Human Immunoglobulin G Antibody Response to the Major Gonococcal Iron-Regulated Protein

MELINDA J. FOHN, TIMOTHY A. MIETZNER, TODD W. HUBBARD, STEPHEN A. MORSE, AND EDWARD W. HOOK III

The Department of Medicine, University of Washington, Seattle, Washington 98105 and Sexually Transmitted Diseases Laboratory Program, Centers for Disease Control, Atlanta, Georgia 30333

Received 27 April 1987/Accepted 10 September 1987

In humans, gonococcal infection occurs in environments limited with respect to free iron. Neisseria gonorrhoeae produces increased quantities of iron-regulated membrane proteins when grown under in vitro conditions which restrict the availability of free iron. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot (immunoblot) techniques, we studied the reactivity of specific antibodies to the 37-kilodalton (kDa) major iron-regulated protein (MIRP) of gonococci grown under iron-limiting conditions. Antibodies reactive with the 37-kDa MIRP were distinguished from those reactive with protein I by using purified 37-kDa MIRP or gonococcal protein preparations. Acute-phase sera from patients with disseminated gonococcal infection (DGI) reacted strongly to both the 37-kDa MIRP and protein I. Acute sera from nine patients with uncomplicated gonorrhea did not exhibit strong reactivity with the 37-kDa MIRP and were indistinguishable from five control sera. When compared with acute-phase sera, convalescent-phase sera from patients with DGI failed to demonstrate increased reactivity, whereas convalescent-phase sera from one of nine patients with uncomplicated gonorrhea developed reactivity to the 37-kDa MIRP. These data indicate that (i) the 37-kDa MIRP is expressed and antigenic in vivo and (ii) humans with DGI consistently develop a systemic antibody response to the 37-kDa MIRP.

In the human host, gonococcal infections occur in a relatively iron-limited environment; the majority of iron present at mucosal sites is infection is complexed to transferrin or lactoferrin (23) and, as a result, is not readily available to support growth of Neisseria gonorrhoeae or other mucosal pathogens. To date, much of the characterization of gonococcal outer membrane proteins (4, 5, 7, 8, 11, 18, 19, 26) has used N. gonorrhoeae cultivated on media designed to optimize bacterial growth; when gonococci are grown under conditions of iron limitation to more closely resemble the in vivo situation, several membrane-associated proteins are produced in increased amounts. In 1984 Mietzner et al. (15) described an iron-regulated, membrane-associated protein with a molecular weight of approximately 37,000 that showed a relative mobility similar to gonococcal protein I (PI) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This protein, the 37-kilodalton (kDa) major iron-regulated protein (MIRP), appears to be the iron-regulated protein produced in largest concentration under conditions of iron limitation and has been demonstrated in all strains of N. gonorrhoeae examined to date (14). Peptide-mapping studies demonstrated that the 37-kDa MIRP was distinct from PI and that the peptide map patterns of the MIRP isolated from multiple gonococcal strains were nearly identical, sharing a large number of common tryptic peptides (15). A similar 37-kDa MIRP has been observed in all serogroups of N. meningitidis (14, 16).

The observations that the 37-kDa MIRP migrated in close association with PI (15) during electrophoresis on SDS-polyacrylamide gels and that humans with acute gonorrhea produce antibody to the 37-kDa MIRP (16; S. A. Morse, unpublished observation) suggested that prior observations that PI was immunogenic for patients with complicated (7, 11) and uncomplicated (11) gonococcal infections (UCG) may have been due, at least in part, to reactivity of immunoglobulins with comigrating 37-kDa MIRP, rather than with PI. In this study we report the development of a system for differentiation of the 37-kDa MIRP from PI by using membrane preparations separated by SDS-PAGE. We also report the use of this system to characterize the prevalence of immunoglobulin G (IgG) antibodies to the 37-kDa MIRP and to PI in patients with UCG and disseminated gonococcal infection (DGI).

MATERIALS AND METHODS

Patient specimens. Acute- and convalescent-phase (3 to 4 weeks after diagnosis and initiation of treatment) sera were collected from eight men and one woman with UCG attending the Seattle King-County Sexually Transmitted Disease Clinic. Gonococcal isolates from these patients were not available. Serum specimens from nine patients (seven men and two women) with DGI were obtained at the time of diagnosis; each patient with DGI had positive blood or joint fluid cultures for N. gonorrhoeae. N. gonorrhoeae isolates from patients with DGI were typed by using the method of Knapp et al. (9); eight isolates belonged to WI (protein I-A), and one isolate belonged to WI/II/III (protein I-B) serovars. Convalescent-phase sera were obtained from three of the DGI patients 3 weeks after the initiation of therapy. Normal human serum specimens were obtained from five volunteers (three male, two female) with no prior history of gonorrhea, other sexually transmitted diseases, or meningococcal infection.

Organisms. N. gonorrhoeae strains were kindly provided as follows: strain FA171 from P. F. Sparling (University of North Carolina, Chapel Hill, N.C.) and strain F62 from R. P.
Purification

The supernatant containing protein IB and belongs to serogroup W1; strain F62 contains protein IB and belongs to serogroup WII. Gonococcal strains were maintained as stock cultures by lyophilization or freezing at -70°C in a solution of 1.5% (wt/vol) Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) containing 1.25% (wt/vol) bovine serum albumin and 6.25% (wt/vol) monosodium glutamate. Cultures were grown on GC medium base agar (Difco Laboratories, Detroit, Mich.) supplemented with 1% (vol/vol) of a growth factor supplement similar in composition to Isovitalex (BBL), except that the glucose and Fe(NO₃)₃ were omitted. This medium was further supplemented with glucose to a final concentration of 0.5% (wt/vol).

Growth conditions. Growth of N. gonorrhoeae in low iron medium has been described previously (15). A basal liquid medium was used with the following components per liter: proteose peptone no. 3 (Difco) 15 g; K₂HPO₄ 4 g; KH₂PO₄ 1 g; NaCl, 5 g; and soluble starch, 1 g. After autoclaving, the medium was supplemented with NaHCO₃ (420 mg/liter), a supplement similar to Isovitalex (1% [vol/vol]), and glucose (0.5% [wt/vol]). To limit the amount of available iron, Deseral mesylate (CIBA-GEIGY Corp., Summit, N.J.), a potent iron chelator, was added to a final concentration of 25 μM. Gonococci do not utilize the iron bound to Deseral (13). This iron chelator has been used previously to study gonococcal proteins expressed under conditions of iron limitation (14, 15, 17, 24, 25). Gonococci grown on GC agar were suspended in the iron-restricted medium described above and used to inoculate 300-ml naphelometers flasks (Bellco Glass Inc., Vineland, N.J.) containing 50 ml of the same medium to a density of ca. 25 Klett units (as monitored with a Klett-Summerson colorimeter [Long Island City, N.Y.] with a no. 54 filter). Upon reaching midlogarithmic phase, the entire suspension was used to inoculate an additional 450 ml of medium. Incubation was continued until the culture reached late logarithmic phase (100 to 120 Klett units), at which time the cells were harvested by centrifugation. The cells from 1 liter of medium were washed once in Davis defined minimal medium, pelleted, and suspended in 10 ml of 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) containing 0.1% (vol/vol) protease inhibitor (10 mM phenylmethylsulfonil fluoride in isopropanol). Cells were stored at -20°C until used for preparation of antigens.

Preparation of gonococcal antigens. For antigen preparation, frozen cell suspensions were thawed, suspended in phosphate-buffered saline (pH 7.2), immersed in an ice bath, and disrupted using sonication at the maximum setting for 4 min. MgCl₂ was added to a final concentration of 100 mM. After overnight incubation at 4°C, the suspension was centrifuged at 12,000 x g for 10 min to remove cellular debris. The supernatant was further clarified by centrifugation at 30,000 x g for 20 min. This resulting supernatant was collected and subjected to a final high-speed centrifugation of 140,000 x g for 1 h at 4°C. The supernatant (140K-S) was collected, the pellet (140K-P) was suspended in 1 ml of phosphate-buffered saline, and each was stored at -70°C until used. Protein concentrations were determined by the method of Lowry et al. (12).

Preparation of rabbit antiserum specific for the 37-kDa protein. Purification of the 37-kDa MIRP from N. gonorrhoeae F62 has been described elsewhere (T. A. Mietzner, G. Bolan, G. K. Schoolnik, and S. A. Morse, submitted for publication). Rabbits were immunized with purified 37-kDa protein according to the procedure of Blake and Gotschlich (3). Purified 37-kDa protein (200 μg) in complete Freund adjuvant (Difco) was injected subcutaneously into female New Zealand White rabbits. After 3 weeks, the rabbits were injected with 200 μg of purified 37-kDa protein in incomplete Freund adjuvant. A final injection of purified 37-kDa protein alone was given 3 weeks later. Control rabbit sera were obtained from the same rabbits before immunization.

SDS-PAGE. 140K-S, 140K-P, or purified MIRP preparations were solubilized in final sample buffer consisting of 0.0625 M Tris hydrochloride (pH 6.8), 2.0% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.001% bromophenol blue, and 5% (vol/vol) 2-mercaptoethanol. Samples were heated at 100°C for 2 min. For SDS-PAGE, membrane preparations were used with 25 μg of protein per lane and purified MIRP was used with 5 μg of protein per lane. Electrophoresis was done on 10% polyacrylamide slab gels (12 cm long and 1.5 mm thick) in the discontinuous Tris-glycine system as described by Laemmli (10). Electrophoresis was performed at 25 mA per gel until the dye front was within 1 cm of the bottom of the gel. Gels were fixed and stained with 0.25% Coomassie brilliant blue or used for Western blotting (immunoblotting). Molecular weight standards (12,400 to 94,700) (Sigma Chemical Co., St. Louis, Mo.) were included in each gel. Approximate molecular weights were determined by the method of Weber and Osborn (22).

Western blots. Electrophoretic transfer (Western blot) of proteins to 0.45-μm nitrocellulose paper (Schleicher & Schuell, Inc., Keene, N.H.) was performed by a modification of the method of Towbin et al. (21) using a Transblot Cell ( Hoefer Scientific Instruments, San Francisco, Calif.). Proteins were transferred at 10 V for 16 to 18 h at 4°C. Efficiency of transfer was determined by staining the nitrocellulose paper with 0.1% amido black (1). Antibodies specific for gonococcal proteins were detected on Western blots as described by Baker-Zander et al. (1), with the exception that phosphate-buffered saline containing 0.05% Tween 20 was used for blocking, washing, and dilution of sera, antibodies, and 125I-labeled staphylococcal protein A. Human sera was used at a dilution of 1:50, rabbit antisera specific for the 37-kDa MIRP at 1:2,000, and monoclonal antibody to PI at 1:5,000. Bound antibody was detected with 125I-labeled staphylococcal protein A. After the blotting procedure, Western blots were exposed to X-ray film (Cromex MRF film [Du Pont Co., Wilmington, Del.]) at ~70°C in cassettes equipped with enhancing screens (20).

RESULTS

SDS-PAGE analysis of separation of the 37-kDa MIRP from PI. SDS-PAGE analysis of sarcosyl-extracted outer membrane preparations from gonococci grown under iron limitation indicated that the 37-kDa MIRP comigrated with PI under normal Laemmli conditions (15). Incorporation of 70 mM NaCl in the lower gel resulted in modest separation of the two proteins (Fig. 1) (15). Early in our studies we observed that additional PI remained in the supernatant during membrane preparation increased the amount of 37-kDa MIRP in the 140K supernatant (Fig. 2). Further experiments to elucidate this observation demonstrated that phosphate buffers containing MgCl₂ at final concentrations of 0, 1, and 10 mM or containing 70 mM NaCl failed to increase the solubilization of the 37-kDa MIRP from the pellet fraction (data not shown). Coomassie blue-stained
SDS-PAGE gels of membrane preparations extracted with 100 mM MgCl₂ showed that PI was retained in the 140K-P fraction as previously noted (15, 16), whereas the majority of the 37-kDa MIRP was present in the 140K-S (Fig. 2). The identity and location of PI and the 37-kDa MIRP were confirmed by Western blotting of SDS-polyacrylamide gels of 140K-S, 140K-P, and purified 37-kDa MIRP followed by probing the blots with monoclonal antibodies to PI (data not shown) and rabbit antisera specific for the 37-kDa MIRP (Fig. 2). The results indicated that a small amount of 37-kDa MIRP remained in the 140K-P from strains FA171 and F62. Also, a band of ca. 41 kDa was observed in the 140K-S preparations from both strains as previously reported by Mietzner et al. (14).

Evaluation of human sera. Human sera from patients with DGI and UCG were evaluated for the presence of antibody to antigens contained in 140K-S and 140K-P preparations derived from N. gonorrhoeae FA171 or F62. Strain FA171 preparations served as antigen for evaluation of sera from patients with DGI caused by gonococcal strains containing protein IA, whereas strain F62 preparations were used for evaluation of sera from patients with UCG and the single patient with DGI caused by a protein I-B-containing gonococcal strain. The protein I-B strain was chosen for evaluation of sera from patients with UCG for whom gonococcal isolates were not available because, at the time the sera were collected, protein I-B-containing serovars constituted 88% of isolates from patients with UCG in Seattle (K. E. Hyde, J. S. Knapp, and E. W. Hook III, unpublished observations). Experiments were standardized to minimize the variability encountered in Western blots. For each gel blotted, lanes of the 140K-S and 140K-P were reacted with previously tested normal human sera as a qualitative control for nonspecific antibody binding.

Faint reactivity to the 37-kDa MIRP was observed in the serum from one of five volunteers with no history of gonococcal or meningococcal infections (Fig. 3; Table 1). Sera from three of five volunteers had detectable IgG anti-

![Image](http://iai.asm.org/)

**FIG. 1.** SDS-PAGE profile of sarcosyl-extracted crude membranes isolated from N. gonorrhoeae F62 on medium without Desferal (−DF) and with Desferal (+ DF). A total of 40 μg of protein was electrophoresed in each lane, and the gel was stained with Coomassie blue. Locations of the 37-kDa MIRP and PI are indicated.

**FIG. 2.** SDS-PAGE and Coomassie blue-stained gel of purified 37-kDa MIRP (A) and the 140K-S (B) and 140K-P (C) protein fractions (25 μg each) of membrane preparations of N. gonorrhoeae FA171 extracted with 100 mM MgCl₂ (SDS-PAGE). Adjacent is a Western blot of rabbit antisera specific for the 37-kDa MIRP reacted with the SDS-PAGE preparations of purified 37-kDa MIRP (A), the 140K-S (B), and 140K-P (C) from N. gonorrhoeae strain FA171 (BLOT). Rabbit antisera was used at a dilution of 1:2,000, and IgG antibody was detected using ¹²⁵I-labeled staphylococcal protein A. Locations of 37-kDa MIRP (37→) and PI are indicated.

body to PI. Patients with UCG (Fig. 3) demonstrated an overall qualitatively weak reactivity to the protein profiles presented. Acute-phase sera collected from nine patients with UCG showed faint reactivity to the 37-kDa MIRP in one patient, and all nine had faintly detectable IgG antibody to PI (Fig. 3). Convalescent-phase sera from the same patients showed little or no increment in reactivity to PI and clearly increased reactivity to the 37-kDa MIRP in one of nine patients (Fig. 3, patient 2).

Acute-phase sera from all nine patients with DGI demonstrated strong IgG reactivity to the 37-kDa MIRP when compared to normal controls or patients with uncomplicated...
gonorrea. Representative Western blots are seen in Fig. 4. A broad band of ca. 28 to 36 kDa in the 140K-P fraction was highly reactive with two of nine acute-phase serum specimens (Fig. 4, patient 1). It is unclear whether reactivity to PI contributes to this broad immunogenic band. Reducing exposure times of autoradiography failed to resolve this broadly reactive band. This 28- to 36-kDa antigen was not seen in Coomassie blue- (Fig. 2) and silver-stained (data not shown) gels of the gonococcal membrane preparations. The seven acute-phase serum specimens not reactive with the broad 28- to 36-kDa band were strongly reactive with PI. In the DGI patients from whom convalescent-phase sera were available, there were no changes in antibody reactivity observed when compared with corresponding acute specimens.

**DISCUSSION**

Initial descriptions of the 37-kDa MIRP noted the tendency of this protein to comigrate with PI when sarcosyl-extracted gonococcal outer membranes were electrophoretically separated (15). In these studies we observed that incubation of sonicated gonococci in 100 mM magnesium chloride followed by high-speed centrifugation resulted in solubilization of most of the 37-kDa MIRP, while PI and the majority of the other membrane-associated proteins remained in the pellet. This solubility difference between the 37-kDa MIRP and gonococcal PI was subsequently used in our studies to characterize the humoral immune response to these proteins. The basis for the observed solubilization of the 37-kDa MIRP in 10 mM HEPES buffer containing 100 mM MgCl₂ is unclear. However, the solubility of the 37-kDa MIRP could not be enhanced to the same degree by using increased concentrations of sodium chloride.

As noted in previous studies (11), serum specimens from individuals with no history of prior gonococcal or meningococcal infections often contained IgG antibody which reacted with gonococcal PI. Similar PI reactivities were noted in sera obtained from patients at the time of diagnosis of UCG and 2 to 4 weeks after diagnosis and therapy. Likewise, in our study, sera from patients with no history of prior gonorrhoea or acute-phase sera from patients with UCG only occasionally reacted to the 37-kDa MIRP by Western blot. Two weeks after the initiation of therapy, however, one of nine paired sera demonstrated a qualitative increase in antibody reactivity to the 37-kDa MIRP. This suggests that, in at least some patients with UCG, the 37-kDa MIRP but not PI, is recognized by the humoral immune system and stimulates antibody production.

Immunoblot analysis of sera from patients with proven DGI revealed marked IgG antibody response (relative to patients with UCG or normal human sera) to a number of gonococcal antigens at the time their infection was identified. Antibody reactivity to gonococcal PI could be definitely demonstrated in seven of nine DGI sera, and all nine sera tested were highly reactive with the 37-kDa MIRP. In two patients with DGI, Western blot analysis of the 140-P fraction demonstrated a highly reactive, broad ‘‘mushroom cap’’-shaped band corresponding to molecular weight 28,000 to 36,000 which was not visible on Coomassie blue- (Fig. 2) or silver-stained gels and which made PI reactivity difficult to assess. The characteristics of this band resemble the pattern of IgG antibody reactivity described for the 23- to 33-kDa antigen observed by Lammel and co-workers in sera from patients with DGI and UCG (11). The characteristics of this band are also compatible with published descriptions of the H-8 antigen previously described in both *N. gonorrhoeae* and *N. meningitidis* (2, 6). Little change in reactivity was observed in the three convalescent-phase sera available for testing. Thus, these data indicate that both PI and 37-kDa MIRP antigens are immunogenic in patients with complicated gonococcal infection and that detectable IgG antibody is present for both antigens in these patients with complicated infections at the time of diagnosis. The observation that IgG antibody to these antigens is present at the time of diagnosis and that little change in reactivity can be demonstrated between acute- and convalescent-phase sera confirms previous observations (7, 11) of the presence of antibody to gonococcal antigens early in the course of clinically apparent complicated gonococcal infections and is of unclear significance with regard to the pathogenesis or pathophysiology of these infections. Antibody to PI has been demonstrated to have bactericidal activity in a limited number of patients (7). However, it is also possible that such antibody could have other, possibly blocking, activity which might play a permissive role in the pathogenesis of DGI. Further studies of the functional activity of antibodies to both PI and

**TABLE 1. Reactivity of human sera with PI and the 37-kDa MIRP**

<table>
<thead>
<tr>
<th>Serum source and type (no. of sera tested)</th>
<th>Reactivity (no. of reactive sera/total tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHS (5)</td>
<td>3/5b</td>
</tr>
<tr>
<td>UCG (9)</td>
<td>1/5b</td>
</tr>
<tr>
<td>Acute (9)</td>
<td>9/9a</td>
</tr>
<tr>
<td>Convalescent (9)</td>
<td>9/9a&lt;sup&gt;°&lt;/sup&gt;</td>
</tr>
<tr>
<td>DGI (9)</td>
<td>1/9b</td>
</tr>
<tr>
<td>Acute (9)</td>
<td>7/7</td>
</tr>
<tr>
<td>Convalescent (3)</td>
<td>2/2&lt;sup&gt;°&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> NHS, Normal human serum.

<sup>b</sup> Weakly reactive.

<sup>c</sup> No change of reactivity in eight of nine patients after therapy. Convalescent-phase serum from one patient with UCG demonstrated increased reactivity to the 37-kDa MIRP.

<sup>d</sup> No change of reactivity in three of three patients after therapy; however, reactivity to PI and the 37-kDa MIRP could be assessed only in convalescent-phase sera of two due to the presence of a broadly reactive 28- to 36-kDa band seen in a Western blot from one patient (see text; Fig. 4).

![Representative Western blots of acute-phase (A) and convalescent-phase (C) sera from two patients with DGI. Each serum was evaluated for reactivity to the 140K-S (s) and 140K-P (p) fractions from membrane preparations of *N. gonorrhoeae* grown under conditions of iron limitation. Locations of the 37-kDa MIRP (37→) and PI are indicated. Locations of molecular weight standards (103) are shown.](http://iai.asm.org/)

Downloaded from [http://iai.asm.org/](http://iai.asm.org/) on November 6, 2017 by guest
the 37-kDa MIRP are necessary to help elucidate the roles of these proteins in gonococcal pathogenesis.

In conclusion, these data suggest that the 37-kDa MIRP is expressed and antigenic in vivo. The results of these studies also confirm the immunogenicity of PI for patients with complicated gonococcal infections. However, the functional activities of antibodies to the 37-kDa MIRP have not been fully investigated and warrant further study. The fact that the 37-kDa MIRP is expressed by all gonococcal stains examined to date, and that at least some patients with UCG, as well as patients with DGI, produce antibody to the 37-kDa MIRP suggest that, should antibody to this antigen have functional activity, further investigation as a potential gonococcal vaccine candidate would be warranted.

LITERATURE CITED