BRL) and the MAbs were purified from hybridoma supernatants by using a column of HA Ultragel (Pharmacia) as previously described (56). HepG2 cells in 24-well plates were washed three times with PBS and then incubated with various concentrations of 4B12 monoclonal immunoglobulin, ranging from 10 to 100 μg in a total volume of 200 μl, for 1 h. Binding of ^{125}I-ASD-LOS to the 4B12-treated cells was carried out in the presence of the immunoglobulin. The 4B12-treated cells were incubated with 2 μg of ^{125}I-ASD-LOS in a total volume of 200 μl for 1 h at 37°C, washed three times with PBS, irradiated, lysed with 0.1 N NaOH–0.5% SDS, and counted. To evaluate the specificity of the 4B12, binding of ^{125}I-ASD-LOS was also carried out in the presence of two other MAbs at the same concentrations: 4D11 (54) and 9A1/A2/G5 (48), which were raised against the gonococcal porin protein. Binding of ^{125}I-ASD-LOS in the presence of 4D11 and 9A1/A2/G5 was performed under the same conditions as for 4B12. Total protein concentrations were determined by the Pierce BCA protein assay as recommended by the manufacturer.

Binding of ^{125}I-ASD-LOS to gonococcal Opa protein (pII). The binding of ^{125}I-ASD-LOS to intact gonococci was assessed as follows. Gonococci of WT strain 1291 demonstrating the opaque colony phenotype were removed from solid agar medium and resuspended in PBS. The gonococci were washed once with PBS and diluted to 10^8 CFU/ml. A 200-μl aliquot was removed and incubated with 2 μg of ^{125}I-ASD-LOS for 45 min at 37°C. The mixture was then irradiated and analyzed by SDS-PAGE and autoradiography. A similar analysis was performed using a purified Opa protein. The gonococcal Opa protein was purified according to our previously published procedure (4). Two micrograms of purified Opa protein was mixed with 2 μg of ^{125}I-ASD-LOS in a total of 200 μl and allowed to incubate for 45 min at 37°C. The mixture was then irradiated and analyzed by SDS-PAGE and autoradiography. Autoradiographies were incubated at −70°C for 24 h.

SDS-PAGE and Western blotting. One-dimensional SDS-PAGE analysis (24) was carried out after denaturation and reduction of cell lysates with SDS and 2-mercaptoethanol, respectively. Proteins were resolved on 12.5% polyacrylamide gels and electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, Mass.). The membranes were then dried and autoradiographed to detect iodinated LOS-binding sites. The autoradiographs were obtained by using Kodak X-Omat XK-1 film and a Dupont cassette with two intensifying screens for 2 to 7 days at −70°C. For immunoblotting, after the electrotransfer and blocking, the Immobilon-P membranes were allowed to react with the monoclonal immunoglobulin 4B12, which was raised against purified gonococcal Opa protein (pII) (1), and developed with alkaline phosphatase-conjugated anti-mouse antibody (Cappel) and reagents (6).

RESULTS

Adherence-invasion of HepG2 cells by gonococci of WT strain 1291 and the isogeneic LOS mutant 1291E. As can be seen in Fig. 1 and has been shown by previous studies, the presence of an Opa protein on gonococci greatly enhanced their ability to adhere to and/or invade HepG2 cells, similar to that which has been shown with other human tissue culture cells (32, 59, 61). However, the influence of the LOS can be seen by comparing gentamicin-resistant organisms of the piliated, Opa$^-$, LOS WT phenotype (lane B) with organisms having the piliated, Opa$^-$, LOS E phenotype (lane D). The LOS

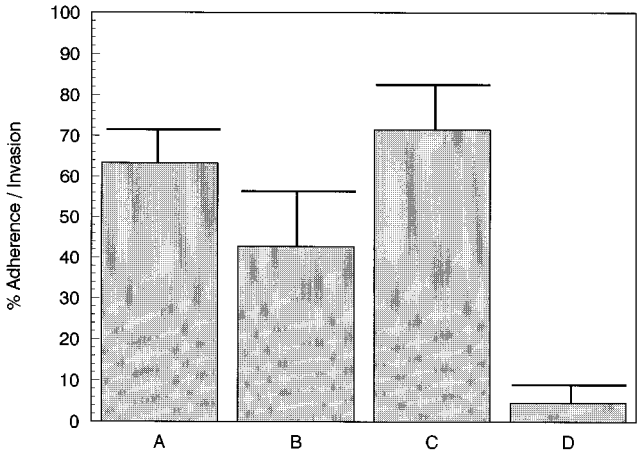

FIG. 1. Adherence-invasion of HepG2 cells by *N. gonorrhoeae* 1291 of different LOS and Opa phenotypes. Adherence-invasion is calculated by the following equation: (CFU of *N. gonorrhoeae* 1291 invading HepG2/total CFU of *N. gonorrhoeae* 1291) × 100. Each column represents the mean results of these assays ($n = 7$). The error bars represent 1 standard deviation from the mean. Column A, 1291 phenotype is Gal(β1-4)GlcNAc$^+$ Opa$^+$; column B, 1291 phenotype is Gal(β1-4)GlcNAc$^+$ Opa$^-$; column C, 1291 phenotype is Gal(β1-4)GlcNAc$^-$ Opa$^+$; column D, 1291 phenotype is Gal(β1-4)GlcNAc$^-$ Opa$^-$. These data suggest that both the Gal(β1-4)GlcNAc LOS structure and the Opa proteins can serve as independent gonococcal surface factors in HepG2 invasion.

of this mutant strain terminates at the diheptose because of a nonfunctional phosphoglucomutase gene (21, 49, 66). The magnitude of this difference in adherence and/or invasion is comparable to that seen with organisms expressing an Opa protein and those which do not. These results suggested to us that this Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc terminal structure present on WT 1291 LOS and lacking on the 1291E LOS is involved in the invasion process.

Demonstration of LOS-binding sites on hepatoma cells. Binding of ^{125}I-ASD-LOS to HepG2 cells was carried out at 4 and 37°C, and representative results are shown in Fig. 2. At both temperatures the autoradiographs reveal one main radiolabeled protein with a molecular mass of approximately 70 kDa (p70) (Fig. 2). The use of a higher concentration of ^{125}I-ASD-LOS (20 μg/ml) for the cross-linking and longer exposure of autoradiographs revealed the presence of other LOS-binding proteins. When the binding was carried out at 4°C (lane A), three minor bands could be detected corresponding to molecular masses of 52, 46, and 30 kDa. Another major high-molecular-mass band appeared at 37°C (lane B) with a molecular mass corresponding to approximately 150 kDa.

Binding of ^{125}I-ASD-LOS to p70: dependence on divalent cations and serum. As shown in Fig. 3 (lanes A and B) specific binding of LOS to p70 is considerably higher in the presence of divalent cations. However, the presence of serum in the binding buffer in these assays caused a considerable decrease, up to 85%, in the binding of LOS to the cells (Fig. 3, lanes C and D). This reduction was due, most probably, to the binding of LOS to serum components, as has been described by others (53, 57). Thus, to achieve optimal binding of LOS to its binding site, experiments were carried out in PBS containing Ca^{2+} and Mg^{2+} and without serum.

Binding of *E. coli* O111:B4 LPS to hepatoma cells. We wanted to determine whether LOS originating from *N. gonorrhoeae* and LPS from *E. coli* would bind to a similar receptor on HepG2 cells. Binding of ^{125}I-ASD-LOS (from *N. gonorrhoeae*) and ^{125}I-ASD-LPS (from *E. coli*) resulted in the labeling of the same or a similar major band, p70, and three minor

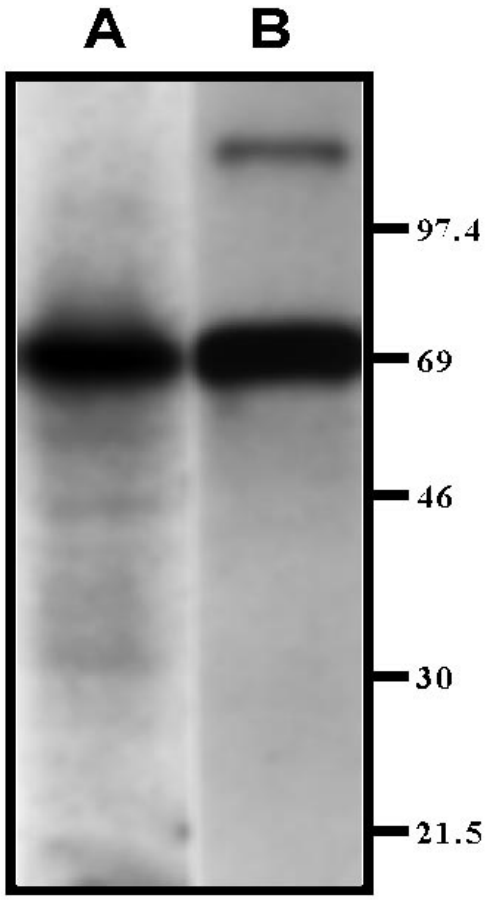

FIG. 2. SDS-PAGE autoradiographs of HepG2 cells after incubation and cross-linking with ^{125}I-ASD-gonococcal LOS. Cells grown in 24-well plates were incubated with 20 μg of ^{125}I-ASD-LOS per ml for 1 h at either 4°C (A) or 37°C (B), photo-cross-linked, and analyzed by SDS-PAGE and autoradiography.

bands at 52, 46, and 30 kDa (Fig. 4). The higher-molecular-mass band (150 kDa) could not be detected when cells were exposed to *E. coli* LPS.

Inhibition of ^{125}I-ASD-LOS binding by *N. gonorrhoeae* LOS and *S. minnesota* LPS. To determine the specificity of binding, HepG2 cells were exposed to ^{125}I-labeled LOS at 4°C in the presence of excess unlabeled LOS of the same origin (LOS from gonococcal WT strain 1291). As shown in Fig. 5, binding of ^{125}I-ASD-LOS was greatly inhibited by unlabeled WT LOS (lane B). When 50-fold-excess unlabeled LOS was added, the counts dropped by 55% (Fig. 6). The decrease in counts in the cell lysates was due to reduced binding of the iodinated LOS to the p70 band. Similar experiments were repeated with *S. minnesota* LPS and LPS from its rough mutant R60. As shown in Fig. 5 and Fig. 6, ^{125}I-ASD-LOS binding to the p70 band decreased by 72% in the presence of 50-fold-excess unlabeled WT *Salmonella* LPS (lane E) and by 54% in the presence of rough (R60) *Salmonella* LPS (lane D). Competition experiments with *Salmonella* LPS were also carried out at 37°C. When the same competition experiments were carried out at physiological temperature, the presence of excess unlabeled *Salmonella* LPS did not inhibit the binding of gonococcal LOS to the cells (data not shown). Similar inhibition experiments were carried out with LPS from *E. coli* O111:B4 and gave results similar to those seen with the *S. minnesota* WT LPS

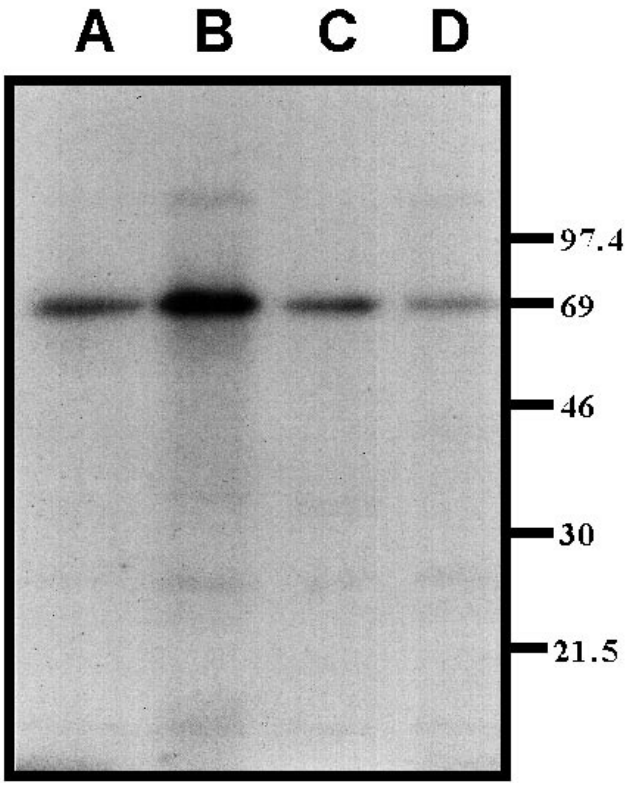

FIG. 3. Binding of ^{125}I-ASD-gonococcal LOS to p70 on HepG2 cells in the presence of divalent cations and serum. SDS-PAGE autoradiographs of HepG2 cells treated with ^{125}I-ASD-LOS in the absence (lane A) or presence (lane B) of 2 mM Ca^{2+} and 1 mM Mg^{2+} and in the absence (lane C) and presence (lane D) of 10% FBS.

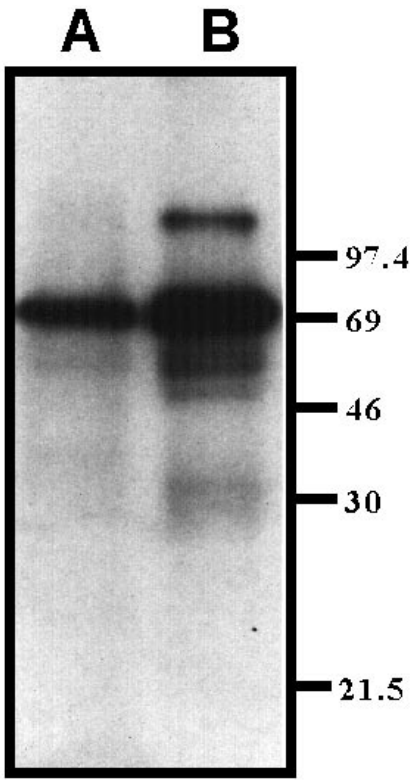

FIG. 4. Binding of *E. coli* O111:B4 LPS to HepG2 cells. SDS-PAGE autoradiographs of HepG2 cells treated with ^{125}I-ASD-*E. coli* LPS (lane A) and ^{125}I-ASD-gonococcal LOS (lane B).

(data not shown). This suggested that all these glycolipids were binding to the identical receptor on the HepG2 cells.

To determine which part of the LOS molecule was important in this binding process, we performed inhibition experiments with LOS purified from the gonococcal mutant 1291E and with synthetic lipid A. As shown in Fig. 6, lipid A had little effect on the specific binding of gonococcal LOS to the cells. The purified LOS from gonococcal strain 1291E was also much less effective in competing with the iodinated WT LOS (Fig. 5, lane C, and Fig. 6) compared with the gonococcal WT LOS or *Salmonella* LPS. The ^{125}I counts decreased by only 25% when 50-fold excess of unlabeled 1291E LOS was present in the labeling solution. Thus, we concluded that the terminal oligosaccharide residues [Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc] on the strain 1291 LOS play an important role in the binding of gonococcal LOS to hepatoma cells.

Specific binding of the MAb 4B12 to p70 on HepG2 cells. In earlier studies, we noticed that when HepG2 lysates were separated by SDS-PAGE and immunoblotted with the monoclonal immunoglobulin 4B12, a single band appeared with a molecular mass corresponding to 70 kDa (Fig. 7). This immunoglobulin was raised against the gonococcal Opa protein (pII) and has been shown to react with all gonococcal and most meningococcal Opa proteins (1). In order to verify whether the 70-kDa protein recognized by this antibody is the p70 protein that binds the iodinated LOS, binding of ^{125}I-ASD-LOS was performed in the presence of increasing concentrations of the 4B12 monoclonal immunoglobulin. As seen in Fig. 8, preincubation of HepG2 cells with the immunoglobulin blocked the binding of iodinated LOS to the cells. The inhibition showed dose dependence (Fig. 8B) and was specific to the 4B12 immunoglobulin, since preincubation with other MAbs (4D11 or 9A1/A2/G5) did not have any effect on LOS binding to the cells (Fig. 8A). These results led us to conclude that the LOS-binding site can either be recognized or hindered sterically by the 4B12 monoclonal immunoglobulin.

Binding of ^{125}I-ASD-LOS to gonococcal Opa protein (pII). To further demonstrate the similarities between the p70 HepG2 protein and the gonococcal Opa proteins, we used the ^{125}I-ASD-LOS in binding assays with intact gonococci (Fig. 9, left panel, lane 1) and purified Opa proteins (Fig. 9, right panel, lane 1). In lane 2 of both panels of Fig. 9, binding of the 4B12 monoclonal immunoglobulin to Opa is also presented. The fact that both Opa and the LOS-binding p70 protein on HepG2 cells bind purified LOS and the 4B12 immunoglobulin suggests that there is some similarity between these proteins. Further experiments (including purification and sequencing of the LOS-binding p70) are needed to prove this point definitely.

DISCUSSION

The success of the gonococcus as a human pathogen suggests that it has redundant mechanisms to assure its ability to establish and sustain infection. Several studies using tissue culture invasion assays have demonstrated the importance of the gonococcal Opa proteins in adherence to and invasion of human cells (32, 59, 61). These investigations have shown that the expression of Opa proteins on the surface of gonococci greatly increases the number of gonococci that are internalized. However, as shown in these studies, a substantial number of gonococci lacking the Opa protein are also endocytosed.

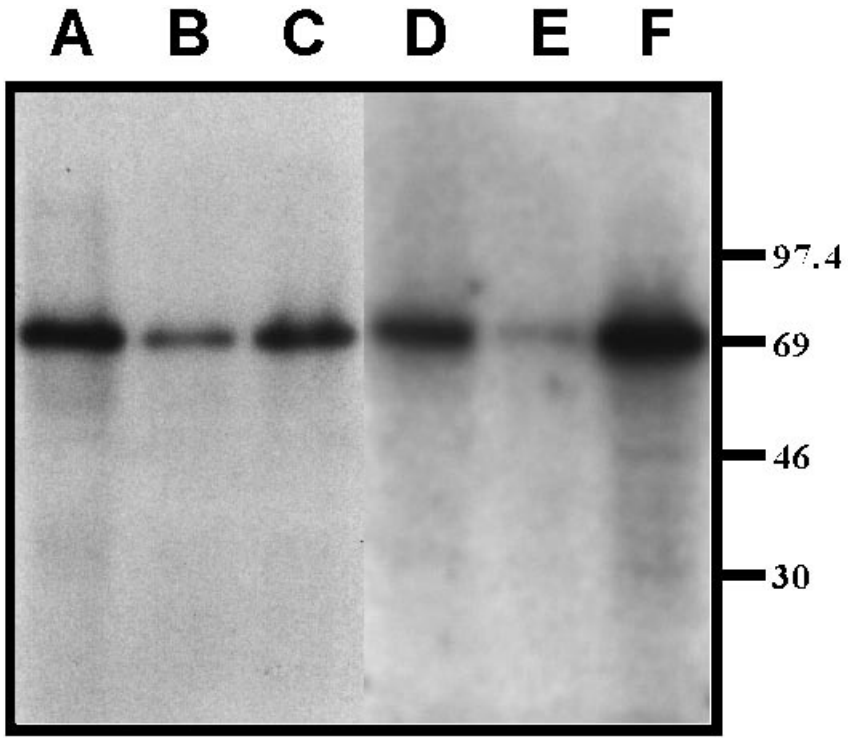

FIG. 5. Effects of various LOSs and LPSs on specific binding of ^{125}I-ASD-gonococcal LOS to HepG2 cells. Binding of ^{125}I-ASD-LOS (2 μg/ml) to HepG2 cells at 4°C was carried out in the presence of 50-fold-excess unlabeled LOS or LPS. Lanes A and F, ^{125}I-ASD-LOS only; lane B, ^{125}I-ASD-LOS in the presence of 100 μg of WT gonococcal 1291 LOS per ml; lane C, ^{125}I-ASD-LOS in the presence of 100 μg of gonococcal 1291E LOS per ml; lane D, ^{125}I-ASD-LOS in the presence of 100 μg of *S. minnesota* R60 LPS per ml; lane E, ^{125}I-ASD-LOS in the presence of 100 μg of *S. minnesota* WT LPS per ml.

With one exception (58), what role the gonococcal LOS plays in these types of assays has never been directly determined. To examine more closely whether the nonreducing terminal portion of gonococcal LOS, i.e., the Gal(β1-4)GlcNAc(β1-3)Gal (β1-4)Glc structure, had an influence on the adherence to and/or invasion of human cells by gonococci, we used piliated variants of the well-characterized WT gonococcal strain 1291 and its isogenic LOS mutant 1291E in invasion assays (10, 21). The results of these assays suggested that in addition to pili, both the Opa protein(s) and the Gal(β1-4)GlcNAc(β1-3)Gal (β1-4)Glc carbohydrate structure on gonococcal LOS affected the association of gonococci with HepG2 cells. As can be seen in Fig. 1, in the absence of the Opa protein(s) and the terminal LOS tetrasaccharide, there was a complete lack of both adherence to and invasion of the HepG2 cells. In contrast, Opa$^-$ gonococci, expressing the WT terminal LOS tetrasaccharide, were found associated with the HepG2 cells at a substantially higher frequency. The presence of Opa protein(s) further enhanced this association, confirming the observations of others (32, 59, 61). These differences suggested the presence of a possible receptor on the surface of the HepG2 cells specific for the Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc structure.

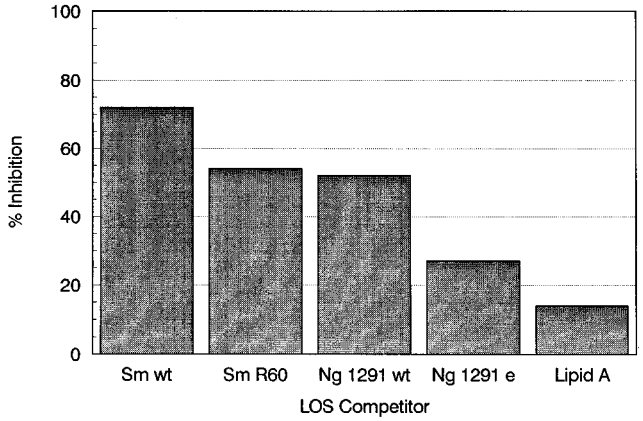

FIG. 6. Effects of various LPSs and lipid A's on binding of ^{125}I-ASD-gonococcal LOS to HepG2 cells. Binding of ^{125}I-ASD-LOS (2 μg/ml) to HepG2 cells was carried out in the presence of a 50-fold excess of either unlabeled LPS or synthetic lipid A. Following incubation at 4°C, cells were washed three times with PBS, irradiated, lysed with 0.1 N NaOH–0.5% SDS, and counted. The results are expressed as percent inhibition versus the control.

Most of the studies on the interactions between LPS and host cells have been directed at understanding the molecular mechanisms involved in how these compounds, at very low concentrations, trigger the physiologic effects of fever, leukocytosis, hypotension, and toxic shock. These effects are related to the release of cytokines, especially tumor necrosis factor alpha, into the host circulation. The host cells examined in these studies were those cells capable of cytokine production, primarily macrophages and lymphocytes. All of these studies would suggest an eventual interaction of the LPS with specific membranous molecules which in turn signal cytokine production. Whether or not various serum proteins are involved in this process and which specific host membrane proteins trigger these reactions remain subjects of intense scrutiny and interest. Our experiments were designed to identify a possible LOS receptor that influenced gonococcal endocytosis. Thus, the studies of Larsen and Sullivan (25), using tritium-labeled LPS from *Salmonella typhi*, which showed that binding of LPS to human peripheral blood monocytes was specific and saturable,

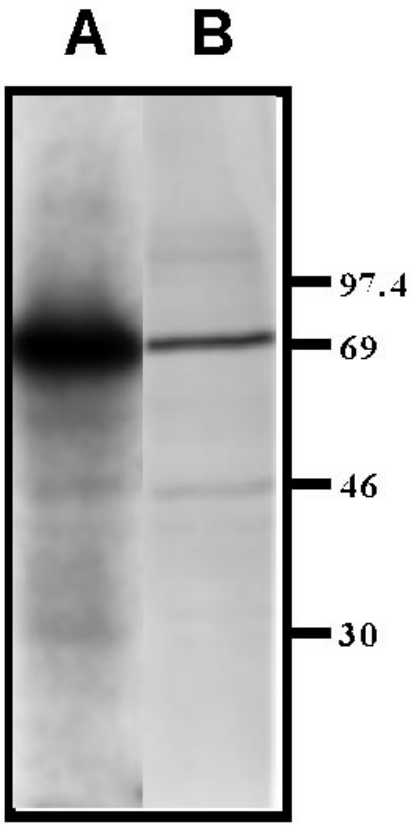

FIG. 7. LOS-binding sites and 4B12-reactive protein on HepG2 cells. Cells grown in 24-well plates were incubated with 2 μg of ^{125}I-ASD-LOS per ml for 1 h at 37°C, photo-cross-linked, and analyzed by SDS-PAGE and autoradiography (lane A). The same membrane was then allowed to react with the monoclonal immunoglobulin 4B12, raised against purified gonococcal Opa protein (pII), and developed with alkaline phosphatase-conjugated anti-mouse antibodies and reagents (lane B).

provided some evidence supporting the concept of specific membrane receptors for LPS.

Recent studies by Lei and coworkers, using photoactive, cross-linking derivatives of LPS in the absence of serum, have provided evidence for the existence of a specific LPS-binding protein, the 73-kDa LPS receptor, on splenic B and T lymphocytes and macrophages (26–28). In addition, Parent had also used a similar ^{125}I-labeled photoactivatable derivative of LPS to identify a 47-kDa integral membrane protein on rat hepatocytes as an LPS receptor (44). We used the same labeling procedure with purified gonococcal LOS in order to identify specific LOS-binding sites in the hepatoma cell line HepG2 which might account for the difference in association seen between the Opa$^-$ WT 1291 organisms and the Opa$^-$ LOS mutant 1291E. As shown in Fig. 2, binding experiments with iodinated LOS from WT 1291 organisms revealed one major binding site corresponding to a molecular mass of 70 kDa. The LOS-binding site on HepG2 cells appears to have the same molecular mass as the 73-kDa band recognized in murine splenocytes by Lei and Morrison (27). When both cell lysates were analyzed in parallel on the same SDS-PAGE gel, the two proteins showed the same R_f (LPS-labeled murine splenocytes were provided by David C. Morrison) (data not shown). Accordingly, the p70 HepG2 receptor could also bind to LPS and, thus, was not inhibited by the polysaccharide (O antigen) region, as shown in the binding experiment using photoactivatable, iodinated *E. coli* LPS (Fig. 4). Other minor binding sites were also detected corresponding to molecular masses of 150, 52, 46, and 30 kDa. While it is possible that binding of LOS to these other proteins has biological significance, we focused on the 70-kDa protein, which was consistently more prominent.

Different serum proteins have been shown to interact with LPS, including lysozyme (41, 42), high-density lipoproteins (29, 39), the acute-phase LPS-binding protein described by Schumann et al. (50), as well as others (see reference 38 for a review). Our results indicate that the binding of gonococcal LOS to the p70 HepG2 LOS receptor was greatly reduced by the inclusion of serum. As shown by Ward et al. (60) and later by McGee and coworkers (37), however, once gonococci are brought into close approximation to the host cell membrane via the actions of pili, the tight interactions between the two membranes exclude other small molecules which might be free in solution. Thus, at this point during the endocytic process it is reasonable to hypothesize a direct interaction between molecules on the gonococcal outer membrane and those of the host cell membrane without the intervention of other molecules such as serum proteins.

Binding of ^{125}I-ASD-LOS to p70 was specific and could be partially inhibited by the addition of a 50-fold excess of unlabeled homologous gonococcal LOS or heterologous *Salmonella* LPS, the extent of inhibition being dependent on the origin of the purified LPS. However, the inhibition was effective only when the experiment was carried out at a low temperature (4°C). Thus, the binding of ^{125}I-ASD-LOS to HepG2 cells carried out at 37°C could not be reversed, even when a high concentration of homologous unlabeled LOS (100 μg/ml) was added to the incubation mixture. These results are in good agreement with the model proposed by Jacobs (19) and by Carr and Morrison (8). According to this model, LPS interacts with cells in a two-stage process. The first step is characterized by its temperature independence, is reversible, and can be inhibited by some polyanions and polycations. As seen in our results, binding of gonococcal LOS could be inhibited when carried out at low temperature. It is thought that this step might involve specific interactions between the negatively charged LPS or LOS molecules and the membrane-protein binding site. On the other hand, the second step is temperature dependent and irreversible. In our observations, binding of gonococcal LOS to the cells could not be reversed at physiological temperature. It has been hypothesized that this step involves hydrophobic interactions between the membrane lipid bilayer and the lipidic region of LOS, leading to the intercalation of the lipid A fatty acids into the plasma membrane. However, from our observations, some sort of covalent temperature-dependent interaction cannot be excluded.

Well-defined LPS receptors which implicate the lipid A region of LPS have been demonstrated in macrophages and lymphocytes (16). However, the involvement of the core oligosaccharide region within the LPS molecule in the binding of LPS to its specific receptor has been widely discussed in the literature. Thus, Parent (44) demonstrated that hepatocytes have high-affinity receptors for LPS which bind both smooth and rough LPS. These hepatocyte interactions with LPS could be inhibited by smooth and rough LPS and heptose residues but not by free KDO residues or by isolated lipid A. These data strongly suggest the existence of an inner-core heptose-specific receptor. Human monocytes and rabbit macrophages may also bind LPS through a lectin-like receptor. Previous studies by Haeffner-Cavaillon and coworkers have shown that the specific binding of [^{3}H]LPS purified from *Neisseria meningitidis* and *Bordetella pertussis* could be inhibited by various LPSs and by isolated core polysaccharides but not by natural or synthetic lipid A (15). The role of the O-specific polysaccharide region in

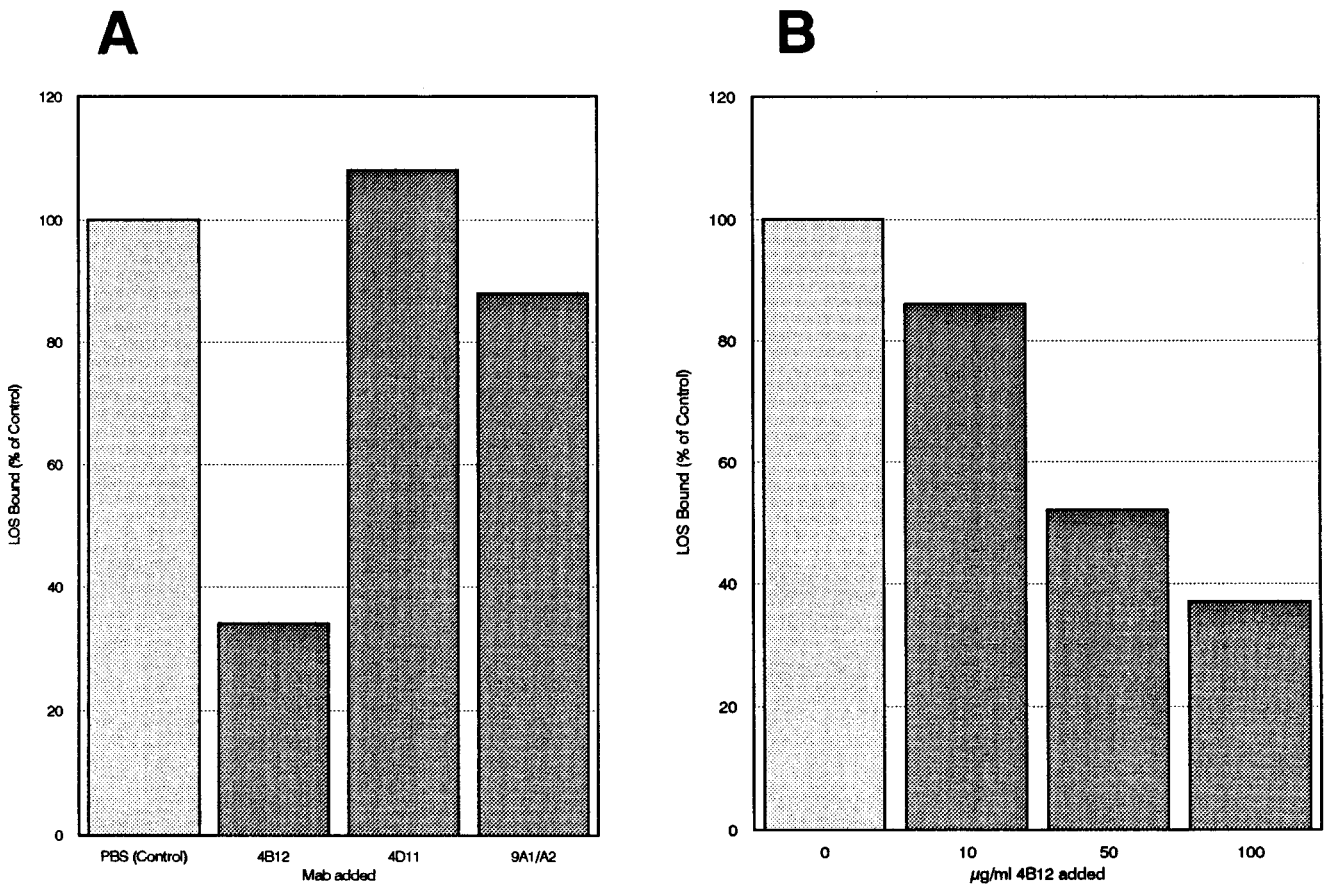

FIG. 8. Inhibition of ^{125}I-ASD-gonococcal LOS binding to HepG2 cells by the monoclonal immunoglobulin 4B12. (A) Specificity of 4B12 binding to HepG2 cells. Sister cultures of HepG2 cells were treated with ^{125}I-ASD-LOS in the presence of MAb 4B12 and two other MAbs, 4D11 and 9A1/A2/G5. Binding of ^{125}I-ASD-LOS in the presence of 4D11 and 9A1/A2/G5 was performed under the same conditions as those for 4B12. The results are expressed as percent binding versus the control. (B) Cells grown in 24-well plates were incubated with various concentrations of the 4B12 monoclonal immunoglobulin, ranging from 10 to 100 μg in a total volume of 200 μl, for 1 h at 37°C. The 4B12-treated cells were then incubated with 2 μg of ^{125}I-ASD-LOS in a total volume of 200 μl for 1 h at 37°C, washed, irradiated, lysed with 0.1 N NaOH–0.5% SDS, and counted. The results are expressed as percent binding versus the control.

the biological activities and the binding characteristics of LPS was emphasized only recently. Otterlei et al. (43) studied the effect of different polysaccharides on cytokine production from human monocytes. They showed that several well-defined polysaccharides (including polymers with different sizes of β1-4-linked D-mannuronic acid and cellulose oxidized in the C-6 position) induce human monocytes to produce tumor necrosis factor alpha. Their results indicate that polyuronic acids and LPS may stimulate monocytes by similar mechanisms and may bind to a common receptor. It is interesting that Dziarski (11, 12) presented additional evidence of a 70-kDa receptor on T lymphocytes and B lymphocytes which binds both bacterial cell wall PGN and LPS. By comparing the chemical structure of PGN and LPS, Dziarski found that the $(GlcNAc\text{-}MurNAc)_n$ backbone of PGN and the GlcNAc part of lipid A are important in the binding of PGN and LPS to cells. In a similar study using both LPS and PGN, Rabin et al. (47) confirmed the studies of Dziarski, implicating a 73-kDa protein on the surface of human blood monocytes in the binding of these two compounds. In a recent publication, Dziarski has suggested that the identity of the 73-kDa protein that he has investigated is cell-bound albumin (13). However, in our hands, the monoclonal immunoglobulin 4B12 which inhibited LOS binding to the p70 HepG2 protein was not reactive to either purified human or bovine serum albumin (data not shown).

Several lines of evidence in this study suggested that the oligosaccharide region of LOS plays an important role in LOS binding to the p70 of HepG2 cells. While the presence of excess WT gonococcal LOS greatly inhibited the specific binding of iodinated WT 1291 LOS to p70, similar concentrations of LOS purified from the gonococcal LOS mutant 1291E were much less effective. Competition experiments with *Salmonella* LPS (Fig. 5 and 6) also revealed that the WT LPS was more effective than the rough LPS in inhibiting the specific binding of gonococcal LOS to p70. It should be noted that the O-polymeric side chains from both the *E. coli* and *Salmonella* LPSs contain substantial amounts of galactose (20, 31). Furthermore, synthetic lipid A had very little effect on the specific binding of iodinated 1291 LOS to p70. We conclude that lipid A and LOS E apparently lack the structural determinants required for the initial high-affinity binding by the p70 HepG2 LOS receptor. Thus, the p70 HepG2 LOS receptor had specific binding characteristics which could explain the results obtained in our adherence-invasion assays (Fig. 1).

One surprising finding in our studies was the interaction between the HepG2 p70 protein and the monoclonal immunoglobulin 4B12. This immunoglobulin was raised against a gonococcal Opa protein and has been shown to react with most neisserial Opa proteins (1). As reported previously (5) and shown here, one of the possible functions of the gonococcal

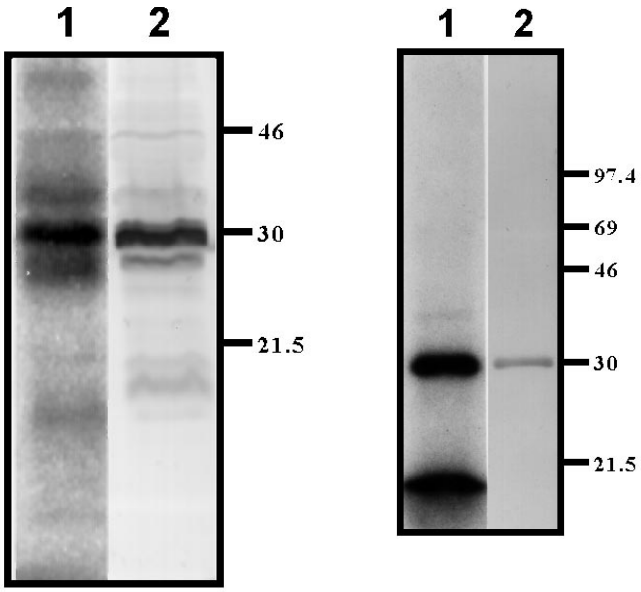

FIG. 9. Binding of ^{125}I-ASD-gonococcal LOS to gonococcal Opa (pII). (Left) Gonococci of WT strain 1291 expressing the opaque colony phenotype were removed from solid agar medium and resuspended in PBS. The gonococci were washed once with PBS and diluted to 10^8 CFU/ml. A 200-μl aliquot was removed and incubated with 2 μg of ^{125}I-ASD-LOS for 45 min at 37°C. The mixture was then irradiated and analyzed by SDS-PAGE and autoradiography (lane 1). The same membrane was then allowed to react with the monoclonal immunoglobulin 4B12 and developed with alkaline phosphatase-conjugated anti-mouse antibodies and reagents (lane 2). (Right) Two micrograms of purified Opa was mixed with 2 μg of ^{125}I-ASD-LOS in a total volume of 200 μl and allowed to incubate for 45 min at 37°C. The mixture was then irradiated and analyzed by SDS-PAGE and autoradiography (lane 1). The same membrane was then allowed to react with the monoclonal immunoglobulin 4B12 and developed with alkaline phosphatase-conjugated anti-mouse antibodies and reagents (lane 2). The lower-molecular-weight species found in lane 1 is unreacted ^{125}I-ASD-LOS.

Opa protein(s) is to bind to LOS. The ability to block the binding of ^{125}I-ASD-LOS by the 4B12 immunoglobulin in a dose-related fashion gave strong evidence that this monoclonal immunoglobulin is reactive and competing for the same p70 protein. It is interesting that both proteins, the prokaryotic Opa protein and the eukaryotic p70 protein, share functional activity in binding to LOS as well as reactivity with the 4B12 immunoglobulin. Determination of how extensive this similarity between the two proteins is awaits further characterization and sequencing of the p70 protein. However, the question is raised of whether the interaction between gonococci and host cells is similar to the adherence mechanism between gonococci of the opaque colony phenotype involving the Opa proteins. Our results point to the importance of the oligosaccharide region in the specific binding of gonococcal LOS to hepatoma cells. Determination of the exact mechanism by which the oligosaccharide region of LOS affects its binding to HepG2 cells and the full characterization of the p70 protein on these cells await further investigation.

In conclusion, it should be reemphasized that the HepG2 cells were used in these studies as a model cell system to investigate the interactions between the galactose-binding receptors on these cells and gonococci containing the lacto-*N*-neotetraose structure as part of their LOS. It remains uncertain whether the p70 protein described in this report or the hepatic asialoglycoprotein receptor is involved in in vivo gonococcal infections. But, it is well known that hepatic involvement may occur during such infections (3, 22, 30) and that gonococci can be recovered from liver biopsies from patients (9). The role of the p70 and the asialoglycoprotein receptor in gonococcal hepatic disease needs to be examined more closely.

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