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Structure and Surface Exposure of Protein IIs of *Neisseria gonorrhoeae* JS3

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Colonies of *Neisseria gonorrhoeae* JS3, each bearing a predominate protein II (PII) type, were derived from a progenitor transparent colony. Five distinct PIIs were identified and isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The PII bands were excised from gels of unlabeled whole cells and from gels containing lysates of surface-radioiodinated bacteria. These were subjected to α-chymotrypsin digestion and two-dimensional peptide mapping, which allowed for a comparison of both the primary structures of the PIIs and the identification of surface-exposed regions of the molecules. The results demonstrated that PIIs are unrelated to either Protein I or Protein III in structure but are closely related to one another, sharing about two-thirds of the peptides generated by α-chymotrypsin. The remaining third of the peptides varied with each PII, resulting in unique portions of the molecule being exposed on the bacterial surface. However, the variable peptides were not always among the exposed peptides, suggesting that the structural differences in the PIIs occur at a discrete site (or sites) of the PII molecule and not randomly throughout the protein. Such alterations can result in the exposure of distant, nonvariant portions of the molecule to the surface, perhaps by conformational changes. These bacteria can thus present a variety of new immunodeterminant sites to the host during the course of disease.

The family of outer membrane (OM) proteins identified as protein II (PII) of *Neisseria gonorrhoeae* has proven to be a most intriguing group of highly variable and diverse proteins. They were first designated as PIIs based on their susceptibility to exogenous proteases, their availability to react with immunoglobin and surface-radiolabeling reagents, their "heat-modifiability" in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and their associations with colony opacity (15, 20). Now, after extensive study, these proteins have been shown to play important roles in adherence (6, 11, 21), susceptibility to killing by serum (2), and possibly in the ability of the bacteria to evade the immune response (19).

Swanson and Barrera (19), in a clever immunological study, demonstrated that despite the ability of animals to generate antibody that recognized common determinants on PIIs from several strains of gonococci if a *denatured* PII was used as an immunogen, PIIs on the surface of intact bacteria elicited immunoglobin that was specific for the homologous PII. Thus, it appears that by varying the PII constituents of the OM, the organism can present a very complex and immunologically heterogenous appearance to the immune system of the host.

In this study, the primary structural relationships and the surface exposure of five PIIs of the *N. gonorrhoeae* JS3 were studied in detail. The results obtained in this study confirm the immunological results of Swanson and Barrera and previous structural data of Swanson (17) and Heckels (5), showing a great deal of primary structural homology among the PIIs. However, differences in the primary structure resulted in unique portions of the PIIs being exposed on the surface. These unique portions of the PIIs putatively represent the immunodeterminants recognized by PII-specific antibody.

MATERIALS AND METHODS

Bacteria. *N. gonorrhoeae* JS3 was the generous gift of John Swanson, Rocky Mountain Laboratories, Hamilton, Mont. Bacteria were grown on clear typing media (16). Isogenic colonies, each bearing a predominant PII type, were derived from a transparent, non-PII-bearing progenitor colony by daily passage. Opaque (PII-bearing) progeny were selected and maintained by daily passage. Five different PIIs (designated PII-a through PII-e) were acquired based on their mobility in SDS-PAGE gels. One PII, PII-a, did not impart opacity to the colony and was found only fortuitously. All other PIIs imparted various degrees of opacity, as clearly detailed by Swanson (18).

SDS-PAGE. Bacteria were solubilized in SDS-PAGE solubilizing solution containing 2-mercaptoethanol at 60 and 100°C as previously described (9). Whole-cell lysates were separated on a 12.5% acrylamide slab gel (acrylamide-to-bisacrylamide ratio of 30:0.8) by using a 5% acrylamide stacking gel. Proteins were stained with Coomassie brilliant blue (9).

Iodination of bacteria. Intact whole cells from colonies bearing a predominant PII type were surface radioiodinated by using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen; Pierce Chemical Co., Rockford, Ill.) as previously described (10). Surface-radiolabeled whole cells were solubilized, the resultant lysates were separated by SDS-PAGE. Labeled proteins were identified by autoradiography and Coomassie blue staining.

Peptide mapping. SDS-PAGE-separated PII bands were identified by Coomassie blue staining and by autoradiography (surface-labeled cells). The appropriate PII bands, as well as protein I (PI) and protein III (PIII), were excised from the gel and subjected to ^{125}I-labeled peptide mapping (bands from unlabeled whole-cell lysates) or surface-peptide mapping as described in great detail elsewhere (1, 3, 9, 10; R. C. Judd, submitted for publication). Briefly, unlabeled protein bands were exhaustively radioiodinated by using chloramide-T (chloro-T) (Sigma Chemical Co, St. Louis, Mo.), and carrier-free ^{125}I (as NaI; ICN Pharmaceuticals, Irvine, Calif.), whereas surface-labeled proteins were sham iodinated by using identical procedures with unlabeled iodine.

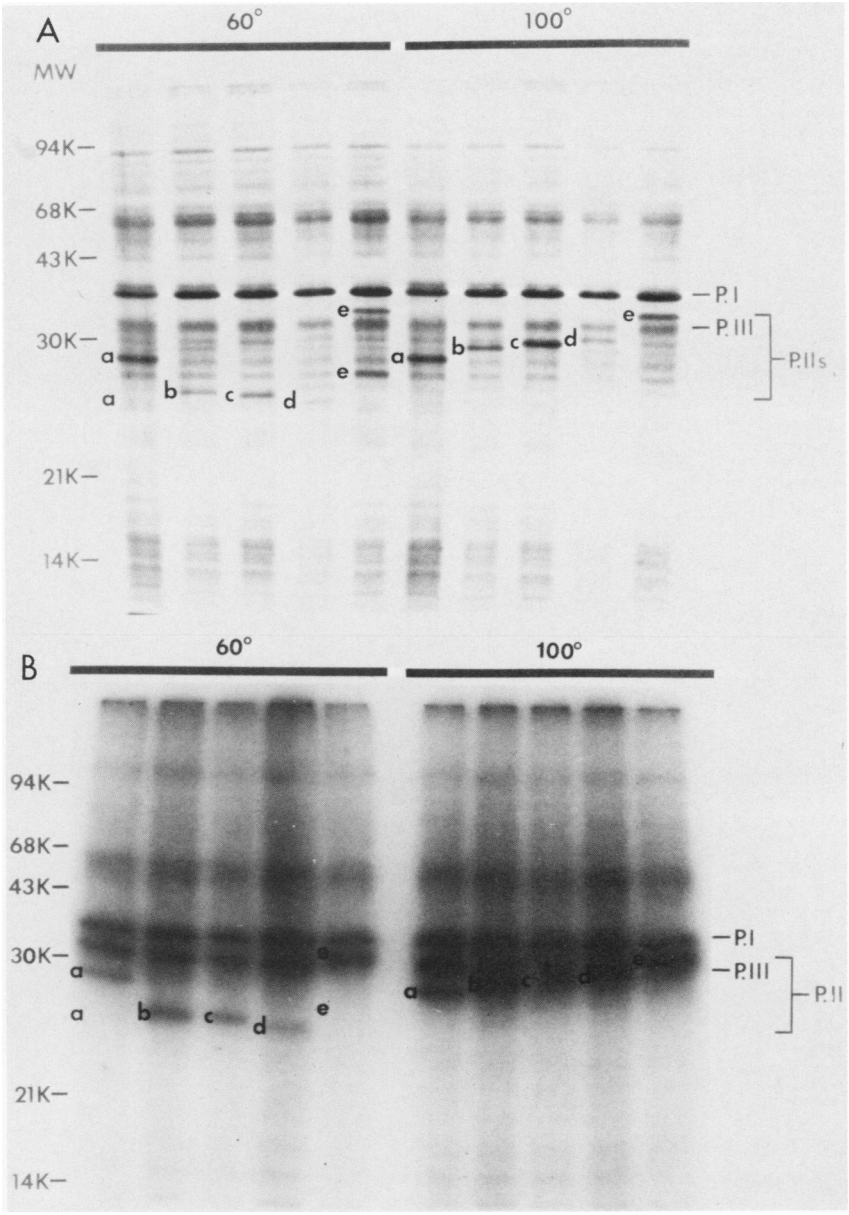

FIG. 1. (A) Isogenic colonies of *N. gonorrhoeae* JS3 were solubilized at 60 or 100°C in SDS solubilizing solution containing 2-mercaptoethanol and separated on a 12.5% acrylamide gel. Each lane shows a whole-cell lysate of colonies containing a prominent PII type (types a through e). Coomassie blue staining demonstrated the increase in an aMW characteristic of PIIs. PII-a and PII-e were partially converted to their higher aMW form at 60°C. PI and PIII showed no variation in aMW. Molecular weight markers were from the Bio-Rad low-molecular-weight marker kit. (B) Isogenic colonies of *N. gonorrhoeae* JS3 as described above were surface radioiodinated by using Iodogen. The whole-cell lysates were separated by SDS-PAGE, and the radiolabeled bands were identified by autoradiography. PI, PII-a through PII-e, and PIII from each lane of unlabeled and radiolabeled cells were excised and subjected to α-chymotrypsin peptide mapping.

The proteins were then cleaved with α-chymotrypsin (Sigma) in 50 mM NH_4HCO_3 buffer (pH 8.5). After multiple washings, the resultant peptides were spotted onto 0.1-mm cellulose sheets and separated by electrophoresis in the first dimension at a constant 1,200 V (thin-layer electrophoresis) in a buffer (pH 3.1) containing water, acetic acid, and pyridine (200:10:1 [vol/vol]) by using a Savant TLE200 (Savant, Hicksville, N.Y.), and then chromatographed in the second dimension in a relatively hydrophobic buffer containing butanol, pyridine, water, and acetic acid (13:10:8:2 [vol/vol]). Peptides were thus separated based on charge in the first dimension and on solubility in the second dimension. The radiolabeled peptides were visualized by autoradiography. Internal amino acid markers (ILE, TYR, ASP) insured consistent migration.

RESULTS

The SDS-PAGE profiles of whole-cell lysates of *N. gonorrhoeae* JS3 are seen in Fig. 1. Figure 1A shows the Coomassie blue-stained proteins, and Fig. 1B shows the autoradiogram of lysates of surface-radiolabeled bacteria. Each lane contains a whole-cell lysate of colonies bearing a predominate PII type solubilized at 60 and 100°C. Note that at 60°C the majority of PII-a is in its higher-apparent-molec-

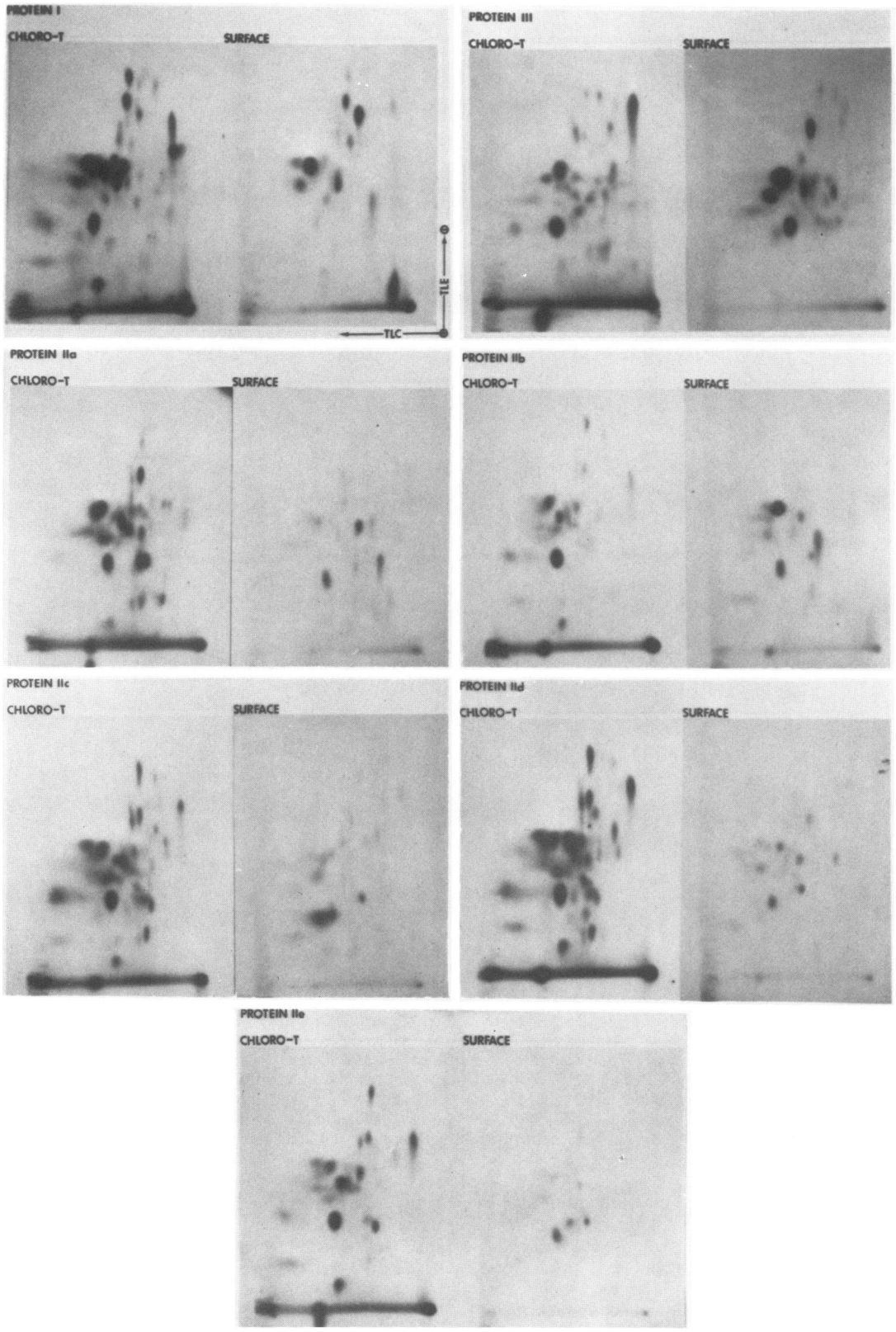

FIG. 2. ^{125}I-labeled peptide maps (chloro-T) and surface-labeled peptide maps (surface) of α-chymotrypsin digests of PI, PII-a through PII-e, and PIII of *N. gonorrhoeae* JS3. Note that both chloro-T and surface maps of PI and PIII are distinct, whereas all the PIIs share many primary structural and several surface-exposed peptides. However, the prominently emitting surface peptides have a unique pattern in each PII surface peptide map, indicating that each PII has a unique surface exposure. TLE, Thin-layer electrophoresis; TLC, thin-layer chromatography.

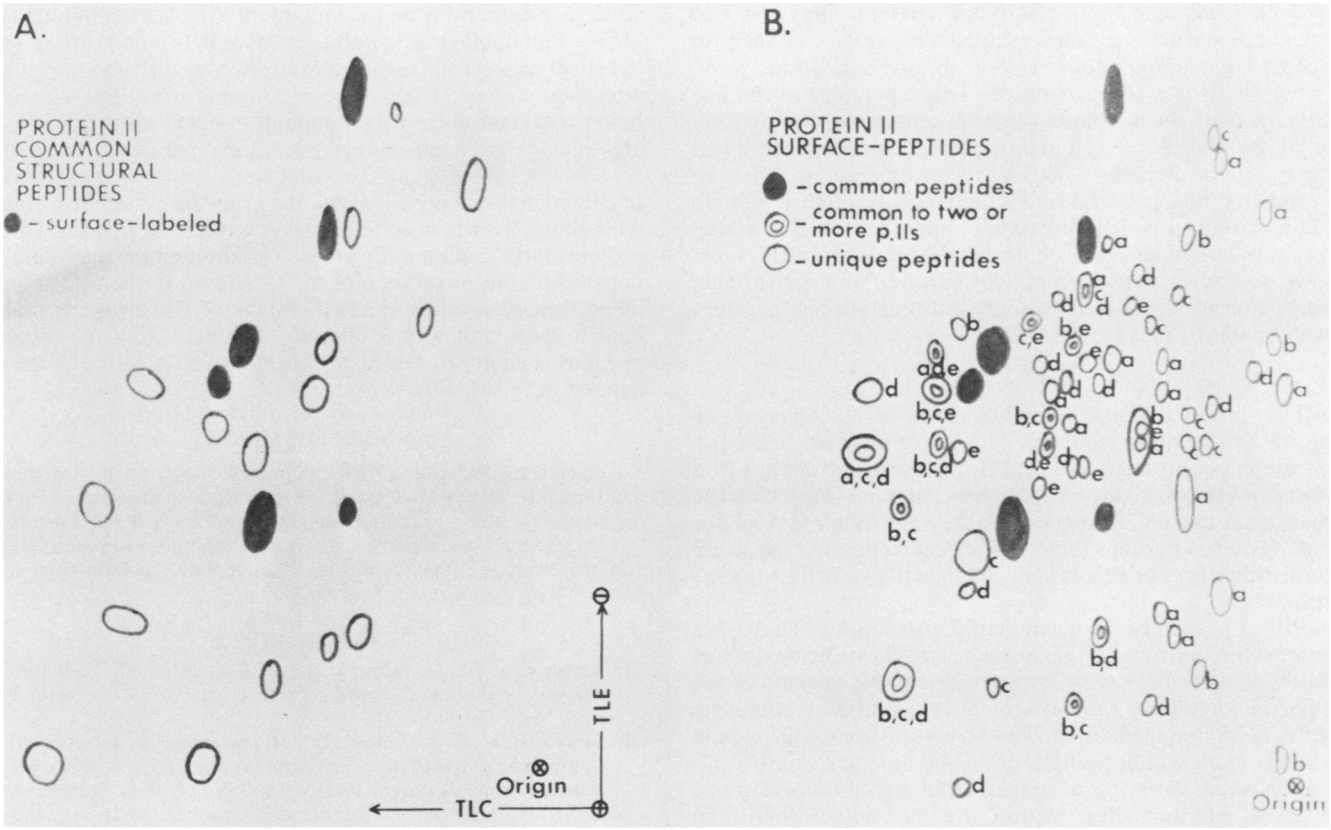

FIG. 3. Diagrams were generated by projecting chloro-T and surface peptide maps of PII-a through PII-e onto paper and tracing peptide outlines. (A) Common structural peptides of PIIs. There are at least 22 shared peptides out of about 35 total peptides seen in each PII or about two-thirds of all PII structural peptides. In contrast, only a single peptide appears to be shared by PI or PIII and the PIIs (peptide shape and relative intensity taken into consideration). TLE, Thin-layer electrophoresis; TLC, thin-layer chromatography. (B) Comparison of surface peptides of PIIs. This diagram clearly demonstrates the widely diverse surface exposure of the PIIs. Each letter refers to the PII designation: e.g., a letter represents the surface peptides of PII-a, etc. There are six common surface peptides which are generally the more weakly emitting surface peptides. Each PII has between 10 and 17 unique surface peptides or about one-third of the total peptides seen in the PIIs.

ular-weight (aMW) form and that PII-e is partially converted at 60°C, whereas all PIIs are converted to their higher-aMW form at 100°C. Bio-Rad molecular weight markers are designated at the left of each gel. Clearly, PIs, PIIs, and PIIIs are the most heavily radiolabeled proteins in these lysates.

Those protein bands designated PI, PII-a through PII-e, and PIII were excised and subjected to peptide mapping. Both the lower-aMW PIIs and their heat-modified higher-aMW forms were mapped and were seen to be identical in both forms, indicating that non-OM proteins which might receive label (12) do not contribute to the peptide patterns. Therefore, only the peptide maps of the higher-aMW forms are presented. Also, PI and PIII from all 10 lysates were shown to be invariant, so only single PI and PIII preparations are displayed.

Figure 2 shows both the primary structural peptide maps (designated chloro-T because these proteins were radioiodinated by using chloro-T) and surface peptide maps (designated "surface") of PI, PIII, and the five PIIs under study. Both PI and PIII had a unique primary structure and surface exposure, which correlates with previously published structural (9, 10) and immunological (19) data. The PIIs shared a good deal of primary structural homology (see Fig. 3). However, when surface- exposures of the PIIs were compared, each PII had a unique peptide pattern. Therefore, PIIs appear to be a homology group of proteins in much the same manner as the PI-a and PI-b subgroups have been shown to be (9, 14), and as was seen with PIs (10), differences in primary structure result in unique portions of the PII molecule being exposed on the surface. Moreover, the structural change can occur either in the surface-exposed region itself or at a site not exposed on the surface, which results in a new region being exposed, perhaps by conformational alterations.

There are a few peptides on the surface peptide maps which have no corresponding peptide in the chloro-T maps. This has been observed in every surface peptide map of gonococcal (10) and chlamydial (3) protein studied in this way. Extended exposure times show these peptides to be present in the chloro-T maps at very weak intensity. Further, high-performance liquid chromatography data (manuscript in preparation) confirms that vast differences in relative intensities of emission of the surface versus chloro-T peptides result from the differences in the Iodogen or lactoperoxidase versus chloro-T labeling procedures.

Figure 3 is a composite of PII peptide maps made by projecting the peptide map autoradiograms onto paper by using a conventional photographic enlarger. The peptides were then traced, and overlapping and unique peptides were identified. Figure 3A shows the 22 peptides that were common to all PIIs, or roughly two-thirds of the total PII peptides. Of these, six appeared in the surface-peptide maps

as well (designated by solid black circles). Only the two common surface peptides nearest the origin seemed to accept large amounts of ^{125}I upon surface iodination.

Figure 3B is a comparison of surface peptides of the five PIIs. Again, the common surface peptides are shown by solid black circles. Each unique peptide is designated by an open circle. Peptides shared by two or more PIIs are designated by dual circles. The letters a through e refer to PII-a through PII-e. Interestingly, some PIIs had common structural peptides exposed on their surface. All PIIs, however, had several unique peptides exposed on their surface, and patterns of the most heavily emitting surface peptides were quite distinct for each PII.

DISCUSSION

The role of PIIs in the pathogenesis of *N. gonorrhoeae* appears to be quite complex. They have been implicated as an adherence structure (6, 11, 21), yet the occurrence of PIIs (as assessed by colony opacity) was shown to vary with the menstrual cycle (7), suggesting they are not required for pathogenesis. Perhaps the variable occurrence and the structural differences of PIIs is a mechanism to evade the immune response.

PIIs appear to be dominant surface structures. They have extensive exposure to the surface, and they elicit vigorous antibody responses (19). By altering various regions of the PII, the organism can present a changing and confusing array of immunogenic sites. The structural homology seen in all PIIs, both within a single strain and among strains (5, 17; unpublished observation) suggests that alterations occur at a discrete site (or sites) within the molecule rather than randomly throughout the protein. Based on the ratio of common structural peptides to unique surface peptides, this region of variation might include about one-third of the protein.

The nature of the response to PIIs tells us a great deal about how the immune system responds to OM proteins. Those sites exposed on the surface which differ elicit vigorous responses, whereas buried sites, which appear to remain unchanged, are not immunogenic. However, the common structural regions are immunogenic if the protein is denatured (19). A possible explanation for this (pure speculation) could be that "nonproductive antibody" (i.e., antibody which is not removed from the system by antigen binding) is suppressed by idiotypic feedback (4, 8) or by suppressor T cells (13) or both, which recognize the idiotype of non-productive antibodies and exert suppressive action against B-cell clones which make antibody directed against buried determinants. It is encouraging to note that at least some homologous regions, as evidenced by the presence of six common surface peptides, appear to be exposed on the surface of the organism. It is tempting to speculate that these common peptides represent regions of the molecule which are in the surface portion of the molecule but, due to folding, are less available for surface radioiodination. Proper manipulation of the molecules may result in generation of antibody that reacts with these common regions and is therefore widely cross-reactive.

Regardless of the immune mechanism, this structural study correlates exactly with immunological information (19) and previous structural data (5, 17). With the procedures described here it is now possible to analyze which portions of the PII-s antibody recognizes and under what conditions can we alter that reactivity. These procedures can also be used to determine whether the presence of two or more PIIs on the bacterial surface interact to create new determinants which are different from the individual PIIs, thus compounding the immunological variability. Also, PIIs appear to be an excellent model to investigate how gram-negative pathogens alter their surface. The pattern of common structural regions being sequestered from the immune system, while the variable regions are exposed, has been demonstrated with PI of *N. gonorrhoeae* (10) and the major outer membrane proteins of chlamydial species (3). Thus, the procedures detailed here will allow us to closely observe which regions of OM proteins are varied and how that variation alters the immunogenic profile of the organism. In addition, peptidic fragments can be used to identify regions of OM proteins that bind to host cells and to identify host receptors for those proteins, helping to elucidate the role of PIIs in the pathogenesis of *N. gonorrhoeae*.

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