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Inhibition of *Neisseria gonorrhoeae* Attachment to HeLa Cells with Monoclonal Antibody Directed Against a Protein II

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This study showed that a protein II (PII) of *Neisseria gonorrhoeae* FA1090 appeared to act as a mediator of attachment to HeLa cells. Two colony variants of FA1090 were selected. Both gonococcal variants were nonpiliated, but one contained a PII and the other did not. A monoclonal antibody (1090-10.1), which was directed against the PII, inhibited the apparent PII-mediated attachment to HeLa cells. Antibodies produced from clone 1035-4, which had no PII specificity, did not inhibit the attachment and were used as controls. Inhibition of gonococcal attachment by the 1090-10.1 monoclonal antibodies was demonstrated by fluorescent microscopy analysis. Monoclonal antibody 1090-10.1 appeared to cause agglutination of the PII-containing organism. To block the clumping caused by the PII-specific monoclonal antibodies, Fab fragments of goat anti-mouse IgG were incubated with gonococci and the 1090-10.1 monoclonal antibodies. The results showed that the goat anti-mouse IgG Fab fragments partially blocked the agglutination caused by the PII-specific monoclonal antibody. The effect of the 1090-10.1 antibodies on attachment was also determined by monitoring the HeLa cells with attached iodinated gonococci. The monoclonal antibody appeared to inhibit the PII-mediated attachment.

In *Neisseria gonorrhoeae*, pili seem to function as primary effectors of attachment to eucaryotic cells (16, 17). The presence of a nonpilus surface protein, protein II (PII), also correlates with attachment (20). A strain of *N. gonorrhoeae* can express several PIIs associated with various degrees of colony opacity (14), and at least one PII appears to be associated with opaque colony phenotype. Opaque colony variants of strain F62 attach better to HeLa 229 and Flow 2000 cells than do transparent colony variants (4). One PII (leukocyte association factor), from strain MS11, promotes attachment to leukocytes (6, 7, 17). Other PIIs promote attachment to other types of eucaryotic cells (9, 15).

The attachment function of a surface protein, such as PII or pili, can be studied by showing that antibodies directed against the protein inhibit attachment (13, 21). Antiserum prepared against leukocyte association factor inhibited gonococcal attachment to polymorphonuclear cells (7). Also, antiserum prepared against pili inhibited attachment to buccal epithelial cells (18).

Our studies have identified a PII in gonococcus strain FA1090 which may act as a mediator of attachment to HeLa cells. We employed a highly specific monoclonal antibody (11) directed against the PII in our attachment assays. The influence of the monoclonal antibodies on attachment was studied by fluorescent microscopy and radiolabel techniques. The results showed that the PII-specific monoclonal antibody inhibited the PII-mediated attachment to HeLa cells.

MATERIALS AND METHODS

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (8). An acrylamide concentration of 12.5% was used in the separating gel. The molecular weight markers (BioRad Laboratories) as well as the *N. gonorrhoeae* FA1090 variants were solubilized at 37 or 100°C in SDS-PAGE sample buffer (0.125 M Tris buffer [pH 6.8], 2.5% SDS, 25% glycerol, 2.5% 2-mercaptoethanol, 0.001% bromophenol blue). The molecular weight markers were: bovine serum albumin, 66.2 kilodaltons (kDa); ovalbumin, 45

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kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and lysozyme, 14.4 kDa.

Iodination procedures. *Staphylococcus aureus* protein A and the SDS-PAGE molecular weight markers were iodinated by the chloramine-T method (1). Free iodine was removed by gel filtration through a Sephadex G-25 column, which was equilibrated in phosphate-buffered saline (PBS). Intact gonococci from the two colony variants of strain FA1090 were iodinated with a lactoperoxidase-catalyzed reaction (19). Briefly, 0.1 mCi of ^{125}I, 5 mM β-D-glucose, 20 μg of lactoperoxidase, and 0.2 U of glucose oxidase in 1 ml of PBS were combined with intact gonococci in a total volume of 1 ml for 10 min at room temperature. The gonococci were centrifuged and washed in PBS to remove unbound iodine.

Hybridoma selection and antibody production. Hybridomas were produced and screened, ascites were induced with hybridoma clones 1090-10.1 and 1035-4, and ascitic fluids were collected as previously described (11).

Antibody specificity assay. Proteins were transferred from SDS-polyacrylamide gels to nitrocellulose paper electrophoretically, overnight, at 100 mA in a Trans-Blot apparatus (BioLad Laboratories). The "Western blotting" procedure of Burnette (2) was used, except that PBS was substituted for Tris-saline. Iodinated molecular weight markers were not incubated with antibody. The X-ray film was exposed to the antigen-antibody-[^{125}I]protein A sandwich for 2 days at −70°C with enhancement by a Dupont Cronex intensifying screen.

Gonococci. *N. gonorrhoeae* FA1090 was grown on agar at 37°C in the presence of 5% CO_2 (5). Strain FA1090 expressed at least six PIIs (W. J. Black, R. S. Schwalbe, I. Nachamkin, and J. G. Cannon, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, D7, p. 48; manuscript in preparation). For our studies, two colony variants of strain FA1090 were selected. One colony type was transparent, whereas the other formed slightly more opaque colonies. Gonococci from the colonies that were slightly more opaque contained a PII (designated strain FA1090 PII$^+$). Gonococci from the other colony variant did not contain a PII (designated strain FA1090 PII$^-$). Colonies were harvested after 16 to 20 h of growth.

N. gonorrhoeae can shift colony phenotype and PII composition with a moderately high frequency (14). Consequently, we monitored the PII composition for the PII$^+$ and PII$^-$ variants used in each experiment. The data reported in this paper are from experiments in which the PII$^+$ variant did have a PII as demonstrated by SDS-PAGE, Western blotting, and autoradiography assays. The PII$^-$ variant did not have PIIs as determined by these assays.

The large-diameter colony types selected for both the PII$^+$ and PII$^-$ variants represented nonpiliated gonococci. Organisms from the same strain FA1090 colony types used in this study were subjected to SDS-PAGE and transferred to nitrocellulose paper for an electroblotting assay. Rabbit antibody directed against gonococcal strain MS11 pilus cyanogen bromide fragment 2 (courtesy of Gary K. Schoolnik, Stanford University) was iodinated and used to react with the strain FA1090 preparations. The resultant autoradiographs showed no evidence of pili. As controls, gonococci from small-colony types (piliated) but not large-colony types (nonpiliated) of many other strains of gonococci were prepared in the same manner. Reactions with pilus protein bands were routinely seen only on autoradiographs of the gonococci from the small-colony types. The minimal amount of strain FA1090 pilus protein required for a positive reaction was not determined.

HeLa cells. HeLa 229 cells were maintained in Eagle minimal essential medium with 10% heat-inactivated calf serum, 2.5 μg of fungizone per ml, 50 μg of gentamicin per ml, and 100 U of penicillin per ml (3). HeLa cells were removed from two 150-cm^2 flasks with 0.1 mg of pancreatin per ml, 0.6 mg of trypsin per ml, and 0.5 mg of EDTA per ml, and then suspended at 1×10^5 to 3×10^5 cells per ml in antibiotic-free minimal medium with serum. Portions (1 ml) of the cells were placed on cover slips in Leighton tubes. The cells were incubated at 37°C in 5% CO_2 and air for 24 h to allow formation of monolayers.

Antibody-binding assay. Duplicate samples of strain FA1090 PII$^+$ and PII$^-$ variants (2×10^7 CFU/ml) were incubated with concentrations ranging between 0.03 and 1.0 mg of ascitic fluid (clone 1090-10.1) per ml. After 90 min at room temperature, the organisms were centrifuged and washed. ^{125}I-labeled protein A (7×10^6 cpm) was added to each tube and incubated for 30 min at room temperature. The tubes were washed twice with medium 199 before being counted. The average number of counts per minute was calculated for each concentration of ascitic fluid.

Fab fragment preparation. Fab fragments of goat anti-mouse IgG (GAM) (Cappel Laboratories) were produced by a pepsin digest as outlined by Nisonoff et al. (12). The peptic digest was treated with 20 mM iodoacetamide for 60 min at room temperature before being purified on a Sephadex G-75 column. Protein concentrations were determined by the method of Lowry et al. (10).

Attachment assays. (i) ^{125}I-labeled gonococci. Four inocula were prepared for each set of experiments. ^{125}I-labeled intact gonococci, strain FA1090 PII$^+$ or FA1090 PII$^-$, were incubated with monoclonal antibody 1090-10.1 or 1035-4 for 90 min at room temperature. The inocula were centrifuged, suspended in medium 199, and diluted 10-fold in medium 199. Cover slips of HeLa cells were washed in medium 199, and then 2 ml of an inoculum was added to each cover slip. The cover slips were incubated without shaking at 37°C for 30 min, the inocula were removed, and the cover slips were washed and counted.

The HeLa cells from one or two cover slips incubated with inoculum or medium alone were scraped off with a rubber policeman and dispersed in 1 ml of the pancreatin-trypsin-EDTA solution. The cells were counted with a hemacytometer, and an average HeLa cell count was determined. There were approximately 10^5 HeLa cells removed per cover slip, which was about one-third the number used to seed the cover slips. The number of HeLa cells incubated with the gonococcal inocula did not vary more than the average ± the standard deviation from the number of HeLa cells incubated with medium alone.

The counts per minute and CFU per milliliter for samples of each of the inocula were also determined. Samples of the organisms before iodination were assayed in the immunoblot assay described above.

(ii) GAM Fab fragment effects on monoclonal anti-

body action. An assay was also performed as outlined above, except that GAM Fab (0.5 mg/ml, final concentration) was added to the four inocula: strains FA1090 PII^+ and PII^- each with monoclonal antibody 1090-10.1 or 1035-4. The GAM Fab inocula were incubated at room temperature for 30 min and vortexed. The remainder of the attachment assay was performed as described above. One GAM Fab preparation was used for two experiments, and a second preparation was used for a third set of experiments.

(iii) Light microscopy analysis. Solutions (2×10^8 CFU/ml) of the strain FA1090 colony variants were prepared in PBS. The gonococci (0.6 ml) were preincubated with 0.025 ml of monoclonal antibody 1090-10.1 or 1035-4 for 90 min at 22°C with periodic shaking. The inocula were diluted 1:10 and incubated with prewashed cover slips coated with nonconfluent HeLa cells (2×10^4) for 30 min at 37°C without shaking. After the cover slips were washed, the HeLa cells and gonococci were fixed in methanol. The cover slips were then stained with 0.005% 3-*bis*dimethylaminoacridinium chloride (acridine orange) in 0.15 M sodium acetate buffer (pH 4.0) at room temperature for 15 min. The numbers of attached gonococci were counted with a microscope with incident fluorescent light at ×1,000 magnification. The CFU per milliliter for each inoculum was determined for each set of experiments.

RESULTS

Two variants of *N. gonorrhoeae* strain FA1090 were selected for study. Based on their SDS-PAGE profiles (Fig. 1), the variant of strain FA1090 that formed slightly more opaque colonies contained a heat-modifiable protein with an apparent molecular weight of 30,000 and was designated PII^+. This PII corresponded with the PIIb-containing variant of strain FA1090 (W. J. Black et al., Abstr. 82nd Annu. Meet. Am. Soc. Microbiol., p. 48).

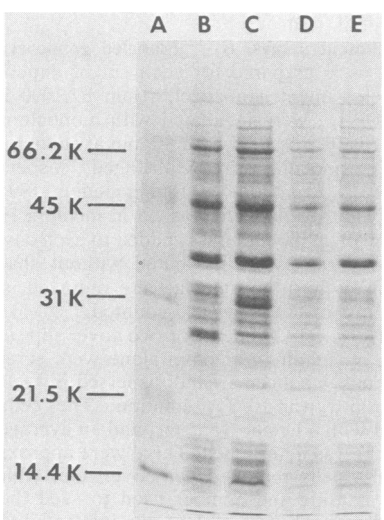

FIG. 1. Coomassie blue R-250-stained, 12.5% SDS-polyacrylamide gel of strain FA1090 organisms. Lanes: A, molecular weight standards; B, PII^+ variant (2 h at 37°C); C, PII^+ variant (5 min at 100°C); D, PII^- variant (2 h at 37°C); E, PII^- variant (5 min at 100°C). The standards and the bacteria were solubilized in SDS-PAGE sample buffer at the temperature and for the time indicated in parentheses.

The lactoperoxidase-catalyzed iodination of intact organisms and their analysis by autoradiography after SDS-PAGE demonstrated that the PII from the PII^+ variant was a surface protein (Fig. 2).

The mouse monoclonal antibody 1090-10.1 was specific for the PII of strain FA1090 PII^+. This was demonstrated by a Western blotting reaction and autoradiography (Fig. 3). There was no reaction between the proteins expressed by strain FA1090 PII^- and monoclonal antibody 1090-10.1, nor was there a reaction between monoclonal antibody 1035-4 and the PII of either variant by the Western blot assay.

The concentration of ascitic fluid protein (clone 1090-10.1) for maximal binding to the PII^+ variant ($\sim 10^7$ CFU) was determined to be 0.34 mg of protein per ml. The PII^- variant did not bind ascitic fluid proteins. At higher concentrations of ascitic fluid, a decrease in the gonococcal-associated ^{125}I counts per minute per CFU was observed. The 0.34-mg/ml concentration of ascitic fluid protein was maintained in the attachment assays. Ascitic fluid protein (clone 1035-4) did not bind to PII^+ organisms in this assay.

The effect of monoclonal antibody 1090-10.1 on the attachment of strain FA1090 organisms to HeLa cells was studied with radiolabeled gonococci. Monoclonal antibody 1090-10.1 either had no effect or appeared to cause an increase in gonococcal binding of the PII^+ organisms in comparison with the control antibody 1035-4 (Table 1). Also, the number of PII^+ organisms that attached when treated with monoclonal antibody 1090-10.1 was greater than the number that attached without it (data not shown). This apparent stimulation of attachment was due to the clumping or agglutination of the bacteria before their attachment to HeLa cells, as evidenced by at least a 50% drop in their numbers in the inoculum for the PII^+ organisms treated with antibody 1090-10.1 as compared with the numbers in antibody 1035-4 treated controls.

To help counteract the clumping, GAM Fab were added to the four inocula before addition to HeLa cells. Monoclonal antibody 1090-10.1-treated strain FA1090 PII^+ cell attachment was significantly decreased ($P < 0.05$) in two of the three experiments compared with the experiments in which strain FA1090 PII^+ was incubated with control monoclonal antibody 1035-4 and GAM Fab (Table 1). Attachment by the control PII^- variant was not affected by either antibody (Table 1).

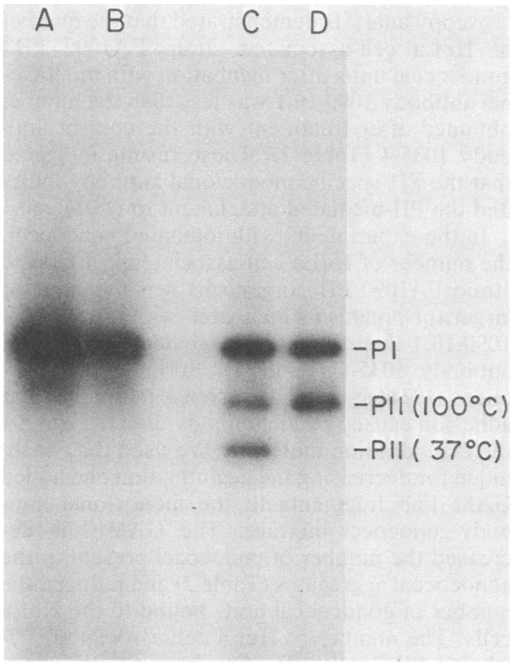

FIG. 2. Autoradiograph of iodinated strain FA1090 PII^+ and FA1090 PII^- gonococci. Lactoperoxidase-catalyzed iodination was performed on whole bacteria in PBS. The gonococci were washed and then solubilized in SDS-PAGE sample buffer before a sample of each variant (10^5 cpm) was applied to a 12.5% SDS-polyacrylamide gel. Labeled proteins were detected by autoradiography (24 h) of the dried gel. Lanes: PII^- solubilized at 37 (A) or 100°C (B); PII^+ solubilized at 37 (C) or 100°C (D).

To quantitate the clumping of strain FA1090 PII^+, HeLa cells and attached gonococci were counted after being stained with acridine orange by using fluorescent microscopy. A gonococcal unit was a single organism, a pair, or a clump. It was apparent that the number of gonococci per clump was greater for monoclonal antibody 1090-10.1-treated strain FA1090 PII^+ than for the other three inocula (Table 2). For example, the percentage of gonococcal units with 16 or more organisms was 15 and 0% for the antibody 1090-10.1-treated PII^+ and the control antibody, respectively. Also, the number of attached PII^+ organisms after treatment with monoclonal antibody 1090-10.1 was decreased (1.66 versus 3.75 U) compared with the number of gonococcal units per HeLa cell after incubation of the organisms with control monoclonal antibody 1035-4 (Table 2). A similar number of strain FA1090 PII^- gonococcal units attached after incubation with either antibody. To inhibit clumping or agglutination due to the PII-specific antibody, GAM Fab were added to the strain FA1090 PII^+ and antibody 1090-10.1 inoculum, yielding a decreased percentage of clumped gonococci as well as a lower number of bound gonococcal units per HeLa cell (Table 2).

DISCUSSION

N. gonorrhoeae PIIs are putative attachment effectors for eucaryotic cells (7, 17, 20). Our investigation has provided evidence that a PII of *N. gonorrhoeae* FA1090 may act as an attachment effector to HeLa cells. The results meet the criteria indicating protein-mediated attachment as outlined by Watt et al. (21). First, the protein must be a surface component. The 30 kDa PII of *N. gonorrhoeae* FA1090 is a surface protein, as evidenced by the ^{125}I labeling of intact organisms (Fig. 2).

A second criterion is that the bacteria which do not have the surface protein do not attach. The number of strain FA1090 PII^- units which attached to the HeLa cells was less than one-half that for the PII^+ variants (1.64 versus 3.75) in the presence of control antibody. The number of attached strain FA1090 PII^- units was not affected by either antibody (Table 2), suggesting that the PII could enhance attachment. The radiolabel studies were not as conclusive, perhaps due to nonspecific or PII-mediated aggregation or clumping of the PII^+ variants. This clumping would have yielded a decreased number of CFU per milliliter, resulting in a decreased number of gonococcal units available for binding while increasing the radioactivity count in the inoculum. Calculations of the percent inoculum bound for PII^+ organisms yielded

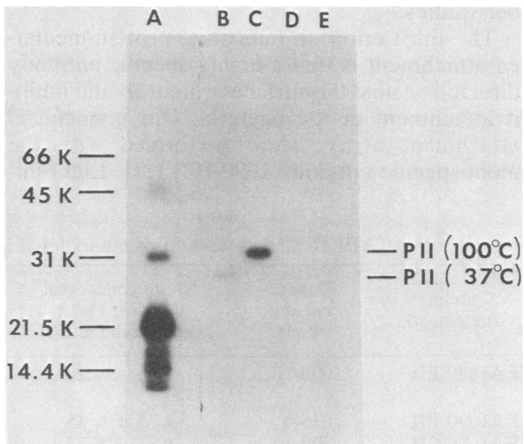

FIG. 3. Autoradiograph of the Western blotting reaction between strain FA1090 organisms and monoclonal antibody 1090-10.1 The strain FA1090 proteins from an SDS-polyacrylamide gel (Fig. 1) were transferred to nitrocellulose paper. The Western blotting assay for reactive antigens employed the PII-specific monoclonal antibody 1090-10.1. Lanes: (A) ^{125}I-labeled molecular weight markers not exposed to antibodies; PII^+ solubilized at (B) 37 or (C) 100°C; and PII^- solubilized at (D) 37 or (E) 100°C.

TABLE 1. Attachment of radiolabeled gonococci to HeLa cells

Strain (treatment)	Expt no.	% Inoculum bound[a] ± SD with monoclonal antibody: 1035-4	1090-10.1
FA1090 PII$^+$	1	1.5 ± 0.21	1.7 ± 0.17
	2	1.3 ± 0.07	1.7 ± 0.04
	3	1.4 ± 0.20	1.2 ± 0.22
FA1090 PII$^-$	1	0.9 ± 0.11	0.9 ± 0.18
	2	1.0 ± 0.13	1.2 ± 0.12
	3	1.0 ± 0.09	0.9 ± 0.16
FA1090 PII$^+$ (GAM Fab)[b]	1	1.9 ± 0.22	1.0 ± 0.38
	2	1.2 ± 0.05	0.7 ± 0.17
	3	2.2 ± 0.19	2.0 ± 0.19
FA1090 PII$^-$ (GAM Fab)	1	0.9 ± 0.16	1.0 ± 0.18
	2	0.8 ± 0.09	0.8 ± 0.12
	3	1.5 ± 0.22	1.5 ± 0.22

[a] Percent inoculum bound values were calculated as ([counts per minute per cover slip] × 100)/([2 ml] [average counts per minute per milliliter of inoculum]). Each value is the average of four to five cover slips.

[b] The difference between the mean percent inoculum bound with antibody 1035-4 and antibody 1090-10.1 was significant by Student's t test for experiments 1 and 2 ($P < 0.025$), but not for experiment 3.

a lower value than would have been obtained had it been possible to count individual bacteria. Thus, a smaller difference in the percent inoculum bound (e.g., 1.0 versus 1.3) between the PII$^-$ and PII$^+$ variants was found in the radiolabel studies.

The third criterion indicating protein-mediated attachment is that a highly specific antibody directed against the surface protein should inhibit attachment of the bacteria. Our gonococcal attachment assays were performed with the monospecific antibody 1090-10.1 (11). Light microscopy analysis demonstrated that the number of HeLa cell-associated strain FA1090 PII$^+$ gonococcal units after incubation with monoclonal antibody 1090-10.1 was less than the number obtained after treatment with the control antibody 1035-4 (Table 2). These results indicated that the PII-specific monoclonal antibody inhibited the PII-mediated attachment to HeLa cells.

In the experiments with iodinated gonococci, the number of HeLa cell-associated ^{125}I-labeled strain FA1090 PII$^+$ organisms was the same or larger for organisms incubated with monoclonal 1090-10.1 than for those incubated with control antibody 1035-4 (Table 1). Springer and Barondes (13) also noted an increase in intercellular adhesion caused by an antibody directed against the cell adhesion molecule. We used their technique for decreasing the agglutination and added GAM Fab fragments to the monoclonal antibody-gonococci mixture. The GAM Fab decreased the number of gonococci present in the gonococcal aggregates (Table 2) and reduced the number of gonococcal units bound to the HeLa cells. The number of HeLa cell-associated ^{125}I-labeled PII$^+$ cells, after incubation with monoclonal antibody 1090-10.1 plus GAM Fab, was significantly lower than that with control antibody 1035-4 plus GAM Fab for two of three experiments (Table 1). The difference in results may have been because a different GAM Fab preparation was used in the third set of experiments, stressing the importance of the specific GAM Fab preparation.

Another source of variation was evident in the experiments done to determine the optimal concentration of ascitic fluid protein for use in the assays. It became apparent that the 1090-10.1 monoclonal antibody incubation and centrifugation steps caused overt aggregation of the organisms. It appeared that the aggregation inhibited the ^{125}I-protein A from binding to the antibody

TABLE 2. Association of monoclonal antibody-treated gonococci with HeLa cells

Strain (treatment)	Monoclonal antibody	No. of gonococcal units per HeLa cells[a] (mean ± SD)	Inoculum (10^8 CFU/ml)	Gonococcal units (% of total no.) with indicated no. of organisms per clump: 1–2	3–5	6–8	9–15	≥16
FA1090 PII$^+$	1090-10.1	1.66 ± 0.20 [b]	2.2	38	21	16	11	15
FA1090 PII$^+$	1035-4	3.75 ± 0.45 [b]	2.7	74	20	3	2	0
FA1090 PII$^-$	1090-10.1	1.81 ± 0.27 [c]	3.6	65	26	7	1	1
FA1090 PII$^-$	1035-4	1.64 ± 0.08 [c,d]	4.2	83	15	2	0	0
FA1090 PII$^+$ (GAM Fab)	1090-10.1	1.09 ± 0.05	1.9	48	27	16	6	3

[a] Each value is the mean of counts from three cover slips with a minimum of 100 LeLa cells counted per cover slip ± standard deviation.

[b] $P < 0.005$ by Student's t test.

[c] No significant difference.

[d] $P < 0.005$ by Student's t test.

molecules and resulted in the drop in counts per minute per CFU at higher ascitic fluid protein concentrations.

The relatively low levels of attachment for the PII^+ variant, 0.7 to 2.2% of the inoculum (Table 1), could be due to the eucaryotic cell substrate used. Previously, Swanson et al. (15) showed that a nonpiliated T_4* colony type with the PII leukocyte association factor was substrate specific. They showed that 3.9% of the T_4* inoculum bound to human leukocytes, whereas only 1.9% of the inoculum bound to HeLa cells. The strain FA1090 PII^+ organism could exhibit this type of substrate specificity; however, we did not examine substrate specificity.

One strain of *N. gonorrhoeae* can make multiple PIIs, and these appear to be antigenically heterogeneous (J. Swanson, Int. Conf. Path. *Neisseria*, Montreal, Canada, August 1982). Additionally, the different PIIs appear to have different eucaryotic cell attachment specificities (7, 20). It is interesting that one strain of *N. gonorrhoeae* can make a series of proteins, such as the PIIs, which may function both to circumvent immune response by antigenic variation and possibly to enhance attachment to mucosal cells at different anatomic sites in men and women. Our studies have shown that monoclonal antibodies are useful reagents for experiments which will further the understanding of these interesting *N. gonorrhoeae* characteristics.

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