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Neisseria gonorrhoeae Utilizes and Enhances the Biosynthesis of the Asialoglycoprotein Receptor Expressed on the Surface of the Hepatic HepG2 Cell Line

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One of the lipooligosaccharide (LOS) structures of *Neisseria gonorrhoeae* contains a terminal Gal(β1-4)GlcNAc residue which is a good candidate to serve as a ligand for human asialoglycoprotein receptors (ASGP-R). These receptors have been shown to be present on macrophages, sperm cells, and hepatocytes. The human tissue culture cell line used most often to study this receptor, HepG2, was used in our investigations only as a model. We also chose *N. gonorrhoeae* 1291 for these studies because, unlike many other gonococcal strains, this strain expresses one main species of LOS. The LOS structure expressed by this strain has also been fully characterized. Using well-established assays for the utilization of the ASGP-R, we found that incubation of HepG2 cells with gonococci expressing the terminal Gal(β1-4)GlcNAc asialo-LOS carbohydrate structure competitively inhibited the ASGP-R from binding to one of its well-known ligands, asialo-α-acid-1-glycoprotein. The inhibition was specific to the ASGP-R, since binding of two other ligands to their specific receptors in the same model cell system was not affected. Immunoblot analysis for the ASGP-R suggested that gonococci seemed to stimulate the HepG2 cells to increase the expression of the major (46-kDa) receptor species. This observation was confirmed both by functional analysis, which showed that the concentration of total receptor molecules, as well as surface receptors, was about 60% higher after incubation with gonococci than in control cells and by Northern (RNA) blot analysis using a cDNA probe of the major human H1 subunit. Poly(A) RNA purified from control and HepG2 cells exposed to gonococci indicated the presence of increased amounts of mRNA coding for the ASGP-R after incubation with gonococci. This result supports the idea that the molecular mechanism controlling the receptor level after gonococcal exposure is under transcriptional regulation.

Many of the lipooligosaccharide (LOS) structures contained in the outer membrane of *Neisseria gonorrhoeae* are now being characterized (26, 39). These glycolipids contain three comparatively short oligosaccharide chains covalently linked through ketodeoxyoctonoic acid to a lipid A component (19). Although at any given time the outer membrane of a gonococcus may contain several different species of LOS, one of these structures in particular has received a great deal of attention. This particular LOS has a nonreducing terminal tetrasaccharide of Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc (26) which has been found in 97% of all gonococcal strains thus far examined (11), and its prevalence has been shown to increase during the course of human experimental gonococcal infections (41). In addition, this particular glycolipid structure can be found in several other mucosal pathogens (11).

Using monoclonal antibodies 3F11 and 6B4, whose specificity for this common LOS structure has been defined (2, 59), Mandrell et al. demonstrated that the epitope recognized by these antibodies is identical to the terminal tetrasaccharide (lacto-*N*-neotetraose) of paraglobosides (35). This is a glycosphingolipid precursor of the major human blood group antigens which terminates in the disaccharide *N*-acetyllactosamine (35). It has been reported that this particular gonococcal LOS structure, like the human counterparts, can become sialylated both in vitro and in vivo (36). Once sialylated, the bacteria are more resistant to the bactericidal activity of normal human serum (40). In addition, studies of urethral exudates from patients with gonococcal infections have shown that while most of the LOS containing this structure is sialylated on the intracellular organisms, approximately 10% of the Gal(β1-4)GlcNAc residues remain unsialylated (3). These studies suggest that this terminal saccharide complex of gonococcal LOS may play an important role in the survival mechanisms of gonococci.

One proposed hypothesis for the importance of this LOS structure is that by mimicking host antigens, gonococci are able to avoid recognition by the human immune system. A second, equally valid hypothesis is that the gonococci synthesize these asialoparagloboside-like structures to be ligands for human cell receptors.

Numerous different lactosamine receptors have been found on human cells. Members of this rather large family of receptors are grouped into two major categories based on the requirement for Ca^{2+} (C-type lectins) or thiol (S-type lectins) for activity. Of the C-type lectins, the asialoglycoprotein receptor (ASGP-R) of human hepatic cells has been cloned and sequenced (47, 48), and the routing pathways for both receptor and ligand have been studied extensively (10, 18, 46). The polypeptides which make up this receptor are contained on several different genes (47, 48) and, depending on the combination of these gene products, differ in the ability to bind carbohydrates or glycoproteins containing terminal galactose. Weigel et al., in their studies of specific carbohydrate recognition by intact cells, were the first to demonstrate that rat hepatocytes bound specifically to ligands containing different structural arrangements of galactose which had been derivat-

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ized onto polyacrylamide surfaces (55). Schwartz (42) and Weiss and Ashwell (57) also indicated that the ASGP-R specifically recognizes ligands with a terminal D-galactose, D-*N*-acetylgalactosamine, and related galactosides. These galactose receptors have been shown to be present on hepatocytes (15), a hepatoma cell line (43), Kupffer cells (where it is known as the fucose receptor) (32, 52), peritoneal macrophages (24), endothelial cells (29), and sperm cells (1, 22).

To determine if this ASGP-R which is presumed to be a part of the normal human scavenging mechanisms could serve as a receptor for the lacto-*N*-neotetrose-containing LOS structure present on the majority of gonococci, we have used the hepatoma cell line HepG2. The assays to determine the utilization of the ASGP-R in this cell line have been well established. In addition, to lessen the complexity of these investigations, we elected to use the wild-type *N. gonorrhoeae* strain 1291. During the course of these studies, it became apparent that HepG2 cells contained at least two receptors which could bind to gonococcal LOS. In this report, we provide evidence which suggests that one of these receptors is the ASGP-R. We will show that exposure of HepG2 cells to gonococci inhibits the binding of an ASGP-R ligand and that the expression of this hepatic cell lectin is modulated as a result of this exposure. The second receptor on HepG2 cells which seems to bind this particular gonococcal LOS much more avidly will be described elsewhere (39a). The data suggest that both of these receptors, separately or in combination, influenced the adherence of *N. gonorrhoeae* 1291 to HepG2 cells.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified. *Vibrio cholerae* neuraminidase was purchased from Calbiochem, La Jolla, Calif. Restriction enzymes and the buffers for their use were from New England Biolabs, Inc., Beverly, Mass. The random-primed DNA labeling kit was from Boehringer Mannheim Inc., Indianapolis, Ind. Kits for isolation cytoplasmic RNA and poly(A) mRNA were from 5 Prime-3 Prime Inc., West Chester, Pa. The GeneClean kit was from Bio 101, La Jolla, Calif. ^{125}I-Bolton-Hunter reagent, ^{3}H-dihydroalprenolol hydrochloride (^{3}H-DHA), and ^{125}I-insulin were from NEN, Boston, Mass. Immobilon-P transfer membranes were from Millipore Corp., Bedford, Mass. Hybond-N^{+} nylon membranes were from Amersham Life Sciences, Arlington Heights, Ill.

Bacteria. *N. gonorrhoeae* 1291 was selected for this study since it produces predominately one species of LOS (14). The saccharide composition, order, and linkages of this LOS have been described in detail elsewhere (26). The gonococci were grown on solid typing medium composed of Protease Peptone no. 3 (15 g; (Difco), K_2HPO_4 (4 g), KH_2PO_4 (1 g), and NaCl (5 g), dissolved in 250 ml of distilled water, and filtered through a 10,000-molecular-weight filter; the flowthrough volume was adjusted to 1 liter by the addition of distilled water, and 10 g of Bacto Agar (Difco) was added; after the mixture was autoclaved and cooled to 45°C, 10 ml of a solution identical to IsoVitaleX (BBL, Baltimore, Md.) was added and mixed, and the mixture was poured into 100-mm-diameter dishes and allowed to cool overnight. Piliated and nonpiliated organisms of the various phenotypes were identified by colony morphology and grown separately.

Tissue culture cells. All experiments were carried out with the human hepatoma cell line HepG2 (28). HepG2 cells from confluent cultures in minimal essential medium (MEM; Gibco-BRL, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, Utah) or 10% dialyzed FBS (Gibco) and 1 mM sodium pyruvate were plated either in 30- or 100-mm-diameter tissue culture dishes or in 24-well microtiter plates (Falcon), depending on the assay. Experiments were carried out on cultures near confluency.

Adherence to and invasion of HepG2 cells by gonococci. HepG2 cells were seeded in 24-well microtiter plates at 10^5 cells per well and allowed to grow for 24 h. Gonococci were grown overnight, harvested, and suspended in MEM buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.3) containing 2 mM $CaCl_2$ (binding medium). The gonococci were diluted, and an aliquot was removed for CFU determination. Approximately 10^5 to 10^6 CFU was placed in each well containing the HepG2 cells. The mixture was incubated for 6 h and washed thoroughly four times, and gentamicin (50 mg/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 phosphate-buffered saline (PBS). Samples of each culture were plated in 10-fold dilutions and, after a 24-h growth period, counted. The results are given as percentages of adherence or invasion, i.e., (CFU after gentamicin selection/total CFU prior to infection) × 100.

Iodination of ASOR. Human orosomucoid was desialylated as previously described (45). Asialoorosomucoid (ASOR) was labeled with ^{125}I-Bolton-Hunter reagent according to the NEN procedure, with some modifications. All manipulations were carried out on ice. Immediately before use, the benzene solvent was evaporated from the reaction vial containing the Bolton-Hunter reagent (250 μCi; specific activity, 2,200 Ci/mmol). ASOR (100 μg) in 100 μl of 0.1 M sodium borate buffer (pH 9.0) was added to the dried iodinated ester, and the reaction mixture was agitated for 2 h at 0°C and an additional 18 h at 4°C. Labeled protein was separated from unreacted ^{125}I-Bolton-Hunter reagent on a Sephadex G-25 column. The specific activity was generally about 6.5×10^7 cpm/nmol of protein.

Competitive binding assay for the ASGP-R on HepG2 cells. All binding assays were carried out in tissue culture dishes (35-mm diameter; Falcon) which were generally seeded (2×10^5 to 5×10^5 cells per dish) 5 to 6 days prior to the assays. The cells were washed twice with MEM and then incubated for 1 h in binding medium. Piliated, opaque (Opa) gonococci which had been grown on agar overnight were harvested and suspended in binding medium. The number of CFU was estimated by optical density measurements at 600 nm (an optical density of 0.1 corresponds to 10^8 organisms per ml). The bacteria were added to the cell culture at a ratio of 20 to 40 CFU per cell. Cells were incubated with gonococci for 2 to 4 h (as indicated in the figure legends) at 37°C in an incubator supplemented with 5% CO_2. Then cells were treated with various concentrations of ^{125}I-ASOR ranging from 0.1 to 2 μg/ml (0.25 to 5 nM) at either 4 or 37°C. Binding was carried out in triplicate for 2 h in binding medium. The medium was then removed, and the cell layer was washed three times with ice-cold PBS. The cells were then dissolved in 1 ml of 0.1 N NaOH containing 0.5% sodium dodecyl sulfate (SDS) and counted in a Packard auto-gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) for 1 min.

Several controls were included in these assays. First, nonspecific binding was determined in the presence of a 400-fold excess of unlabeled ASOR, and these counts were subtracted from the bound counts of the samples. Second, binding of ^{125}I-ASOR to gonococci was determined as follows. The bacteria (10^8 CFU per sample) were suspended in binding medium and treated with 2 μg of ^{125}I-ASOR per ml at either 4 or 37°C; following a 2-h incubation with the labeled ligand, the gonococci were washed twice by centrifugation with ice-cold PBS, dissolved in 1 ml of 0.1 N NaOH containing 0.5% SDS, and counted. Third, the binding of ^{125}I-insulin and ^{3}H-DHA was used to evaluate the specificity of the gonococcal inhibition of ASOR binding to the ASGP-R. Binding of ^{125}I-insulin was carried out in the same conditions as the ^{125}I-ASOR binding assay, with ^{125}I-insulin concentrations ranging from 0.1 to 0.5 μg/ml. Nonspecific binding was determined in the presence of a 100-fold excess of unlabeled insulin. Binding of ^{3}H-DHA, a β-adrenergic antagonist, was carried out as described by Munnich et al. (37). The ^{3}H-DHA concentration used was 10 nM, and the nonspecific binding was determined in the presence of 0.2 mM alprenolol. The interaction of ^{125}I-insulin and ^{3}H-DHA with gonococci was also determined by incubating 10^8 bacterial CFU with the labeled ligands under the conditions used to label the HepG2 cells, after which the samples were washed and counted as described above.

The determination of the total number of cellular ^{125}I-ASOR binding sites available either after the incubation with gonococci or on control cells was performed by the well-established methods of Fallon and Schwartz (16). The cells were first permeabilized with 0.1% saponin for 30 min at 4°C. Cells were washed free of detergent with binding medium, and the ^{125}I-ASOR binding assay was performed as described above. The number of ^{125}I-ASOR binding sites which were previously occupied and unavailable in the first assay was established by the acid-stripping procedure (16). With this technique, any ligand bound to the ASGP-R was selectively removed by incubating either intact cells or saponin-permeabilized cells for 4 min at 4°C in PBS containing no Ca^{2+} and 10 mM EDTA at pH 5.0, after which the cells were washed with binding medium and the ^{125}I-ASOR binding assay was performed as described above. In some instances, the HepG2 cells were treated with cycloheximide for 1 h at 37°C in binding medium containing 0.4 mM cycloheximide prior to the addition of the bacteria, and this concentration was maintained throughout the binding assay. Total protein concentrations were determined by the Pierce bicinchoninic acid protein assay as recommended by the manufacturer (Pierce, Rockford, Ill.).

Immunoblotting. One-dimensional SDS-polyacrylamide gel electrophoresis analysis was carried out by the method of Laemmli (30). Proteins were resolved on 12.5% polyacrylamide gels, electrotransfered to Immobilon-P membranes, allowed to react with a polyclonal rabbit antiserum raised against the purified human ASGP-R (kindly provided by Richard J. Stockert, Albert Einstein College of Medicine, New York, N.Y.), and developed with alkaline phosphatase-conjugated anti-antibodies and reagents (8).

RNA isolation and Northern (RNA) blot analysis. Total cytoplasmic RNA was isolated from approximately 10^8 HepG2 cells, using the isolation kit manufactured by 5 Prime-3 Prime Inc. Purification of poly(A) mRNA was carried out with the use of an oligo(dT)-cellulose spin column (5 Prime-3 Prime Inc.). The yield from 1.5 mg of cytoplasmic RNA loaded on the column was approximately 20 μg of poly(A) mRNA. The purity of the poly(A) RNA samples was checked by the A_{260}/A_{280} ratio and was always higher than 1.90. The samples were then equalized according to their A_{260}. For Northern blot analysis, RNA samples were electrophoresed on horizontal denaturing formaldehyde agarose gels (1%) and transferred to positively charged nylon membranes (Hybond-N^{+}; Amersham). Hybridization was performed as described by the manufacturer. The hybridiza-

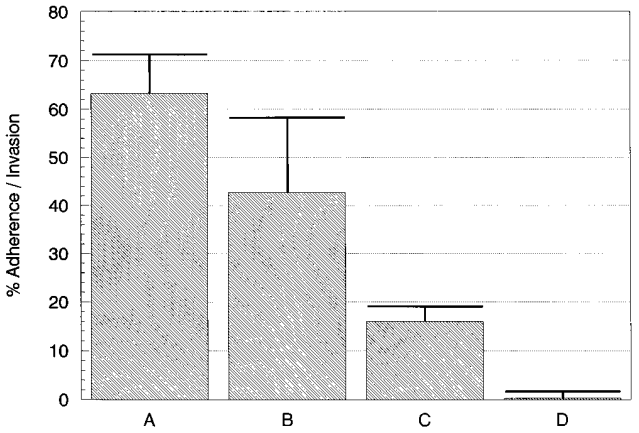

FIG. 1. Adherence to and invasion of HepG2 cells by *N. gonorrhoeae* 1291. Close adherence or invasion is calculated by the equation (CFU of *N. gonorrhoeae* 1291 invading HepG2 cells and resistant to gentamicin selection/total input CFU of *N. gonorrhoeae* 1291) × 100. Each column represents the mean results of seven different assays. *N. gonorrhoeae* 1291 phenotypes: column A, Opa^+, $pili^+$; column B, Opa^-, $pili^+$; column C, Opa^+ $Pili^-$; column D, Opa^- $Pili^-$.

tion solution contained 50% formamide. Both the incubation with the labeled probe and the washings were carried out at 42°C.

Probe for hybridization. Plasmid pSA1 was kindly provided by Michael Spiess, Biozentrum, Basel, Switzerland, and contained a full-length cDNA (1,210 bp) encoding the major form of the human ASGP-R (48). The plasmid was transformed into *Escherichia coli* MC1061, extracted, purified by standard methods (5), and cut with restriction endonucleases *Eco*RI and *Hin*dIII. The cut fragments were run on a 1% agarose gel, and the 1,210-bp fragment was identified, excised from the gel, and extracted from the agarose by using a GeneClean kit (Bio 101) as described by the manufacturer. The fragment was labeled with [^{32}P]CTP by using a random-primed DNA labeling kit (Boehringer Mannheim Inc.).

RESULTS

Adherence to and invasion of HepG2 cells by gonococci. The assays were carried out to determine what influence the presence of the terminal Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc tetrasaccharide had on the adherence and invasion of piliated and nonpiliated gonococci expressing either the Opa or the transparent phenotype. *N. gonorrhoeae* 1291 is unusual in that it produces predominately only one species of LOS (14) containing this tetrasaccharide (26). As noted previously by Shaw and Falkow (44), within the time frame of our studies, these assays would not differentiate between closely adherent organisms and those internalized. As can be seen in Fig. 1 and has been shown by previous studies, gonococci expressing both pili and Opa proteins had greatly enhanced ability to adhere to and/or invade HepG2 cells (34, 53, 54). In addition, in comparison with these other studies, which used different tissue culture cells and gonococcal strains, we found that the wild-type gonococci adhered to the HepG2 cells in greater numbers. As will be reported elsewhere (39a), the lack of the terminal LOS tetrasaccharide greatly reduced the number of adherent or invasive cells of both gonococci expressing Opa proteins and those lacking such expression. Thus, we used piliated Opa bacteria to infect HepG2 cells in all further experiments described in this report.

^{125}I-ASOR binding to the ASGP-R on HepG2 cells in the presence of *N. gonorrhoeae*. Binding of gonococci to the ASGP-R was studied by competition experiments, using ^{125}I-ASOR as the specific ligand. HepG2 cells were exposed to wild-type, piliated, Opa gonococci for 3 h at 37°C. The cells were then transferred to 4°C for 30 min prior to the addition of

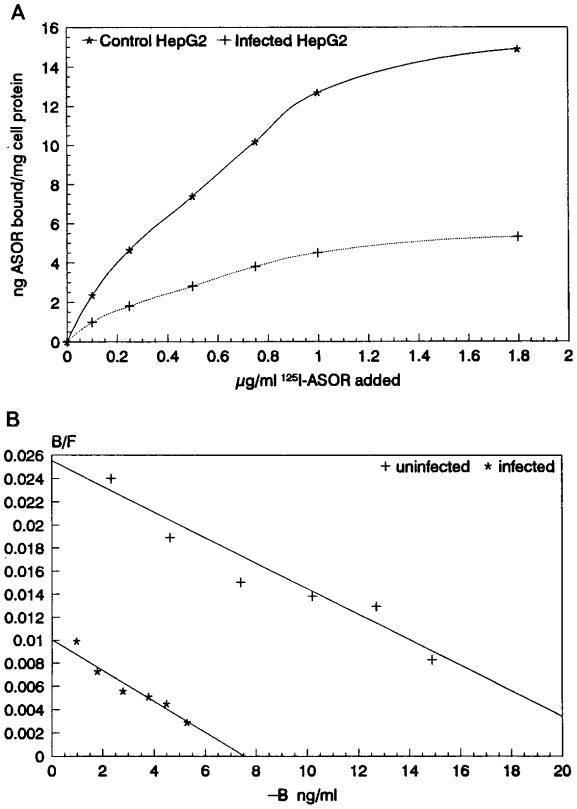

FIG. 2. (A) Effect of gonococci on the binding of ^{125}I-ASOR to HepG2 cells at 4°C. HepG2 cells near confluency were infected with piliated, Opa *N. gonorrhoeae* 1291 for 4 h at 37°C in binding medium. Triplicate dishes were incubated with various concentrations of ^{125}I-ASOR for 2 h at 4°C. The medium was then removed, and the cell layer was washed three times with ice-cold PBS, dissolved in 1 ml of 0.1 N NaOH containing 0.5% SDS, and counted. Nonspecific binding was determined in the presence of 400-fold-excess unlabeled ASOR and subtracted. The results are from a representative experiment. Three other experiments yielded similar data. (B) Scatchard plot analysis of ASOR binding to uninfected and infected HepG2 cells. The curves are plotted from the data represented in panel A. B, bound; F, free.

different concentrations of ^{125}I-ASOR. Incubation of the cells in the presence of gonococci resulted in 60 to 70% inhibition in the specific binding of ^{125}I-ASOR (Fig. 2A). Calculations based on Scatchard plots (Fig. 2B) yielded a dissociation constant of 2.0×10^{-8} M for uninfected control cells as well as for HepG2 cells infected with gonococci, a value in good agreement with data reported previously by Steer et al. (49). As illustrated in the Scatchard plots, exposure to gonococci resulted in an apparent decrease in available surface receptors (intercept on the B axis is maximal binding) with no detectable change in affinity (slope = $-1/K_d$). In all experiments, the nonspecific binding of the ligand was always less than 13% of the total binding.

Initially, it was somewhat disturbing that no inhibition of ^{125}I-ASOR binding to the cells could be detected in experiments carried out in the presence of 100 μg of purified wild-type LOS per ml. However, this observation led us to the conclusion that a second receptor with a higher affinity for LOS must be present on HepG2 cells. Our observations with respect to this second HepG2 receptor which binds LOS will be described elsewhere (39a). Concentrations of purified LOS needed to saturate both this higher-affinity receptor and the ASGP-R became toxic to the HepG2 cells and eliminated the possibility of further evaluation.

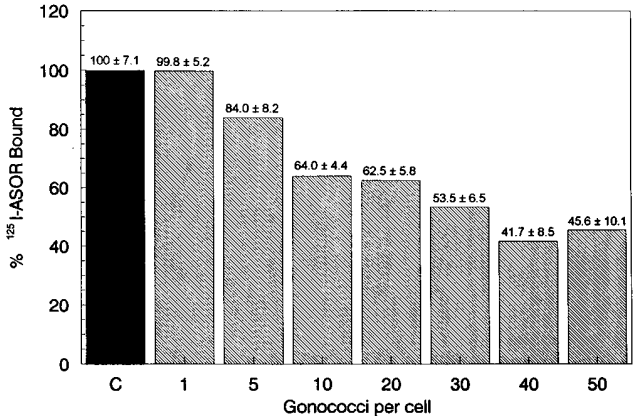

FIG. 3. Effect of bacterial cell number on the binding of ^{125}I-ASOR to HepG2 cells. HepG2 cells near confluency were infected with *N. gonorrhoeae* at various concentrations for 3 h at 37°C in binding medium. Triplicate dishes were incubated with 0.5 μg of ^{125}I-ASOR per ml for 1 h at 4°C. The medium was then removed, and the cells were washed three times with ice-cold PBS, dissolved, and counted. Binding is expressed as a percentage of the control value.

To rule out a possible gonococcus-ASOR interaction, which itself may cause an apparent inhibition of ASOR binding to the hepatocytes, the same binding experiments were carried out with gonococci suspended in binding medium at the concentration used to infect the HepG2 cells. The amount of radioactivity measured in the intact bacterial samples was always less than 2% of the total binding.

We next examined the effect of bacterial cell number on ASGP-R activity in HepG2 cells (Fig. 3). The dose-response curve revealed a decrease in the binding capacity of the receptor as a function of bacterial cell number, reaching a maximal inhibition at a ratio of 40 CFU per cell. Even with a higher ratio of bacteria to cell (100 CFU per cell), greater inhibition could not be achieved (data not shown).

To abolish the possibility of new receptor synthesis, the same experiments were carried out in the presence of 0.4 mM cycloheximide (Fig. 4). The addition of cycloheximide did not change the degree of inhibition of ^{125}I-ASOR binding in the presence of gonococci, and the inhibition by the bacteria never reached more than 70% even under these conditions.

The competition experiments were repeated at 37°C. Specific binding of ^{125}I-ASOR to the cells at 37°C after gonococcal adherence was inhibited by 50 to 60% (Fig. 4), a bit less than the inhibition found at 4°C. The 5.7-fold increase of ^{125}I-ASOR binding at 37°C over what was observed at 4°C was well within the range of the observations made by others (49) and represents active recycling of internal pools of the ASGP-R to the cell surface. A similar comparison of HepG2 cells exposed to gonococci demonstrated 7.3-fold-greater ^{125}I-ASOR binding activity at 37°C than at 4°C. This finding suggested that HepG2 cells exposed to gonococci synthesized more ASGP-R than did unexposed control cells.

Gonococci bind specifically to the ASGP-R on HepG2 cells. The specificity of the binding of gonococcal surface components to the ASGP-R was evident by comparing the binding report above with that of two other ligands, ^{125}I-insulin and ^{3}H-DHA, to their specific receptors on infected HepG2 cells. The presence of gonococci resulted in only a 12.7 ± 10.3% inhibition of the binding of the iodinated insulin, with nonspecific binding accounting for less than 10% of the total counts. Similarly, binding of ^{3}H-DHA to the β-adrenergic receptor on infected HepG2 cells was not inhibited by the presence of gonococci. In this case, nonspecific binding accounted for less than 26% of the total binding. However, in contrast to the insulin assay, the gonococcal infection of HepG2 cells seemed to result in even a slight increase (113% of the control value) in the amount of ^{3}H-DHA associated with the cells. The same phenomenon was found when the experiment was performed in cell culture medium. It was determined that this increase was not due to bacterial binding of ^{3}H-DHA, since binding of this ligand to intact bacteria in solution was always less than

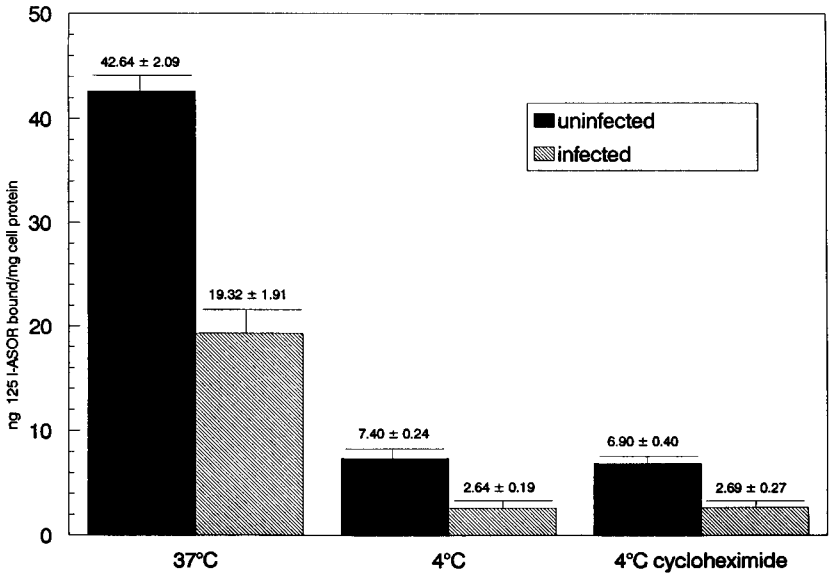

FIG. 4. Inhibition of ^{125}I-ASOR binding to HepG2 cells by *N. gonorrhoeae* at 4 and 37°C in the presence and absence of cycloheximide. HepG2 cells near confluency were infected with *N. gonorrhoeae* for 4 h at 37°C in binding medium. Triplicate dishes were incubated with 0.5 μg of ^{125}I-ASOR per ml for 2 h at either 4 or 37°C. The medium was then removed, and the cell layer was washed with ice-cold PBS, dissolved in 1 ml of 0.1 N NaOH containing 0.5% SDS, and counted. Cells treated with cycloheximide were preincubated for 1 h at 37°C in binding medium containing 0.4 mM cycloheximide and then infected with gonococci. Binding of ^{125}I-ASOR was also carried out in the presence of 0.4 mM cycloheximide.

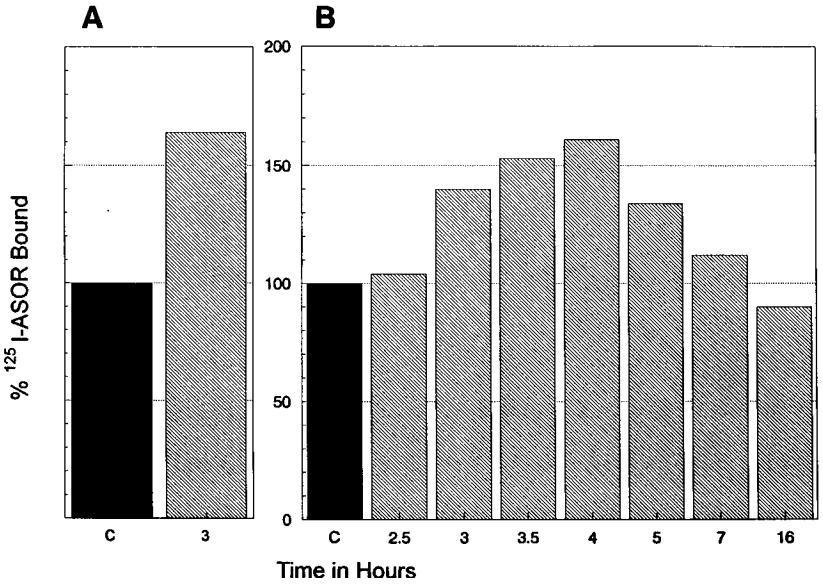

FIG. 5. Effect of gonococcal infection on ^{125}I-ASOR binding to intact and permeabilized HepG2 cells. (A) Following a 3-h infection with gonococci, infected and control HepG2 cells were washed with ice-cold PBS and permeabilized with 0.1% saponin for 30 min at 4°C. The permeabilized cells were then treated with 10 mM EDTA in PBS lacking Ca^{2+}, pH 5.0 (acid stripping), prior to the addition of 0.5 μg of ^{125}I-ASOR per ml. Radioligand binding was carried out at 4°C for 1 h. (B) For determination of receptors on the cell surface, cells infected for 2.5 h were washed from bacteria and allowed to further incubated at 37°C for various time periods up to 16 h. Immediately after the removal of the bacteria (2.5 h) and at various times during the incubation without bacteria, cell cultures were cooled by washing with ice-cold PBS, acid stripped, and then labeled with 0.5 μg of ^{125}I-ASOR per ml for 1 h at 4°C.

5% of the total binding. Further studies of this phenomenon are being carried out. However, the very low decrease (12.7%) in insulin binding to its receptor in the presence of bacteria, together with the fact that the bacteria did not inhibit the ^{3}H-DHA binding, strengthened our hypothesis that gonococci bind specifically to the ASGP-R on HepG2 cells.

Inhibition of ligand binding to the ASGP-R is due to receptor occupancy by the gonococci. To rule out the possibility that the observed inhibition of ^{125}I-ASOR binding to infected cells was due to receptor damage by the bacteria rather than the bacteria simply occupying the receptor, we examined the ability to recover receptor activity in the infected cells by means of an acid-stripping procedure (16). By this technique, the ligands bound to the ASGP-R are selectively removed by treating the cells with PBS containing no Ca^{2+} and 10 mM EDTA at pH 5.0. The total cell receptor number could be determined in saponin-permeabilized HepG2 cells, both infected and uninfected.

Use of this acid-stripping method preceding the ^{125}I-ASOR binding assay on saponin-permeabilized cells revealed that not only could the ASGP-R activity be recovered after exposure to gonococci, but in addition there was a demonstrable 64% increase in the total amount of ASGP-R associated with these cells (Fig. 5A). This increase could be detected immediately after the 3-h incubation with the bacteria. To determine how many of these additional receptors found their way to the cell surface and the time period involved, we examined intact HepG2 cells after infection by using the same acid-stripping method at different time intervals up to 16 h. The amount of cell surface ASGP-R showed only a slight increase immediately after infection (about a 4% increase after 2.5 h; Fig. 5B) but continued to increase, reaching a maximum level at 4 h. The number of surface receptors in the infected cells then decreased, returning to normal levels by 16 h. The change in total receptor number as a function of time was not determined.

Gonococci modulate the expression of the ASGP-R. The relative abundance of the ASGP-R after exposure to gonococci was also determined by immunoblot analysis using a polyclonal antibody raised against the purified human ASGP-R (Fig. 6). Previously, it was shown that HepG2 cells grown in tissue culture express three distinctive ASGP-R polypeptides: a 46-kDa species, corresponding to the major subunit (H1) of the human ASGP-R; a 40-kDa species, known to be the H1 precursor (45), and a 35-kDa degradation product of the H2 subunit (58). Binding of gonococci to HepG2 cells seemed to result in an increase in the 46-kDa species, while the intensities of the two other species were reduced. Thus, it appeared that the exposure of HepG2 cells to gonococci resulted in an increased concentration of the major ASGP-R subunit, while the levels of the precursor molecules and degradation products decreased. This apparent increase of ASGP-R on immunoblot analysis seemed in good agreement with results of the binding

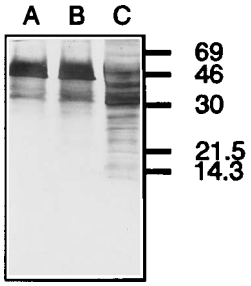

FIG. 6. Effect of gonococcal infection on the concentration of ASGP-R. HepG2 cells were infected with *N. gonorrhoeae* for 4 and 7 h. Cell lysates were electrophoresed on SDS–12.5% polyacrylamide gels, electroblotted, reacted with a polyclonal antiserum raised against the purified human ASGP-R, and detected with alkaline phosphatase-conjugated anti-antibodies and reagents. Lane A, cells infected for 7 h; lane B, cells infected for 4 h; lane C, uninfected control cells. Sizes are given in kilodaltons.

TABLE 1. Effect of gonococcal infection on total cell receptors and cell surface receptor concentrations, determined by ligand binding to HepG2 cells grown in MEM containing nondialyzed and dialyzed FBS[a]

Expt	FBS	Mean ng of ^{125}I-ASOR/10^7 cells ± SD	
		Noninfected	Infected
A			
Total cell receptors	Control	21.16 ± 1.23	33.55 ± 7.25
Saponin permeabilized	Dialyzed	15.47 ± 2.70	21.87 ± 1.97
B			
Cell surface receptors	Control	14.75 ± 1.24	6.92 ± 1.56
Nonpermeabilized	Dialyzed	14.40 ± 0.48	8.70 ± 0.76

[a] Quadruplicate HepG2 cells in six-well plates were infected with gonococci for 4 h at 37°C in binding medium. Cells were then washed with ice-cold PBS, chilled to 4°C, permeabilized with 0.1% saponin (experiment A) in binding medium for 30 min at 4°C, and rinsed with ice-cold binding medium. The level of total cell receptors was then determined by incubation with 1.0 μg of ^{125}I-ASOR per ml for 1 h at 4°C as described in Materials and Methods. Nonpermeabilized cells (experiment B) were incubated with 1.0 μg of ^{125}I-ASOR per ml at 4°C for 1 h, then washed and lysed with 0.1 N NaOH containing 0.5% SDS, and counted.

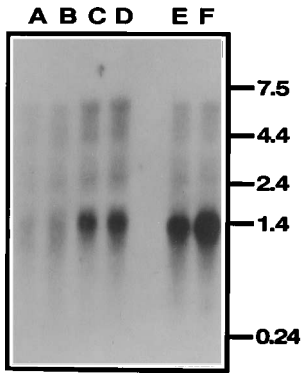

FIG. 7. ASGP-R transcripts in normal and infected HepG2 cells. Cytoplasmic RNA and poly(A) RNA were purified from uninfected HepG2 cells, and cells were infected for 4 h with *N. gonorrhoeae*. RNA was then fractionated on a denaturing formaldehyde–1% agarose gel (3 μg per lane) and transferred to a positively charged nylon membrane. The specific transcript for ASGP-R was detected with a full-length cDNA (1,210 bp) encoding the major form of the human ASGP-R. Lanes A and B, cytoplasmic RNA from uninfected cells; lanes C and D, cytoplasmic RNA from infected cells; lane E, poly(A) RNA from uninfected cells; lane F, poly(A) RNA from infected cells. Sizes are given in kilodaltons.

assays, which suggested that there was a 64% increase in total ^{125}I-ASOR binding after HepG2 cells were exposed to gonococci.

ASGP-R activity in infected HepG2 cells grown in dialyzed and undialyzed FBS. Stockert and colleagues (51) have shown that HepG2 cells grown in dialyzed FBS (depleted of biotin) express reduced amounts of the ASGP-R. With the addition of biotin to the dialyzed FBS, the amounts of the ASGP-R returned to control levels. The added biotin was correlated with reduced intracellular levels of cylic cGMP (cGMP) and thus related to translational control. Neither biotin or increased ligand had any effect on the transcriptional level in the amounts of specific mRNA detected. We attempted to determine whether gonococcal exposure of HepG2 cells, cultured in dialyzed FBS, would attain the same increased ASGP-R levels as observed in infected HepG2 cells grown in undialyzed FBS. The results (Table 1) show, as expected, increased ^{125}I-ASOR binding in the saponin-treated cells relative to nonpermeabilized cells (21.16 ng in uninfected permeabilized cells, compared with 14.75 ng in nonpermeabilized cells). Cells grown in dialyzed FBS demonstrated reduced levels of total receptor activity (15.47 ng), with most of it (14.4 ng) located on the cell surface.

The amount of ^{125}I-ASOR bound to the surface of infected cells grown in dialyzed FBS was about the same as that bound to infected cells grown in undialyzed FBS (6.92 ng in infected cells grown in normal FBS and 8.70 ng in cells grown in dialyzed FBS). The total cell receptor number (in the saponin-permeabilized cells) showed a significant increase after gonococcal infection. This demonstrable increase in receptor levels was observed in infected cells grown in either undialyzed or dialyzed FBS. However, the increased levels of total receptor molecules expected in infected cells grown in dialyzed FBS did not attain the levels seen in infected cells grown in undialyzed FBS. Thus, gonococcal infection did initiate an upregulation of the ASGP-R in cells grown in dialyzed FBS but did not restore the production of this receptor to the levels seen in cells grown in normal FBS.

Transcriptional regulation of the ASGP-R. The molecular mechanism involved in the increased expression of the ASGP-R in HepG2 cells infected with gonococci was also studied at the transcriptional level to determine whether gonococcal infection of HepG2 cells would result in a higher level of the specific transcript for the major (H1) receptor subunit. The relative concentrations of the specific mRNA for the H1 receptor subunit from both the infected and noninfected cells was determined by Northern blot analysis using a radiolabeled probe of the H1 form of the receptor (Fig. 7). The message detected by this probe corresponded to that previously reported for the H1 subunit in HepG2 cells (47) and migrated at a position of approximately 1.4 kb. Northern blot analysis indicated that greater amounts of ASGP-R mRNA were produced in HepG2 cells after infection with gonococci than in uninfected cells. From the higher H1 mRNA levels observed in the infected HepG2 cells, we concluded that at least part of the increase in ASGP-R protein expression could be accounted for by an effect of the bacteria on the cells at the transcriptional level. Any additional effects of the gonococci on the posttranscriptional production of the ASGP-R in HepG2 cells were not directly evaluated.

DISCUSSION

Whether human cells express a receptor(s) which can bind gonococcal ligands has never been explored. We thought that the Gal(β1-4)GlcNAc residues found on particular gonococcal LOS moieties and common to most strains of gonococci might serve as a ligand for the human ASGP-R. The ASGP-R, an integral membrane protein, was originally discovered in hepatic parenchymal cell membranes (4, 6). This hepatic lectin removes serum glycoproteins that have lost their terminal sialic acid residues, suggesting a physiological function for this receptor in the turnover of serum glycoproteins.

The HepG2 cell line was chosen as a model cell system since the ASGP-R had been studied most extensively in these cells and the functional assays for this receptor had been well established. Our results suggest that the ASGP-R on HepG2 cells can serve as a receptor for gonococci. Binding of the ASGP-R ligand, ^{125}I-ASOR, to HepG2 cells was partially blocked in the presence of gonococci. This inhibition was specific to the ASGP-R, since the binding of two other ligand-receptor pairs, insulin and the insulin receptor as well as the β-adrenergic blocker dihydroalprenolol and its receptor, was

only slightly affected by the presence of gonococci. The inhibition of ^{125}I-ASOR binding to the ASGP-R by *N. gonorrhoeae* 1291 ranged from 50 to 70%, depending on the temperature at which the binding was measured. At 4°C, a temperature at which recycling, synthesis, and degradation of ASGP-R were suppressed, the bacteria blocked 60 to 70% of the receptor molecules on the cell surface. Even with a higher ratio of gonococci to cells, the bacteria could achieve only partial inhibition of ^{125}I-ASOR binding and higher inhibition could not be demonstrated. In attempts to inhibit the ASGP-R with purified gonococcal LOS, a second, higher-affinity receptor on HepG2 cells was discovered and will be described elsewhere (39a). LOS concentrations needed to saturate the more avid receptor became toxic to the tissue culture cells and eliminated the possibility of further evaluation of the ASGP-R with purified material.

Partial occupation of the ASGP-R, as observed in our studies, was been reported in the early studies of Weigel et al. (56). These authors studied the binding of intact rat hepatocytes to specific carbohydrates immobilized on polyacrylamide gels. They demonstrated that cell adhesion was remarkably dependent on the sugar concentration and conformation on the synthetic surface, exhibiting a threshold-binding phenomenon. Also, Lee (31) described a model whereby two classes of noninteracting receptor binding sites are organized in a distinct geometrical arrangement, each having a specific attachment to different terminal galactose-containing compounds. Such arguments could explain these frequently observed partial inhibitions of the ASGP-R. This arrangement would result in heterogeneous ligand-lectin interactions depending on the nature of the ligand in use. Thus, the number of terminal Gal or GalNAc residues in gonococcal LOS, as well as their degree of branching and geometrical arrangement, may play a crucial role in their affinity to the ASGP-R. This may account for the partial inhibition (60 to 70%) of ASOR binding to the receptor caused by the presence of the bacteria.

To show that receptor activity lost from the surface of infected HepG2 cells resulted from their occupancy by the bacteria rather than receptor inactivation or damage, we reversed the binding of the bacteria to the ASGP-R by treating the infected cells with a calcium chelator (10 mM EDTA [pH 5.0]). When intact cells were acid stripped immediately after infection, 104% of the receptor molecules could be recovered on the cell surface (Fig. 5B). Further incubation of the infected cells (at 37°C) in the absence of gonococci followed by acid stripping resulted in increased surface receptor activity. These results suggest that the 60 to 70% reduction in the ability of infected cells to bind exogenous ASOR (Fig. 2A) was due to receptor occupancy by the bacteria. In addition, these results suggest that the binding of the gonococcal LOS to the second high-affinity receptor did not substantially influence binding to the ASGP-R. Although acid stripping removes bound ligand from the ASGP-R, it does not remove gonococcal LOS that has been bound to the high-affinity receptor at 37°C (39a). Thus, in these acid-stripping experiments, the high-affinity LOS receptor remains occupied and yet does not seem to interfere with the subsequent binding of ASOR. The two receptors seemed to act independently of each other. However, the increased number of cell surface ASGP-R during exposure to gonococci makes it impossible to totally rule out some interactions between the two receptors or some other mechanisms.

We next examined how the infectious process affected the number of total receptor molecules in the cells. Immunoblot analysis with polyclonal antibody raised against the purified human receptor gave evidence that exposure to gonococci resulted in an increased expression of the major (46-kDa) receptor species. The functional ^{125}I-ASOR binding assay substantiated this finding, demonstrating that the concentration of total receptor molecules (measured in cells treated with 0.1% saponin) increased by 60% after gonococcal infection compared with the level in unexposed controls (Table 1). The same percentage of increase was observed in infected cells which were first permeabilized and then acid stripped (Fig. 6A). Thus, we concluded that exposure of HepG2 cells to gonococci caused an increased expression of the ASGP-R.

Modulation of ASGP-R expression is well documented in hepatocellular development, proliferation, and disease (for a recent review, see reference 50). The molecular mechanism governing the changes in receptor expression is still unresolved. In particular, the question of whether transcriptional and/or posttranscriptional regulation is involved in the modulation of receptor levels is under intensive investigation. Huber and coworkers (23) studied the regulation of the ASGP-R synthesis in fetal, regenerating, tumor-promoted, and transformed rat hepatocytes. These authors have presented data which demonstrate that ASGP-R activity can be modulated at both the transcriptional and posttranscriptional levels.

We attempted to understand the molecular mechanism controlling modulation of the ASGP-R after gonococcal infection. Poly(A) RNA was examined by Northern blot analysis using a cDNA probe of the major human H1 subunit. This assay indicated an increased amount of mRNA coding for the ASGP-R in HepG2 cells after infection with gonococci, which is consistent with the higher amount of receptor molecules observed both in the immunoblot analysis and in the functional binding of ^{125}I-ASOR to permeabilized and nonpermeabilized infected cells. This result supports the idea that at least part of the molecular mechanism controlling the receptor level is under transcriptional control.

The possibility of posttranscriptional regulation mediated by a second messenger was also considered. Collins et al. (12) have demonstrated the effect of growing the cells in dialyzed FBS, lacking biotin, on the level of the human ASGP-R in HepG2 cells. In their proposed mechanism, biotin regulates expression of the ASGP-R by the activation of guanylate cyclase, which results in increased concentrations of intracellular cGMP. No change in the steady-state concentrations of H1- or H2-specific mRNA could be detected when cells were grown in the presence or absence of cGMP, which led the authors to conclude that this regulation takes place at a posttranscriptional level. We checked whether infection of HepG2 cells, grown in dialyzed FBS, with gonococci would restore the level of the ASGP-R to the level found in infected cells grown in undialyzed FBS, much as Collins et al. had shown (12). Our results indicated that infection with gonococci resulted in an increased level of the total number of receptor molecules by 41% but, unlike the results of Collins et al., did not reach the levels seen in infected cells grown in undialyzed FBS. In infected cells grown in normal FBS, the amount of receptor was 60% greater than in noninfected cells. Thus, the increased receptor level in the infected cells was in the same range regardless of whether the cells were grown in normal FBS or in dialyzed FBS. No additional increase in the ASGP-R level could be detected in the infected cells grown in dialyzed FBS.

Many other posttranscriptional mechanisms affecting receptor expression and activity are documented in the literature. For example, phosphorylation appears to be a potent regulatory mechanism in many receptor systems. Ligand phosphorylation by hepatocytes, subsequent to recognition by the specific receptor, was thought to be involved in the trapping and immobilization step of receptor-mediated endocytosis (9). On

the other hand, studies on receptor phosphorylation in hepatoma cells during basal conditions and exposure to phorbol esters suggested that this step leads to intracellular sequestering of receptor molecules, defining a stable, nonrecycling pool of intracellular receptors (17). Other steps in the endocytic pathway were found to be modulated by selected pharmacological agents and thus are considered to be important in the regulation mechanism of the receptor.

In this report, we demonstrated that gonococcal infection of HepG2 cells elicited an increase in the synthesis of new receptor molecules. How much of this increased receptor production is due to the increased levels of specific mRNA as opposed to posttranscriptional events remains unclear. We think that the increased expression of the ASGP-R after gonococcal exposure is controlled, at least in part, at the level of transcription.

Whether the ASGP-R is involved in in vivo gonococcal infections is not known. Although hepatic involvement is well known to occur during such infections (7, 13, 20, 33, 38) and gonococci can be recovered from liver biopsies (27), it has not been directly examined whether hepatic ASGP-R plays a role in this syndrome, nor has the role of the hepatic ASGP-R in disseminated gonococcal infections been examined. Recently, Abdullah and Kierszenbaum (1) reported the presence of the minor form (RHL-2/3) of the ASGP-R on sperm, although its function on these cells has not been established. Huber (22) has also demonstrated the expression of functional RHL-1 ASGP-R in late-stage mature mouse and rat spermatides. In 1971, Howard had speculated that gonococci might attach to human sperm and thus be transported through the female and male reproductive systems to establish infections in the fallopian tubes or the epididymis (21). Specific attachment of piliated and nonpiliated gonococci to human sperm cells was demonstrated by James-Holmquest and coworkers (25), suggesting that factors other than pili must be involved in the attachment process. We are currently examining gonococcal utilization of the ASGP-R on human sperm cells, and initial studies suggest that, as found in the present study, gonococci are able to bind to this receptor. As stated previously, the specific lactosamine-containing LOS on which we focused in this study as well as our current studies with sperm can also become sialylated both in vivo and in vitro. Such sialylation would block the interaction between the ASGP-R and gonococci containing this specific LOS. Although studies have shown that at least some of these specific LOS moieties remain unsialylated (3), there has not been a systematic examination of when, where, and how much sialylation of gonococcal LOS occurs in vivo. Thus, further studies are necessary to clarify these issues, including whether the ASGP-R has a role in gonorrheal disease. However, the present study does point out the fact that human cell surface receptors can interact with gonococci and that gonococci can influence the array of proteins and/or receptors on these human cells.

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