

# Bacterial Entry and Intracellular Processing of *Neisseria gonorrhoeae* in Epithelial Cells: Immunomorphological Evidence for Alterations in the Major Outer Membrane Protein P.IB

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## Summary

The fate of the major outer membrane protein of the gonococcus, P.IB, during the adherence, entry, and intracellular processing of the bacteria in infected epithelial cells was investigated using post-embedding immunoelectron microscopy. Various domains of the P.IB molecule were probed at different stages in the infection. These studies revealed that P.IB epitope exposure remained unaltered during the initial attachment of the bacteria to the host cells. In contrast, upon secondary attachment of the bacteria to the eukaryotic cells, apparent zones of adhesion were formed between the gonococci and the host cell membrane, which were characterized by loss of a defined P.IB epitope. These zones of adhesion with the altered P.IB immunoreactivity continued to exist and increased in number during cellular penetration, suggesting that they were essential to bacterial invasion into the eukaryotic cells. After bacterial entry, two classes of gonococci could be recognized; morphologically intact, P.IB-positive bacteria and disintegrated organisms that showed a change in, and, in a later stage, a complete loss of P.IB immunoreactivity. The intracellular alterations in the P.IB antigen could be prevented by treatment of the host cells with the lysosomotropic agent chloroquine. These observations point to a mechanism by which a subpopulation of intracellular gonococci can escape the epithelial cell defense by preventing or resisting exposure to host cell proteolytic activity.

*Neisseria gonorrhoeae* is an exclusively human pathogen that colonizes a diverse array of mucosal surfaces and can give rise to local and disseminated infections with potentially serious sequelae, including destructive arthritis and, when the fallopian tubes are infected, infertility. Histopathological examination of infected mucosal tissue indicates that, during the course of infection, the bacteria adhere to, invade, and pass through the mucosal barrier (1, 2). The intracellular localization of the bacteria may, together with the observed molecular mimicry (3) and variation (4, 5) of surface antigens, contribute to their evasion of the host immune defense and, thus, to persistence of the infection.

At the molecular level, the pathogenesis of the disease is not well understood. The establishment of an infection requires the presence of pili, which are thought to mediate the initial attachment of the gonococci to the host cells (4). The subsequent entry into the mucosal cells probably involves bacteria-directed endocytosis (6), but the mechanism of invasion as well as the intracellular pathway(s) of the bacteria are unknown. One bacterial component that has been demon-

strated to influence host cell function is the principal outer membrane protein, and candidate vaccine antigen of the gonococcus P.I. This pore-forming protein, which is antigenetically conserved within a strain and which is expressed by a particular strain in either of two structurally different forms (type P.IA or type P.IB) (7, 8), can translocate from the gonococcus into artificial lipid bilayers and plasma membranes of mammalian cells with maintenance of its voltage conducting activity (9, 10) and, furthermore, is capable of binding the intracellular regulator calmodulin (11). When added to human neutrophils, this protein gives rise to a transient change in the membrane potential and inhibits host cell granule exocytosis (12), events that might influence cellular invasion and the intracellular fate of the bacteria. The propensity of P.I to insert into foreign membranes differs among strains and correlates with the clinical manifestations of the disease (9).

In the present study, we have investigated the gonococcal adherence, their entry and the intracellular processing of the bacteria, and the fate of P.I during these events by immunoelectron microscopic analyses of infected epithelial cells using

P.IB-specific antibodies as probes. This approach, which allows ultrastructural localization of single epitopes, has greatly contributed to the understanding of molecular events such as receptor recycling and antigen presentation (13), and has been successfully applied in determining gonococcal antigen variation during the course of infection (14, 15). The invasive gonococcal strain used in our infection experiments expressed the P.IB phenotype, and the antibodies that were used as probes were selected for recognition of distinct surface and non-surface exposed parts of P.IB, in order to optimize the detection of alterations in the P.IB antigen that might occur during infection. Our results demonstrate that gonococcal invasion into epithelial cells is accompanied by characteristic changes in the P.IB antigen and that additional alterations in P.IB immunoreactivity occur during intracellular processing of the bacteria. Furthermore, we present immunomorphological evidence that a subpopulation of the intracellular bacteria is able to evade the host cell defense machinery.

## Materials and Methods

**Bacterial Strain.** *Neisseria gonorrhoeae* strain 5590 (pil<sup>+</sup>, Opa<sup>+</sup>, P.IB, serotype 5), isolated from a patient with a local infection, was grown for 16 h on GC agar base (Difco Laboratories, Detroit, MI) containing 1% Vitox (Oxoid GmbH, Wesel, Germany) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Colony morphology was judged with a plate microscope (Leitz, Wetzlar, Germany).

**Preparation and Characterization of the P.IB- and LOS-specific Polyclonal Antisera.** The P.IB-specific polyclonal antiserum VK-207 was generously provided by Dr. T. Teerlink (RIVM, Bilthoven, the Netherlands). The serum was raised by immunizing mice subcutaneously with iscoms (saponin-lipid complexes) containing 2.5 µg purified P.IB followed by a booster after 4 wk. Purification of P.IB and incorporation of the protein into iscoms have been described previously (16, 17). Immunoblotting experiments with lithium acetate-extracted gonococcal outer membranes, purified P.IB in its native trimeric and in its denatured monomeric form, and cyanogen bromide cleavage fragments of the protein (CB-1, -2, -3, counting from the NH<sub>2</sub> terminus, prepared as described [16]) as antigens, revealed that the antiserum reacted with the trimeric (not shown) and the monomeric forms as well as with all the cyanogen bromide cleavage fragments of the protein (Fig. 1 b). No reaction was observed with lipooligosaccharide (LOS),<sup>1</sup> even at a low dilution of the antiserum. The SDS-PAGE and immunoblotting procedure have been described (16). The LOS-specific polyclonal antiserum K3-048 was raised by immunizing rabbits intravenously with formalin killed and washed *Neisseria meningitidis* group B cells as described (18). The serum K3-048 crossreacted with gonococcal LOS, but not with other outer membrane components of gonococcal strain 5590 (not shown).

**Preparation and Characterization of P.IB-specific mAbs.** P.IB-specific mAbs were raised by immunizing BALB/c mice intraperitoneally at days 1, 8, and 15 with either lithium acetate-extracted gonococcal outer membranes (20 µg protein) or cyanogen bromide cleavage fragments of the protein (10 µg dose) that had been purified by preparative SDS-PAGE (16). On day 18, spleen cells were fused with NS-1 myeloma cells as described by Tam et al. (19). Antibody-

producing hybridomas were selected by ELISA using microtiter plates coated with outer membrane fractions (50 µg/plate), or with P.IB cyanogen bromide cleavage fragments (0.5–1.0 µg/plate). The mAbs were further characterized by either gel immunoradioassay (20), which allows analysis of the trimeric configuration of the protein, or by Western blot analysis. mAb 4D9A specifically recognized the trimeric form of P.IB (Fig. 1 a). The mAbs C12.43 and B5.36 (kindly provided by H. Versantvoort; RIVM, Bilthoven, the Netherlands) recognized epitopes located at the CB-1 (mAb C12.43) and CB-3 (mAb B5.36) fragment of the protein (Fig. 1 b), but were also reactive with outer membrane blebs (see Results), in which the protein is in its native trimeric state as judged from non-denaturing SDS gels (not shown) and from their reactivity with mAb 4D9A (see Results).

**Antigen Detection on Intact Bacteria.** Detection of P.IB epitopes in a whole cell binding assay was carried out by fixing gonococci in 0.1 M cacodylate buffer containing 140 mM glucose, 0–2% paraformaldehyde (PFA), and 0–2% glutaraldehyde (GA) (30 min, 20°C), washing the cells with 0.1 M NH<sub>4</sub>Cl to quench free aldehyde groups, coating them onto microtiter plates (10<sup>8</sup> bacteria/well in PBS, 20 h, 37°C), and incubating them with anti-P.IB-specific antibodies (30 min, 20°C). After washing to remove the unbound Ig, the antibody binding was quantitated using horseradish peroxidase-conjugated protein-A with 3 amino-9-ethylcarbazole as a substrate. The results were read at 492 nm with an ELISA reader (Titertek Multiscan Laboratories, McLean, VA).

The same suspensions of unfixed and fixed gonococci used in the whole cell binding assay were subjected to immunoelectron microscopy. The bacteria were mounted onto Formvar-coated grids, incubated (30 min) with the appropriate antibodies, and, after washing with PBS, with gold-labeled protein-A (prepared as described [21]). All immunolabeling steps were performed at 20°C. The bacteria were viewed in an EM 300 electron microscope at 80 kV (Philips Electronics, Eindhoven, the Netherlands).

**Cell Culture.** Chang conjunctiva epithelial cells (Flow Laboratories, Irvine, Ayrshire, UK) (mycoplasma free) were seeded at a density of 3 × 10<sup>3</sup>/cm<sup>2</sup> and grown in 25-cm<sup>2</sup> culture flasks in DMEM supplemented with 5% bovine serum (tissue culture medium) in a CO<sub>2</sub> incubator at 37°C. 5 d after seeding, when confluence had been reached, the cells were used in the infection experiments.

**Infection of the Monolayer.** Confluent Chang conjunctiva epithelial cells were infected with *N. gonorrhoeae* strain 5590 at a bacteria/host cell ratio of 15:1. After 6 h of infection, the unbound bacteria were removed and fresh tissue culture medium was added. This procedure was necessary since rapid multiplication of the bacteria in the infection system resulted in nutrient deprivation and poor morphology of the eukaryotic cells. At appropriate times, the infection was stopped by washing the monolayer three times with 0.1 M cacodylate buffer containing 140 mM glucose (pH 7.2), followed by fixation with the cacodylate buffer supplemented with 2% PFA for 30 min (20°C), unless otherwise indicated. After fixation, the cells were removed with a rubber policeman, embedded in 2% gelatin (final concentration; Sigma Chemical Co., St. Louis, MO), pelleted in a centrifuge (Eppendorf, Stuttgart, Germany) (10,000 g, 15 s, 4°C), and stored in fixation solution (4°C) for a maximum of 1 wk until further processing for electron microscopy.

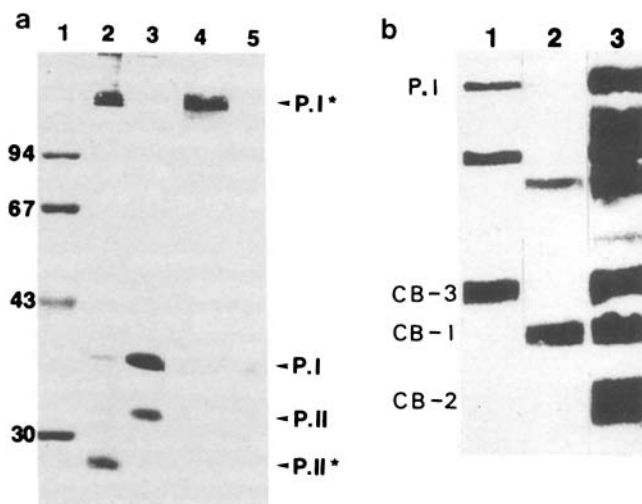
**Post-embedding Immunoelectron Microscopy.** The fixed specimens were frozen and sectioned (70–100-nm sections) with an ultracycrotome as described previously (22). The immunolabeling was performed by incubating the grids with pure hybridoma supernatant and/or with the polyclonal sera VK 207 (1/100 diluted in PBS) or K3-048 (1/10,000 dilution in PBS) for 30 min. The grids were

<sup>1</sup> Abbreviations used in this paper: GA, glutaraldehyde; LOS, lipooligosaccharide; PFA, paraformaldehyde.

then washed with PBS (four times for 5 min) and incubated for another 30 min with gold-conjugated protein-A (10 nm), which was diluted in PBS containing 1% gelatin to an optical density of 0.06 at 520 nm. When a second epitope was probed, the grids were then washed with PBS (four times for 5 min), incubated with unconjugated protein-A (10 µg/ml) to saturate possible free Fc binding sites, washed again, and exposed to the second antibody. These antibodies were marked by 5-nm protein-A gold particles. After the gold labeling, the grids were washed with PBS (four times for 5 min) and distilled water (three times for 3 min), and finally adsorption stained as described by Tokayasu (23). All incubations were performed at 20°C. The gold particles and the gold conjugates were prepared according to Slot and Geuze (21). The sections were viewed in an EM 201 or 300 electron microscope at 60 kV (Philips Electronics, Eindhoven, the Netherlands).

## Results

**Preservation Accessibility, and Ultrastructural Localization of Gonococcal Protein IB Epitopes.** Immunomorphological localization of epitopes in cryosectioned cells (post-embedding immunoelectron microscopy) involves fixation, embedding, and sectioning of the specimen, incubation of the sections with specific antisera, and marking of the antibodies with gold spheres. Since our goal was to detect possible alterations in the P.IB antigen during infection of epithelial cells, it was essential to ascertain that the native accessibility of the antigen was maintained during the specimen manipulation. The effect of fixation on P.IB epitope exposure was in-

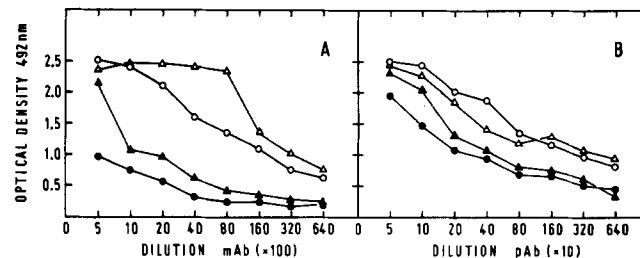


**Figure 1.** Specificity of the P.IB specific antibodies. (a) SDS-PAGE (lanes 2 and 3) and immuno-reactivity of mAb 4D9A in a gel-immunoradioassay (lanes 4 and 5) of lithium acetate-extracted outer membranes of *N. gonorrhoeae* strain 5590 solubilized at 37°C (lanes 2 and 4) and 100°C (lanes 3 and 5). Note the characteristic heat-modifiable migration of the PI and PII (opacity) proteins. mAb 4D9A strongly reacts with the trimeric but not with the monomeric form of P.IB. (Lane 1) Molecular weight markers. (b) Immunoblot demonstrating the reactivity of the P.IB-specific antibodies to the different cyanogen bromide cleavage fragments of the P.IB molecule. Purified P.IB was digested as described (16). Lane 1, mAb B5.36 reacts with an epitope on the CB-3 fragment of the molecule; lane 2, mAb C12.43 recognizes an epitope on the CB-1 fragment; lane 3, pAb VK 207 reacts with all three cleavage fragments. mAb 4D9A did not react with any of the fragments (not shown).

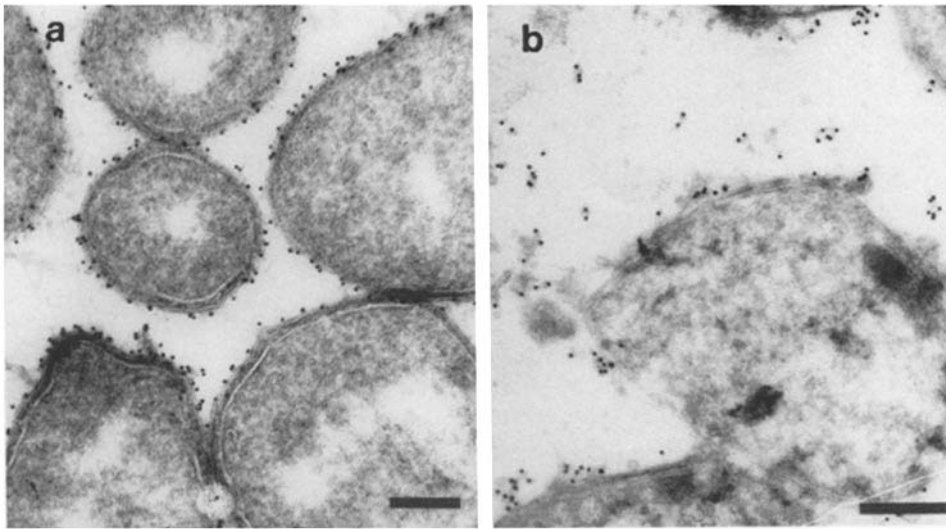
vestigated by comparing antibody reactivity against native and fixed bacteria in a whole cell binding assay. Probing of *N. gonorrhoeae* strain 5590 with P.IB-specific polyclonal antiserum VK 207 and with mAb 4D9A, both of which recognize the native trimeric configuration of the protein, revealed selective damage to some P.IB epitopes by the commonly used crosslinking agent GA (Fig. 2). This loss of epitopes, which was confirmed with immunoelectron microscopy, was much less with short PFA fixation of the bacteria (Fig. 2). With PFA as a fixative, successful immunolabeling could be achieved with both native and fixed cells (see below).

Comparison of the P.IB immunolabeling pattern in native and cryosectioned microorganisms in the electron microscope revealed no further alterations in the accessibility of the P.IB molecule during embedding and sectioning of the specimens. Probing of the surface-exposed 4D9A epitope resulted in both cases in extensive gold labeling of all gonococcal membranes, including membrane blebs (Fig. 3 a). In contrast, mAb C12.43, which recognizes an epitope on the 13-kD cyanogen bromide cleavage fragment of the protein in Western blots (CB-1 fragment; Fig. 1), was reactive with only a few specific areas of the gonococcal cell surface (Fig. 3 b). Similarly, mAb B5.36, specific for an epitope on the 15-kD cyanogen bromide cleavage fragment of the molecule (CB-3 fragment; Fig. 1), was only weakly reactive with the gonococcal membranes (not shown), suggesting that this epitope was also poorly accessible in both native and cryosectioned bacteria. Interestingly, both the CB-1- and the CB-3-specific antibodies reacted with membrane blebs, indicating that the probed epitopes were accessible in blebs (Fig. 3 b) and, hence, that the exposure of P.IB in blebs differs from that in intact bacteria. The specificity of the immunolabeling was demonstrated by the absence of gold spheres after incubating the bacteria with mAb 7B1E, which reacts with LOS of several gonococcal strains (14), but not with that of strain 5590 (not shown).

**P.IB Epitope Exposure during the Attachment of Gonococci to Cultured Epithelial Cells.** Infection of Chang conjunctiva epithelial cells with gonococci results in: (a) loose adherence of the bacteria to the cell surface, in particular to extending microvilli; (b) the formation of localized intimate contact be-



**Figure 2.** Effect of fixation on the immunoreactivity of the P.IB molecule. P.IB epitope preservation upon fixation of the cells was tested in a whole cell binding assay using the mAb 4D9A (a) and the pAb VK207 (b) as antibodies and either unfixed ( $\Delta$ ), 2% PFA-treated ( $\circ$ ), 2% PFA + 0.05% GA-treated ( $\blacktriangle$ ), or 2% PFA + 0.5% GA-treated ( $\bullet$ ) gonococci as antigens. The use of GA resulted in a loss of 4D9A reactivity and a slight reduction of VK 207 reactivity. The loss of immunoreactivity was much less when PFA was used as a fixative.



**Figure 3.** P.IB immunolabeling in cryosectioned gonococci. Cryosections of *N. gonorrhoeae* strain 5590 were incubated with mAb 4D9A and the CB-1-specific mAb C12.43, and subsequently with the gold conjugate. mAb 4D9A labeled the gonococcal membranes randomly (a). mAb C12.43 preferentially labeled membrane blebs (b). Similar immunolabeling was observed with whole, unfixed organisms (not shown). Bars, 0.25  $\mu\text{m}$ .

tween the bacterial and host cell membranes; and (c) internalization of the attached bacteria into endocytic vacuoles (14, 22). Probing of various domains in the P.IB molecule in cryosections of 6-h infected epithelial cells revealed no alterations in P.IB labeling after primary attachment of the bacteria to the host cells. The 4D9A epitope was still found to be randomly distributed over the bacterial membranes (Fig. 4), and the gold labeling of the probed CB-1 and CB-3 epitopes remained restricted to membrane blebs (not shown). No labeling of host cell components was observed.

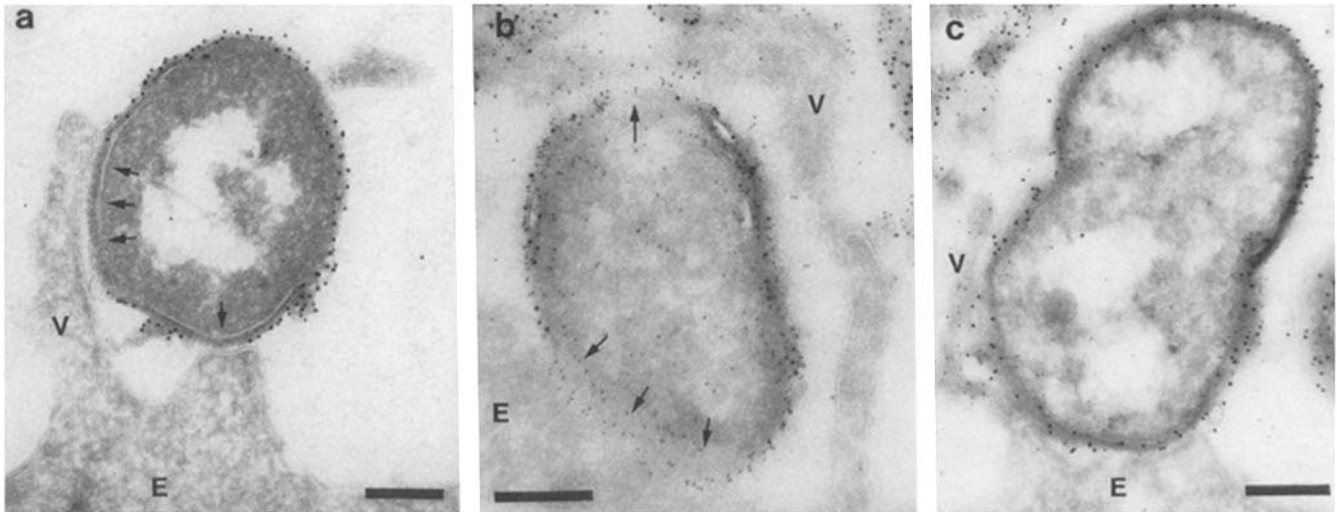
In the second stage of the infection, once intimate contact between the bacteria and the host cells had developed, changes



**Figure 4.** Immunoaccessibility and localization of the P.IB epitope 4D9A during the initial gonococcal–host cell contact. Cryosections of 6-h infected epithelial cells were incubated with the mAb 4D9A and thereafter with the gold conjugate. The bacteria were mainly attached to host cell microvilli. The gold particles, representing the 4D9A epitopes, were specifically localized at the bacterial membranes. V, microvillus; bar, 0.25  $\mu\text{m}$ .

in immunolabeling of the probed P.IB epitopes were observed. At bacterial membranes that were not in contact with the host cell, the 4D9A epitope was still abundantly expressed, but at regions of apparent intense interaction between the bacterial and the host cell plasma membrane, no gold particles were seen (Fig. 5 a). This localized loss in P.IB immunoreactivity was not found when the P.IB-specific polyclonal antiserum VK-207 or the LOS-specific polyclonal antiserum K3-048 were used as probes (Fig. 5, b–c). These observations indicate that both P.IB and LOS were present at the site of interaction, and, hence, that the apparent alteration in the P.IB antigen was epitope restricted. The C12.43 (CB-1-specific) and BC5.36 (CB-3-specific) epitopes remained inaccessible at this stage of infection (not shown). The altered P.IB immunoreactivity was found exclusively in regions of intense bacteria–host cell membrane contact; at zones of adhesion between bacteria (24), such loss in 4D9A labeling was not observed.

*Probing of P.IB Epitopes during Cellular Invasion.* Bacterial entry into the host cells was characterized by a gradual engulfment of the strongly attached bacteria by epithelial cell protrusions together with an apparent retraction of the organisms into the host cells. The engulfment was accompanied by the continuous formation of zones of intimate contact between the bacteria and the host cells next to areas with clearly distinguishable bacterial and host cell membranes, suggesting that these are the sites at which the bacteria trigger their internalization and/or anchor to the plasma membrane of the host cells during entry. Immunoelectron microscopic analyses of P.IB epitopes demonstrated the characteristic altered P.IB immunoreactivity at these sites of interaction. All zones of adhesion were found to be 4D9A negative, whereas this epitope was abundantly expressed elsewhere on the gonococcal membrane (Fig. 6 a). Again, the gold labeling with the polyclonal antiserum VK-207 was not influenced by membrane contact (Fig. 6 b), and the probed CB-1 and CB-3 epitopes were still masked, except in blebs (not shown). Taken together, these data strongly suggest that the cellular invasion proceeds by a sequential interaction between the bac-

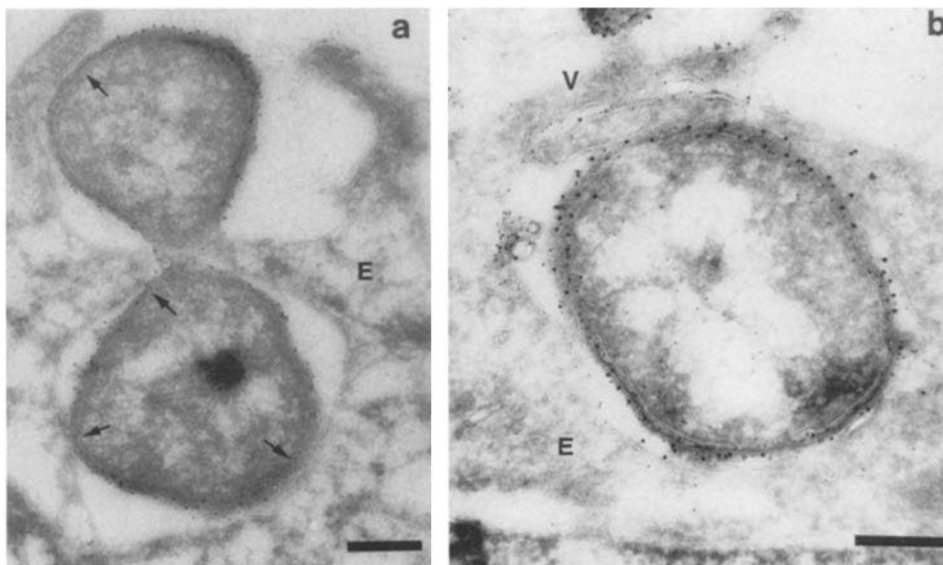


**Figure 5.** Loss of some P.IB epitopes at sites of intimate bacteria–host cell contact. Cryosections of 12-h infected epithelial cells were incubated with the P.IB-specific mAb 4D9A, the P.IB-specific pAb VK207, and/or with the LOS-specific pAb K3-048, and subsequently with protein A–gold. At sites of intense membrane contact between the bacteria and the host cell membrane, no 4D9A epitopes could be detected (*a* and *b*; arrows). Simultaneous probing of the 4D9A epitope (large gold particles) and the K3-048 LOS epitopes (small gold particles) (*b*) or the VK 207 epitopes (large gold particles) and the K3-048 epitopes (small gold particles) (*c*) showed that only the 4D9A epitope was absent at the sites of the interaction. Note that LOS, but not P.IB, could also be detected on the host cell microvilli. E, epithelial cell; V, microvillus; bars, 0.25  $\mu\text{m}$ .

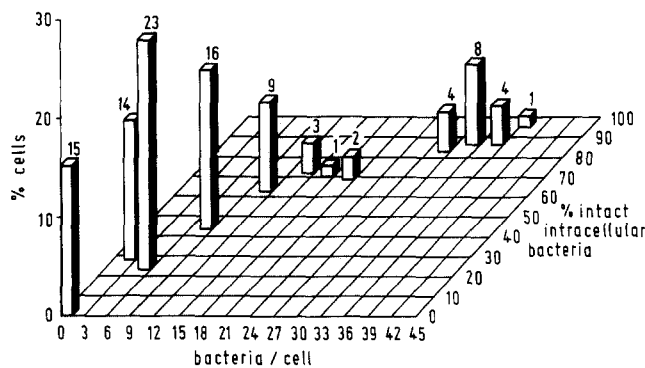
terial and the host cell membranes with a concomitant change in the P.IB epitope exposure.

*Alterations in the P.IB Immunoreactivity Associated with the Intracellular Fate of the Bacteria.* At 18 h post-infection, nearly all epithelial cells contained gonococci, but the bacteria were not equally distributed among the cells. Most of the cells ( $\sim 75\%$ ) had taken up to one to eight bacteria per cell, whereas a minority ( $\sim 15\%$ ) contained  $\sim 30$ – $36$  bacteria per cell (Fig. 7; bacteria/cell vs. percent cells). All microorganisms seemed to be contained in membrane-bound vacuoles, but both morphologically well-preserved and apparently disintegrating bacteria with ruffled membranes and a condensed

cytoplasm were observed. The intracellular localization of the bacteria was confirmed by adding 2-nm gold spheres to the infected cells. These particles penetrate nearly all spaces that are in open contact with the extracellular environment (Fig. 8). Probing of the P.IB antigen at this stage of infection revealed that all intracellular bacteria that were morphologically intact labeled for the 4D9A epitope, except at the sites of intimate contact with the vacuole membrane (Fig. 9 *b*). The polyclonal antiserum VK-207 gave random gold labeling, irrespective of membrane interactions (Fig. 9 *a*), whereas the CB-1- and CB-3-specific mAbs were weakly reactive (not shown). In contrast, in the morphologically disintegrating



**Figure 6.** Epitope restricted loss of P.IB epitopes upon bacterial entry into the epithelial cells. Gonococcal entry was accompanied by a sequential increase in the number of zones of intimate bacterial–host cell contact. These electronmicrographs show cryosections of 18-h infected cells after incubation with the P.IB-specific mAb 4D9A (*a*), the P.IB-specific pAb VK207 (*b*; large gold particles), and the LOS-specific pAb K3-048 (*b*; small gold particles). The gonococci are at different stages of entering into the host cells. Note that the 4D9A epitope, but not the other probed epitopes, was lost at the sites of intimate contact between the bacteria and the host cell membrane (arrows). The host cell microvilli labeled for LOS, but not for P.IB. E, epithelial cell; V, microvilli; bars, 0.25  $\mu\text{m}$ .

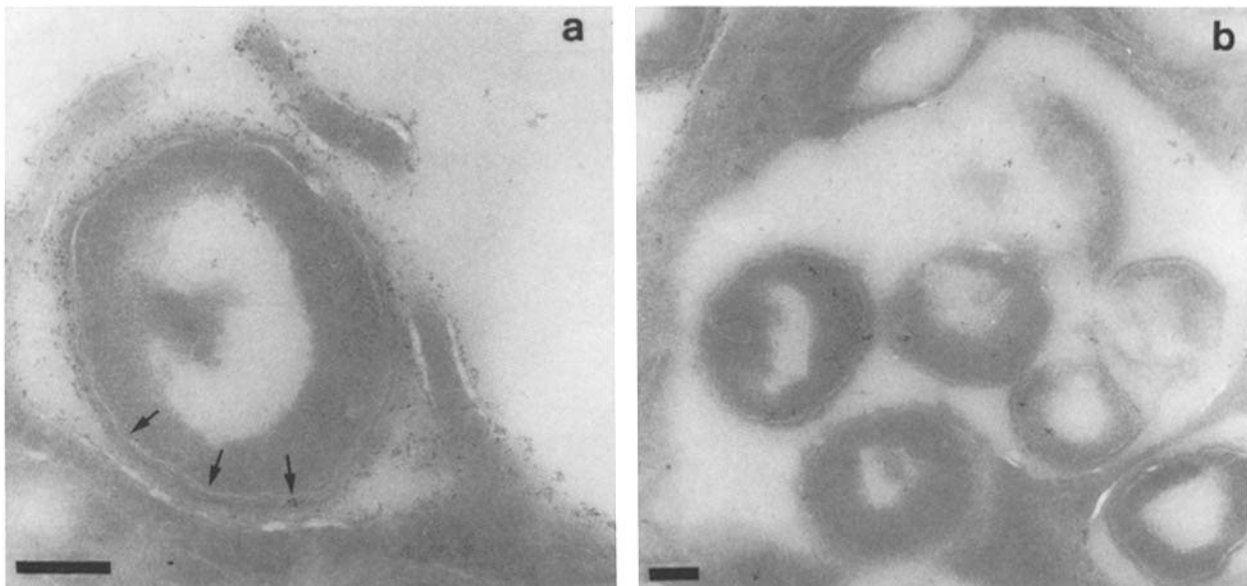


**Figure 7.** Distribution and immunomorphological fate of intracellular gonococci in infected epithelial cells. The number of internalized bacteria in individual host cells and their immunomorphological status (intact or disintegrated) were estimated by counting 500 cryosectioned and immunolabeled (mAb 4D9A) 18-h infected cells. The figure shows: the uneven distribution of the intracellular bacteria among the host cells (bacteria/cell vs. percent cells); and the relationship between the number of bacteria within individual cells and their immunomorphological fate (bacteria/cell vs. percent intact intracellular bacteria). The majority of the epithelial cells (the percentages are indicated on top of the bars) contain only a few (one to eight) bacteria and most of these organisms are immunomorphologically disintegrated. A minority of the cells (~15%) contains high numbers of bacteria (30–36), most of which are immunomorphologically intact.

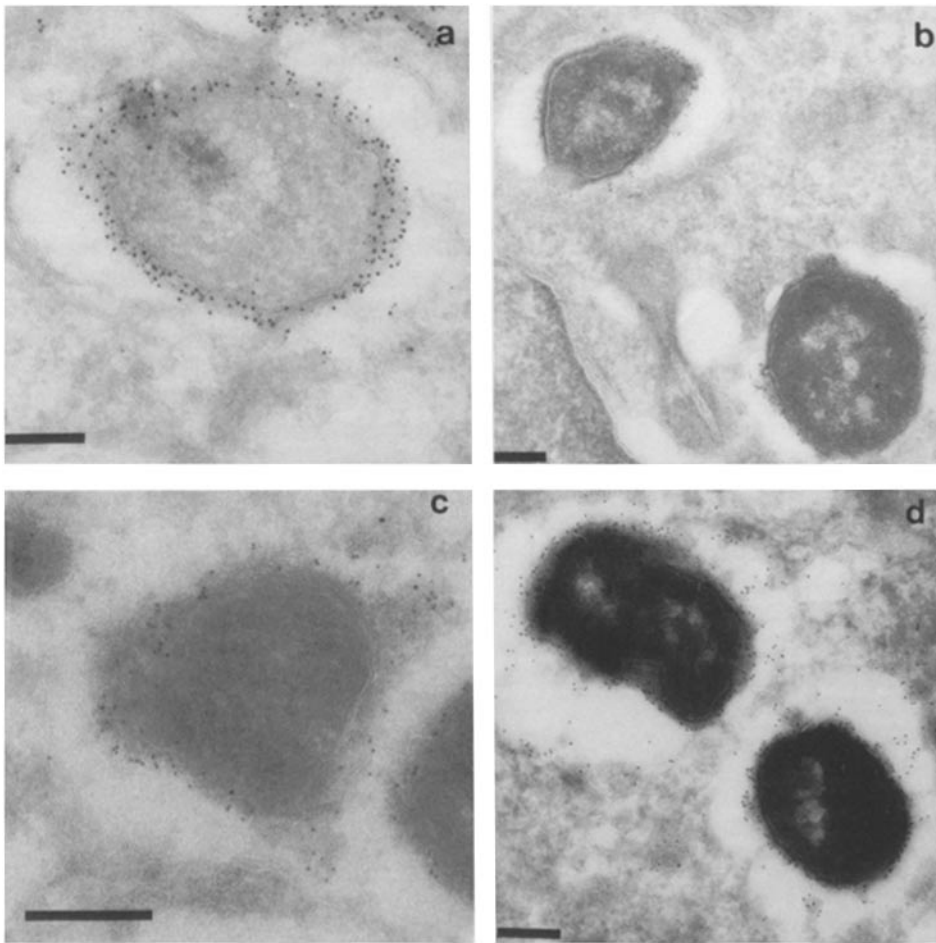
bacteria, the 4D9A epitope could not be identified, irrespective of the presence or absence of contact between the bacteria and the vacuole membrane (Fig. 9 c). The polyclonal antiserum VK-207 and also the LOS-specific antiserum K3-048 were, however, still reactive and, surprisingly, an abundant labeling of the probed CB-1 and CB-3 epitopes was observed (Fig.

9 c). Double-labeling experiments using the mAb 4D9A and the CB-1- and CB-3-specific mAbs demonstrated an inverse relationship between the labeling of the 4D9A epitope and those of the other two epitopes (Fig. 9 c). At 30 h of infection, the labeling of the immunomorphologically well-preserved bacteria was unchanged (not shown), but by this time a subpopulation of the disintegrating bacteria had lost not only the 4D9A epitope but also the previously unmasked CB-1 and CB-3 epitopes and all the epitopes that were recognized by the polyclonal antiserum VK 207, suggesting a total breakdown of the P.IB antigen (Fig. 9 d). Probing of the LOS epitopes at this stage of the infection resulted in an extensive gold labeling of gonococcal remnants, indicating that not all outer membrane constituents had been degraded (Fig. 9 d).

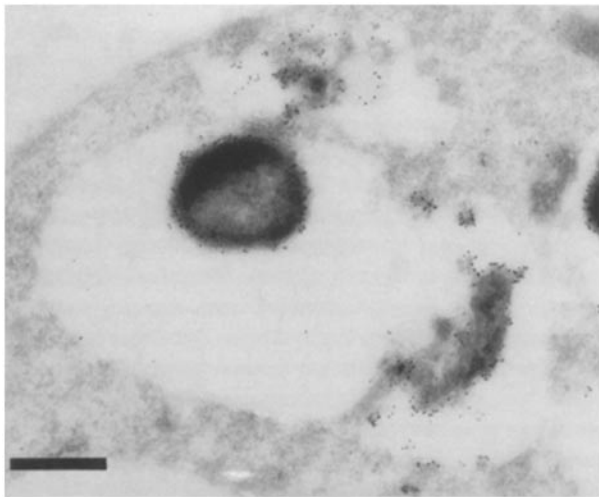
Interestingly, quantitation of the numbers of morphologically well-preserved and apparently disintegrating bacteria in individual epithelial cells revealed that in cells containing only a few bacteria, most of the gonococci (80%) showed signs of immunomorphological disintegration with a condensation of the cytoplasm, a loss of 4D9A reactivity, and either an unmasking or, at a later stage, a total loss of the CB-1 and CB-3 labeling, whereas in cells containing numerous bacteria, only a few of the gonococci seemed to be degraded (Fig. 7). We made several attempts to modulate this association between the number of intracellular bacteria in individual cells and their intracellular fate. Viable intracellular bacteria were isolated after selective antibiotic killing of the extracellular bacteria (25) and used directly, or after one passage on a plate, as an inoculum in new infection experiments. Every time, similar results were achieved. This finding suggests that if the different intracellular fate of the bacteria was due to



**Figure 8.** Evidence for the intracellular localization of the bacteria. 18-h infected epithelial cells were fixed, incubated with 2-nm unconjugated gold spheres to mark all the spaces that were in contact with the extracellular environment, and cryosectioned. Both extracellular and intracellular bacteria were observed. The gold particles were strictly localized at the plasma membrane of the epithelial cells and membranes of extracellular bacteria (a). Note the absence of gold particles at the site of intimate membrane contact between the bacterium and the epithelial cell surface (arrows). No gold particles were found at the vacuole membrane and the outer membranes of the morphologically intact or disintegrated intracellular organisms (b). Bars, 0.25  $\mu$ m.



**Figure 9.** Differential processing of the intracellular bacteria. Cryosections of 18-h (a-c) and 30-h (d) infected cells were incubated with the P.IB-specific antibodies 4D9A, C12.43, and VK 207, and/or with the LOS-specific pAb K3-048, and subsequently with protein A-gold. Immunomorphologically, two classes of intracellular bacteria could be recognized: morphologically intact bacteria that were positive for the VK 207 P.IB epitopes (a, large gold particles) and the K3-048 LOS epitopes (a, small gold particles) and, except for at the sites of intense membrane contact, the 4D9A epitope (b); and morphologically disintegrated bacteria that showed no 4D9A labeling (c, large gold particles) but that labeled for the previously masked C12.43 epitope (c, small gold particles). At a later stage of disintegration, no P.IB epitopes could be detected (d, large gold particles), whereas LOS labeling was unaffected (d, small gold particles). Bars a, c, and d, 0.25  $\mu\text{m}$ ; bar b, 0.5  $\mu\text{m}$ .



**Figure 10.** Effect of chloroquine on the immunomorphology of intracellular gonococci. Epithelial cells infected for 10 h and then treated with chloroquine for an additional 8 h were cryosectioned and incubated with the various P.IB-specific antibodies and protein A-gold. This electron micrograph shows a morphologically disintegrated bacterium lying within a host cell vacuole, with intact P.IB epitopes (mAb 4D9A). It illustrates the differential effect of chloroquine on the bacterial morphology and the P.IB antigen. Bar, 1.0  $\mu\text{m}$ .

population heterogeneity, the causative factor was unstable under the conditions used.

*Prevention of the Intracellular Alterations in the P.IB Antigen by Chloroquine Treatment of the Host Cells.* The molecular mechanism underlying the intracellular alterations in P.IB was further investigated by adding the lysosomotropic agent chloroquine to the infection system. Chloroquine interferes with phagosome acidification and intracellular vesicle trafficking, thus preventing phagosome-lysosome fusion (26). The addition of this compound (45  $\mu\text{M}$ ) at the start of infection did not interfere with attachment or entry of the bacteria into the host cells (not shown). In the first 8 h of infection, the pattern of P.IB labeling was identical to that observed in the untreated cells. Exposure of the epithelial cells to chloroquine for >8 h, however, resulted in a marked vacuolization of the host cell cytoplasm, probably a result of the disordering of host cell vesicle processing. Since the intracellular alterations in P.IB and morphological disintegration of the bacteria first became apparent after 10 h of infection, we also added chloroquine later in the infection. When chloroquine was added 10 h after the start of infection and remained present during the next 8 h, the total loss in 4D9A labeling and the increase in the immunoaccessibility of the probed CB-1 and CB-3 epitopes that previously accompanied the early morpholog-

ical disintegration of the intracellular microorganisms was prevented. The morphological disintegration of the bacteria, however, was not affected (Fig. 10). This uncoupling of the alterations in the P.IB antigen and in bacterial morphology suggests that the immunomorphological degradation of the bacteria is a two-step process, involving a chloroquine-insensitive morphological disintegration and a chloroquine-sensitive immunological breakdown of the P.IB antigen. The immunomorphologically intact bacteria apparently have the ability to evade these processes.

## Discussion

Gonococcal infection of epithelial cells leads to a sequence of events that can be defined as primary attachment, strong attachment and invasion, and intracellular processing of the bacteria. In the present study, we have used post-embedding immunoelectron microscopy to investigate these processes at the ultrastructural level. By correlating cell morphology with the reaction pattern of antibodies that recognize different epitopes of the major outer membrane protein of the gonococcus (P.I) in cryosections of infected epithelial cells, we have been able to demonstrate that the process of gonococcal entry into the host cells involves a sequential interaction between the bacteria and the plasma membrane of the eukaryotic cells accompanied by changes in the P.IB antigen. In addition, we found, using P.I as a marker, that a percentage of the intracellular gonococci can evade the host defense by preventing or resisting exposure to host cell proteolytic enzyme activity.

Immunoelectron microscopy greatly contributes to understanding of bacteria-host cell interactions, because of the ability to follow the fate of defined epitopes. Gonococcal protein P.IB is an integral outer membrane porin with surface-exposed loop structures (27, 28) that vary in immunoaccessibility depending upon bacterial growth conditions, probably the result of alterations in LOS (29). The anti-P.IB antibodies that we have used in the present study recognize epitopes that are invariantly at either the surface- or the nonsurface-exposed parts of the molecule under various growth conditions, including those used in our infection assay. The epitopes recognized by the pAb VK 207 and the mAb 4D9A were accessible in all bacteria and outer membrane vesicles (blebs), while the probed C12.43 (CB-1-specific) and B5.36 (CB-3-specific) epitopes were only reactive with blebs. Importantly, this pattern of immunolabeling was maintained after cryosectioning of the bacteria. Unmasking or loss of epitopes (30) were not observed, provided that glutaraldehyde was omitted from the fixation protocol. The maintenance of the native accessibility of the probed epitopes is essential in order to be able to translate the immunoelectron microscopic observations into molecular events occurring during the interaction between gonococci and epithelial cells.

Our immunomorphological data demonstrate that, during gonococcal infection of epithelial cells, neither the exposure of the bacteria to the host cell environment nor the initial contact with the host cells, which is thought to involve the recognition by gonococcal pili of specific host cell surface receptors (4), are accompanied by alterations in P.IB labeling.

A similar stable immunoreactivity of epitopes has been found for LOS (14) and the major outer membrane protein P.II (opacity protein) (J.F.L. Weel and J.P.M. van Putten, unpublished observations). These data suggest that primary attachment does not result in marked alterations in the outer membrane architecture.

The next stage in the infection process is characterized by the development of a localized intimate membrane contact between the bacteria and the host cells. This event, which also occurs during *in vivo* infection (2), probably contributes to a stronger attachment of the bacteria to the host cells. The factors responsible for these zones of adhesion are unknown, but opacity protein (P.II) might be involved (25). Interestingly, this second step in the infection process was accompanied by a localized loss of immunorecognition of the 4D9A epitope but not of all surface-exposed P.IB epitopes, indicative of an alteration but not a complete absence of the P.IB antigen at the site of interaction. The loss of 4D9A labeling associated with host cell membrane contact can be explained either by binding of the protein or an adjacent component to host cell receptors, or by partial degradation of the molecule. Alternatively, it is possible that the intimate contact leads to a partial insertion of the P.IB molecule into the host cell membrane with a concomitant masking or change of epitopes. Both purified and native PI have been reported to translocate vectorially into host cell membranes (31, 32). This process, which results in a transient membrane hyperpolarization and an inactivation of degranulation in polymorphonuclear cells (12), has been suggested to play an important role in the initiation of the internalization process (31, 33). Although occasionally P.IB epitopes are located at the host cell membrane (Figs. 6 and 9), our data do not provide conclusive immunomorphological evidence for insertion of the P.IB molecule into the host cell plasma membrane. Attempts to unmask the possibly inserted 4D9A epitope by phospholipase treatment of the cryosections have not been successful (data not shown).

Though the molecular basis of the altered P.IB immunoreactivity in areas of contact with the host cell membrane is not clear, electron microscopy of many infected cells gives the impression that the apparent localized fusion of bacterial and host cell membranes is an important event in the bacterial entry of the host cell. The increasing number of zones of adhesion next to regions with clearly distinguishable bacterial and host cell membranes observed during the engulfment of the bacteria suggests that the gonococcal entry process proceeds by a sequential and circumferential interaction of bacterial and host cell membrane components. A similar "zipper" mechanism of phagocytosis has been proposed for the uptake of particles into professional phagocytes (34). In these cells, the uptake may occur via a two-step process of integrin binding and induction of an activation signal (35). This information makes it tempting to speculate that the formation of zones of adhesion between gonococci and the host cell membrane, in combination with the possible transfer of PI with its channel-inserting and calmodulin-binding activity, promotes the phagocytic behavior of the infected epithelial cells.

Detailed knowledge about the intracellular processing of



gonococci in epithelial cells is not available. It has been described that during *in vitro* infection, gonococci can be found within endocytic vacuoles (2, 14, 22, 36). Furthermore, in fallopian tubes (37), but not in human corneas in organ culture (38), bacteria have been observed in the subepithelial space possibly as a result of transcytosis (37). The present study confirms that, in infected Chang epithelial cells, all intracellular bacteria are contained within endocytic vacuoles, and furthermore demonstrates that two classes of immunomorphologically distinct bacteria can be recognized. One class consists of morphologically well-preserved gonococci that are 4D9A positive at sites where no intimate contact with the vacuole membrane exists and are negative when probed with the CB-1- and CB-3-specific mAbs. A second class is characterized by morphological disintegration and, at prolonged infection, a total loss of P.IB immunoreactivity. Several mechanisms may account for this apparent differential processing of bacteria. One is that all the intracellular gonococci are contained in similar cellular compartments (e.g., phagosomes) but that they are heterogeneous in adapting to the host cell environment, resulting in autolysis of the part of the bacterial population that is unable to carry out proper metabolic function with a loss of the P.IB antigen. Degradation of P.IB by endogenous gonococcal enzymes has been reported to occur *in vitro* (39). However, our observation that chloroquine, which interferes with phagosome acidification, phagolysosomal fusion, and sorting of lysosomal proteins (26), was apparently able to modulate the intracellular fate of the P.IB antigen, points to the involvement of host cell proteolytic activity rather than endogenous gonococcal enzyme activity. A second possibility is that the gonococci are in the same type of compartment (e.g., lysosomes) but that they differ in susceptibility to host cell proteolytic activity, a process that might be operative in infected polymorphonuclear cells (40). In this case, however, it remains unexplained why chloroquine treatment prevented the alterations in the P.IB antigen but not those in bacterial morphology. A more attractive explanation, which does fit with our data, is that the two classes of bacteria reside in cellular compartments that differ in host cell proteolytic activity as a result of heterogeneity in adaptation to the host cell environment. Thus, a subpopulation of gonococci can interfere with the cellular events that lead to phagosome acidification and phagosome-lysosome fusion, a well-known mode of action for several obligate intracellular parasites (*Legionella pneumophila* [41, 42]), *Toxoplasma* [43]), while the remainder cannot and are subject to a chloroquine-insensitive autolysis and, after fusion of phagosomes and lysosomes, chloroquine-sensitive breakdown of the P.IB antigen. A totally different explanation for our findings is that the bacteria are at a different stage of intracellular processing, be-

cause of limited capacity of epithelial cells to degrade microorganisms. In this case, the intracellular fate of the bacteria depends on the balance between the invasiveness of the gonococcal strain and the defense of the host cells. This idea is supported by the observed inverse relationship between the number of internalized bacteria within an individual cell and the number of immunomorphologically disintegrated bacteria, but determination of the capacity of the epithelial cell lysosomal machinery, and identification of gonococcal components that can interfere with the host cell vesicle trafficking, are necessary to address this point adequately. Our unsuccessful attempts to enrich for bacteria that are able to resist the host cell environment do not provide information at this point.

Another interesting observation in our studies is that the P.IB-specific mAbs can be used to map the degradation of the P.IB antigen inside eukaryotic cells. At first there is a loss of the surface-exposed 4D9A epitope and an unmasking of the CB-1 and CB-3 epitopes. In a later stage, all probed P.IB epitopes are degraded but LOS epitopes can still be detected (Fig. 10). These observations suggest that first the highly protease sensitive epitopes are degraded, resulting in an increased immuno-accessibility of normally nonsurface exposed epitopes. At a later stage these epitopes also become degraded, leaving gonococcal remnants still surrounded by LOS. Such a sequence of events is in agreement with the reported sensitivity of P.IB to lysosomal enzyme activity (40, 44, 45) and the dependence of the cleavage of P.IB on the outer membrane structure (28). The availability of specific mAbs that can be used to follow the degradation of the gonococcal P.I antigen within cells makes P.IB an excellent marker for studies concerning the mechanisms regulating the intracellular trafficking of gonococci.

Altogether, based on our immunomorphological observations, we hypothesize that gonococcal infection of epithelial cells involves (a) initial contact of the bacteria to the host cells without marked alterations in the outer membrane structure; (b) localized intimate contact of the bacterial and host cell membrane accompanied by a change in the P.IB antigen; (c) engulfment of the bacteria by a zipper-like mechanism, whereby the sequential interaction of the bacteria and the plasma membrane is also characterized by a change in P.IB immunoreactivity; (d) differential intracellular processing of the bacteria, varying with the number of intracellular gonococci, and resulting in either apparent survival or degradation of the bacteria. The precise role of the P.IB antigen in the mechanism of bacterial entry, and whether the internalized bacteria are capable of intracellular multiplication or are in the process of transcytosis to deeper tissues, remain the subjects of future studies.

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