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Immunoelectron Microscopic Localization of Outer Membrane Proteins II on the Surface of *Neisseria gonorrhoeae*

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To determine the ultrastructural distribution and immunological accessibility of proteins II (P.IIs) on the surfaces of whole gonococci, anti-P.II gold probes were developed and used in electron microscopic studies of viable P.II-expressing variants of *Neisseria gonorrhoeae* FA1090. Anti-P.II probes clearly marked the surfaces of organisms and their associated outer membrane blebs. The surface-exposed portion of P.II is antigenically variable. With the use of two different sizes of gold probes, it was demonstrated that individual gonococcal cells can express more than one antigenic type of P.II simultaneously.

The surface proteins of gonococci, which are probably important in the interaction of the organisms with mucosal cell surfaces, have been extensively characterized (3, 5, 8, 19). Some of the proteins, such as outer membrane protein II (P.II), demonstrate marked interstrain and intrastrain structural and antigenic diversity (2, 7, 9, 11, 17, 20). Variants of a single gonococcal strain can express antigenically different P.II proteins, and changes in expression of the protein occur at high frequencies (16, 17). The high rate of P.II switching results in gonococcal populations that are always heterogeneous in P.II expression, and it has been difficult to characterize expression of the proteins in individual organisms.

Recent advances in immunoelectron microscopy using gold spheres conjugated to specific antibodies allow visualization of the ultrastructural distribution and immunological accessibility of native antigens on the surfaces of viable bacteria (12–15). Application of this technique to the study of gonococcal surface proteins has revealed unexpected heterogeneity in labeling with antibodies specific for protein I and the common neisserial antigen H.8 (12). These components are produced by all gonococcal strains tested, but H.8- or protein I-specific gold probes label only some of the organisms in a population. A possible explanation for this is that the antigens are sometimes covered or masked by another surface component that is variably present, such as P.II. In this study, we used monoclonal antibodies specific for different P.IIs to determine the distribution and accessibility of P.II on the surfaces of whole gonococci and the ability of single organisms to express more than one P.II simultaneously. We also tested the possibility that changes in the expression of P.II affect the surface accessibility of the H.8 antigen.

The microorganisms used in this study were P.II-expressing phenotypes of *Neisseria gonorrhoeae* FA1090 (10). FA1090 is a serum-resistant, prototrophic strain originally isolated from a patient with probable disseminated gonococcal infection. Single-colony isolates were passaged on solid agar and chosen for expression of different molecular weight and antigenic types of P.II as previously described (2). The P.IIs were designated P.IIa through P.IIf in order of increasing molecular weight, as demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2). Cultures with six phenotypes of FA1090, each expressing predominantly a single P.II, and one phenotype that did not express any P.II were used.

Cultures with the P.II phenotypes displayed colony morphologies with various degrees of opacity and transparency, depending on the P.II expressed (18). After 18 to 20 h of incubation in a 2% CO_2 incubator at 37°C, organisms were harvested from clear agar medium and used in immunoelectron microscopic studies (12).

Culture supernatants containing monoclonal antibodies specific for strain FA1090 organisms with P.II phenotypes were prepared and characterized as previously described (10). The anti-P.II monoclonal antibody preparations used in this study have the following designations, with the P.II specificities given in parentheses: H.138.1 (anti-P.IIa), H.4.1 (anti-P.IIb and anti-P.IId), H.157.1.8 (anti-P.IIc), H.164 (anti-P.IIe), and H.156.2 (anti-P.IIf). Monoclonal anti-H.8 antibody was prepared and characterized as previously described (6).

Specific monoclonal P.II and H.8 antibodies were individually conjugated to electron-dense gold spheres, and the resultant gold probes were used to mark whole gonococci as previously described in detail (12, 15). All specimens were examined by transmission electron microscopy (JOEL 100S, Tokyo, Japan).

Anti-P.II gold probes clearly marked the surfaces of P.II-expressing gonococci and their associated outer membrane blebs (Fig. 1). However, within each of these highly select gonococcal variants there were organisms which were not labeled by the homologous anti-P.II probes (Fig. 2). When organisms of the P.IIa-expressing phenotype were exposed to probes directed against the other antigenic types of P.II, a small percentage (1 to 6%) of the cells was marked by each of the heterologous immunologic probes. Similarly, when organisms of the P.II culture were exposed to each of the anti-P.II probes, a small percentage (1 to 15%) of the cells was marked. Thus, although most of the cells from these cultures expressed one antigenic type of P.II predominantly, cultures of gonococcal P.II phenotypes contained

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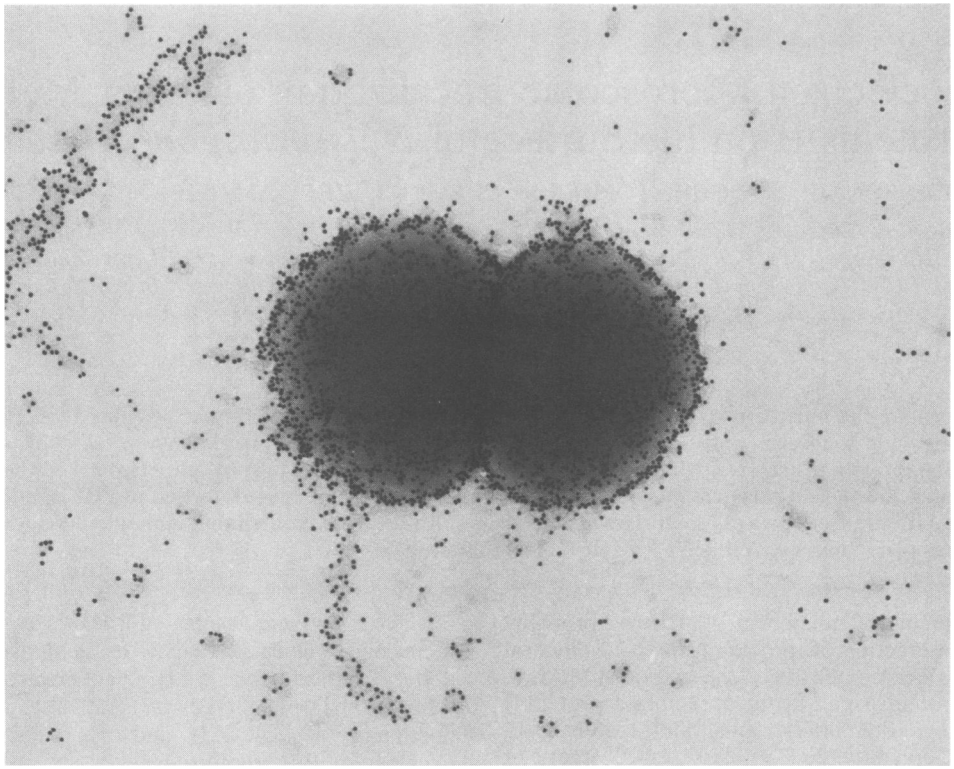

FIG. 1. Ultrastructural localization of accessible outer membrane protein IIa on the surface of *N. gonorrhoeae* FA1090 (phenotype P.IIa), as detected by gold spheres complexed with a P.IIa-specific monoclonal antibody. Note the heavy attachment of gold spheres to the surfaces of viable organisms and the outer membrane blebs associated with them. Similar labeling of the surfaces of organisms and outer membrane blebs was seen when other P.II variants were examined with homologous anti-P.II-specific gold probes. Few organisms of cultures of this P.IIa-expressing variant were labeled with heterologous anti-P.II gold probes. Thus, the surface-exposed portions of P.IIs, as detected by anti-P.II antibody-gold probes, appear to be highly antigenically variable. Negative stain was used. Magnification, × 36,920.

"contaminating" gonococci expressing different antigenic types of P.II.

To determine whether a single gonococcal cell is capable of expressing more than one antigenic type of P.II simultaneously, organisms of the P.IIa-expressing variant were exposed in sequence to large anti-P.IIe probes, followed by small anti-P.IIa probes (Fig. 3). Very few cells were double labeled, and most cells were labeled by the small anti-P.IIa probe alone. Since this P.IIa phenotype had been chosen for its expression of a single P.II type, the reduced expression of P.IIe by cells of this culture, and thus the limited number of cells marked by two P.II probes, was not surprising.

To increase the expression of antigenic types of P.II other than P.IIa, the P.IIa-expressing culture was passaged nonselectively on five successive days and frozen in defibrinated sheep blood. This frozen stock was then thawed and plated on clear typing agar. The organisms from this nonselectively passaged culture were first examined in single-label experiments to assess the proportion of cells expressing each type of P.II. The percentage of cells labeled by each anti-P.II probe increased dramatically in comparison with the earlier P.IIa-selected culture. Since the greatest number of gonococci in this culture was labeled by the P.IIa probe (~94%) or the P.IIb/P.IId probe (~34%), these probes were chosen for use in double-label experiments. The organisms were exposed to small anti-P.IIa probes, followed by large anti-P.IIb/P.IId probes. As predicted by the single-label experiments, virtually all of the cells were labeled with anti-P.IIa probe. One-quarter of these cells were marked with the anti-P.IIb/P.IId probe as well. Very few cells were marked by the anti-P.IIb/P.IId probe alone or by neither probe. Thus, a single gonococcal organism is capable of expressing more than one P.II antigenic type simultaneously on its surface.

To determine whether antibody accessibility of the H.8 antigen is affected by differences in P.II expression, FA1090 P.II phenotype organisms were exposed to a single anti-H.8 probe. No significant differences in anti-H.8 probe labeling were evident among the gonococcal variants. Each culture (including the P.II variant) contained cells which were (~4 to 16%) and were not labeled with the single anti-H.8 probes. Double-label experiments were then performed with small anti-P.II probes and large anti-H.8 probes. Within each of the cultures of P.II variants, there were gonococci which were labeled with each probe alone, neither probe, or both probes (data not shown). Thus, surface expression of P.II did not preclude or predict surface expression of the neisserial H.8 antigen in strain FA1090.

By using gold immunological probes to examine viable gonococci, we confirmed, at the level of individual organisms, some of the predictions based on studies of gonococcal colony phenotypes that differ in predominant P.II expression. The monoclonal antibodies used in this study bind to variable determinants of the six identified P.II proteins of strain FA1090 (4, 10). Each of the antibodies recognized only a single P.II protein, except for antibody H.4.1, which binds to both P.IIb and P.IId. The epitopes recognized by the monoclonal antibodies are not common to all gonococcal strains, although they are not limited exclusively to strain FA1090 (1).

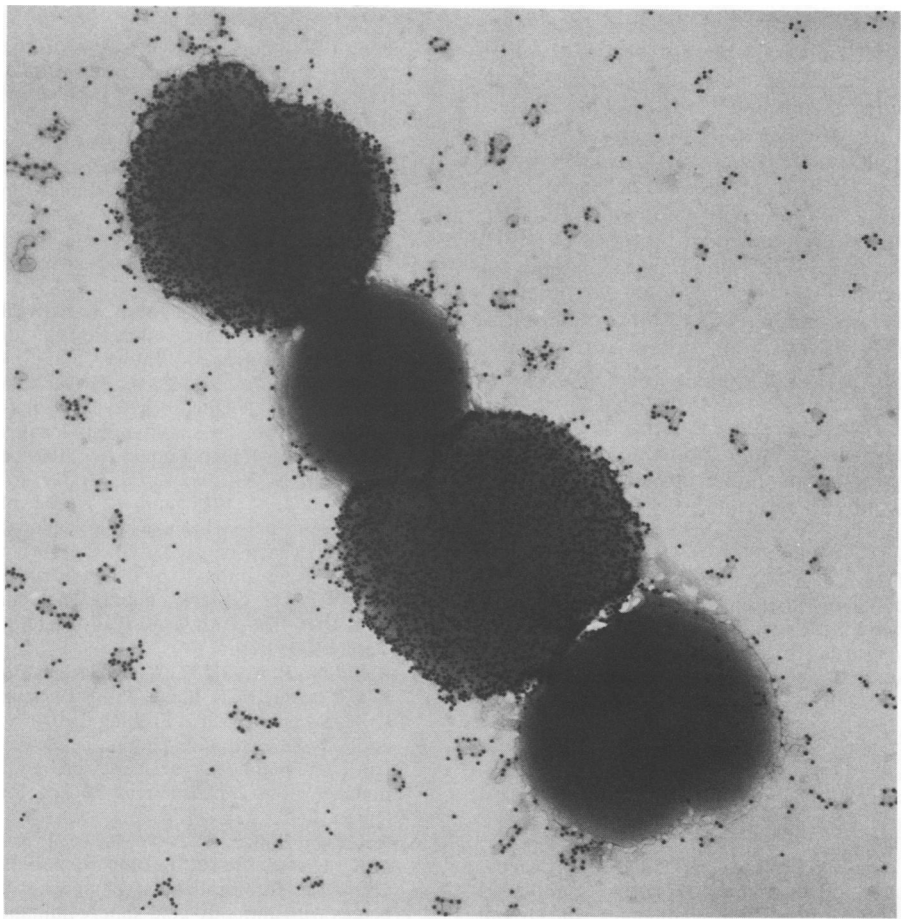

FIG. 2. Variation in the expression of P.IIa on the surface of *N. gonorrhoeae* FA1090 (phenotype P.IIa), as detected by gold spheres complexed with a P.IIa-specific monoclonal antibody. Note the markedly diminished amount of surface labeling by gold probes of several of the organisms in comparison with adjacent gonococci. Negative stain was used. Magnification, × 24,360.

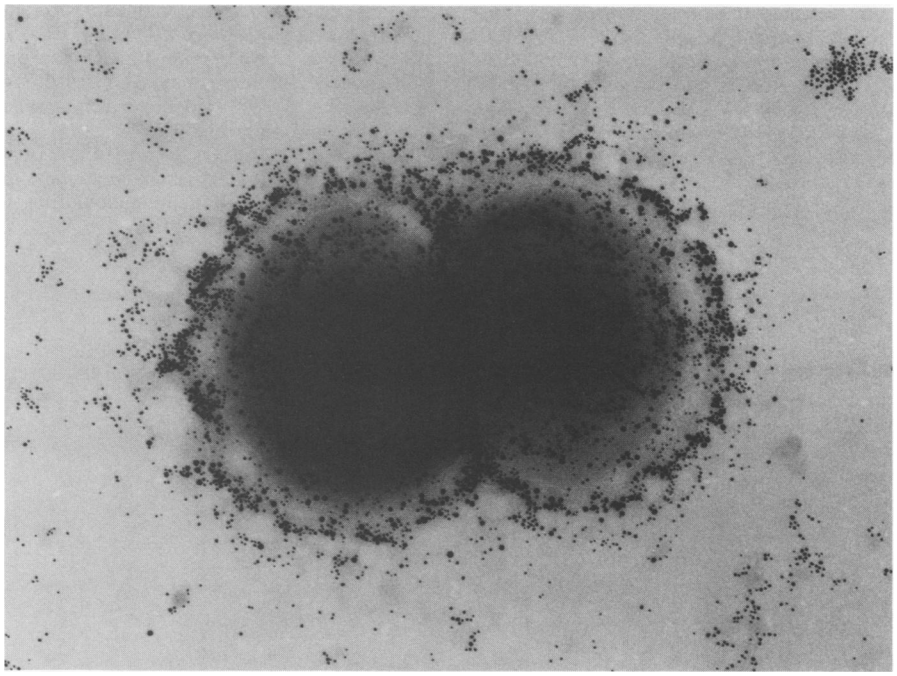

FIG. 3. Simultaneous localization of two P.IIs on the surface of *N. gonorrhoeae* FA1090, as detected by large anti-P.IIe gold probes, followed by small anti-P.IIa gold probes. Note the attachment of both sizes of gold probes to the surface of the diplococcus. Thus, a single gonococcal organism can produce more than one antigenic type of P.II simultaneously. Negative stain was used. Magnification, × 44,160.

We found that antigenically variable portions of the P.II proteins of FA1090 were exposed on the surfaces of bacteria and on elaborated outer membrane blebs. Colony phenotypes that expressed a particular P.II protein always contained a minority population of organisms that was labeled with probes for the other P.II proteins. For example, a variant chosen for predominant expression of P.IIa contained cells (≤11%) that were labeled with probes for P.IIb and P.IId, P.IIc, P.IIe, and P.IIf. The number of gonococci labeled by the heterologous P.II probes could be doubled by nonselective passage over a few days. Thus, preferential expression of one type of P.II by gonococci in vitro was not a stable trait. As demonstrated in double-label experiments, a single gonococcal cell is capable of expressing more than one type of P.II simultaneously. These results emphasize the heterogeneity of P.II expression in gonococcal colonies, which may be a complication in studies attempting to clarify the functions of different P.II proteins.

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