Effect of Anti-Pilus Antibodies on Survival of Gonococci within Guinea Pig Subcutaneous Chambers

PAUL R. LAMBDEN, JOHN E. HECKELS,* AND PETER J. WATT

Department of Microbiology, Southampton University Medical School, Southampton General Hospital, Southampton S09 4XY Hampshire, England

Guinea pigs were immunized with either α or β pili from *Neisseria gonorrhoeae* P9 before the inoculation of subcutaneous chambers with a mixture of variants having α, β, γ, or δ pili. Animals immunized with α pili showed significant protection, but those immunized with β pili did not. The degree of protection could be correlated with the cross-reactivity of the antibodies produced to the heterologous pilus types.

The role of pili in the pathogenesis of gonorrhea has been of interest since the original observations that virulent colonial types differed from avirulent types in their possession of pili (6, 16). Recent work has focused attention on the contribution of anti-pilus antibodies to immunity to gonococcal infection and on the possible use of pili in a future human vaccine (1, 2).

The use of the guinea pig subcutaneous chamber model of gonococcal infection (4) has enabled the investigation of some of the factors influencing gonococcal survival in vivo. In particular, resistance to complement-mediated serum killing (15) and resistance to phagocytosis (19) enhance survival within chambers. Previous studies have suggested that, although pili are crucial determinants of gonococcal virulence in human infection, anti-pilus antibodies give only limited protection against chamber infection (4, 18). The implication is that the critical role of pili in the virulence of gonococci for humans is to function as adhesins to mucosal surfaces. However, since anti-pilus antibodies are responsible for immune-enhanced phagocytosis in vitro (7), they would be expected to contribute to immunity in guinea pig chambers. Gonococcal pili are capable of antigenic variations within a single strain (10); thus, particular pilus types may be selected during growth in vivo which do not correspond to the original immunizing type. This possibility is strengthened by the observation that when chambers were infected with a mixture of colonial variants of strain P9 with two pilus types (α and β), survivors were isolated with two further novel pilus types (γ and δ) (9). In this report, we demonstrate that pre-immunization with α or β pili does, indeed, produce a selection pressure for pilus variation in vivo and that the degree of chamber protection is dependent on the cross-reactivity of the antibodies formed for the other pilus types.

MATERIALS AND METHODS

Growth of gonococcal variants and preparation of pili. Transparent piliated colonial variants of *Neisseria gonorrheae* P9 were grown on clear typing medium (8), and pili were purified as described previously (1, 9). Organisms were subjected to mild shearing in ethanolamine buffer (0.15 M, pH 10.5) to remove pili, which were recovered by differential centrifugation and ammonium sulfate fractionation. Gonococcal α or β pili used for immunization were free from detectable outer membrane protein and lipopolysaccharide contamination as revealed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and staining with Coomassie blue or Stainsall (10).

Immunization and challenge of guinea pig chambers. Guinea pigs were inoculated intramuscularly in each hind leg with 50 μg of purified pili in complete Freund adjuvant (Difco Laboratories). The dose was repeated after 2 weeks, using pili in Freund incomplete adjuvant at several intradermal sites. Two weeks later, chambers were inoculated as described below. Each group—α, β, and nonimmunized control—contained six infected chambers.

Guinea pig subcutaneous chambers were implanted and inoculated as previously described (11). Two open-ended polypropylene chambers were implanted in each animal and left for 60 days to encapsulate. Chambers were infected with a standardized suspension of a mixture of gonococci P9-2 (α pili), P9-20 (β pili), P9-35 (γ pili), and P9-37 (δ pili). Suspensions of each of the variants in phosphate-buffered saline (Oxoid Ltd.) were adjusted to 2.8×10^8 colony-forming units per ml. Equal volumes of each of the suspensions were mixed, and 0.5 ml of the mixture was injected into each chamber. Chambers were sampled at regular intervals by withdrawing 0.2 ml of fluid, and samples were plated directly onto typing agar for viable counting. Gonococci recovered from chambers were subcultured once to obtain sufficient material for the isolation
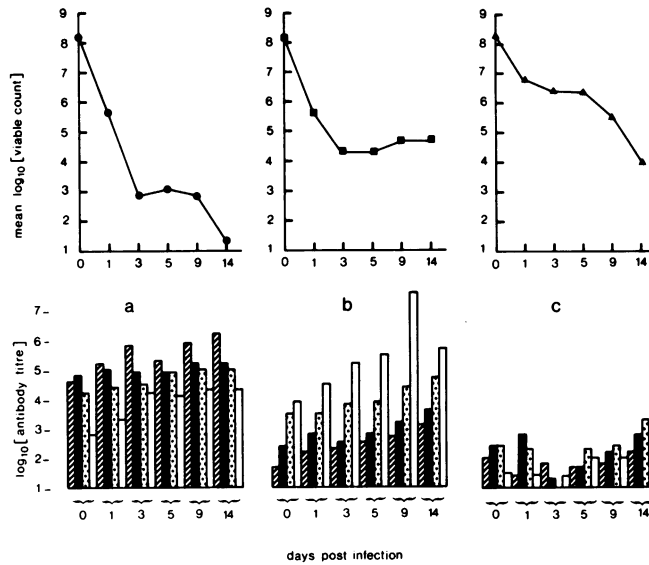
FIG. 1. Survival of piliated variants in guinea pig subcutaneous chambers, together with the antibody response for each group of animals. The graphs (top) show the changes in the viable count in the chambers during the infection; symbols: ●, animals immunized with α pili; ■, animals immunized with β pili; ▲, control animals. The corresponding histograms (bottom) show the ELISA titer for homologous and heterologous pili on the same days. Anti-pilus titers to the different types of pili are represented in the histogram as follows: ▨, anti-α pili; ■, anti-γ pili; ▨, anti-δ pili; □, anti-β pili. Antibody titers are expressed as ELISA units, which were recorded as the serum dilution giving an increase in the optical density at 490 nm of 0.1/h. An ELISA using lipopolysaccharide as the antigen showed a titer of less than 2 in immunized and control animals.

of pili. Preparations of purified pili were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on linear gradients of 10 to 25% (wt/vol) acrylamide as previously described (5); the pilus type present was determined by comparison with standards of purified α, β, γ, and δ pili.

Antibody detection in chamber fluid. Anti-pilus antibodies were detected in chamber fluid after centrifugation at 10,000 × g to remove gonococci. Samples were examined for the presence of anti-pilus antibodies by an enzyme-linked immunosorbent assay (ELISA) by the method of Buchanan (3). Wells of polystyrene microtiter plates coated with purified α, β, γ, or δ pili were incubated with serial dilutions of chamber fluid. After the plates were washed, bound antibody was detected with peroxidase-conjugated goat anti-guinea pig immunoglobulin (Miles, Stoke Poges, England) using o-phenylene diamine as the substrate.

RESULTS AND DISCUSSION

The effect of pre-immunization with purified pili on infection of the chambers is shown in Fig. 1. The control group (c) showed a progressive fall in viable count throughout the experiment. For the first 24 h after the infection of the chambers, the β-immunized group (b) eliminated organisms from the chambers at a rate similar to that of the α-immunized group, but then maintained the infection at a constant level such that by day 14 the viable count was similar to that of the control. In contrast, the α-immunized group

(a) showed a rapid elimination of organisms from the chambers throughout the experiment. By day 14, the difference between the α- and β-immunized groups was highly significant (log viable count α, 1.35 ± 0.4; log viable count β, 4.71 ± 0.4; $P < 0.001$, Student t test).

The detection of antibodies in chamber fluid showed a considerable difference in cross-reactivity between the α- and β-immunized groups (Fig. 1). With the α group, antibodies present on day 0 showed considerable cross-reactivity with the heterologous pilus types in order α ≥ γ > δ ≫ β, and both homologous and heterologous titers increased 10- to 100-fold during the experiment. The β-immunized group also showed an increase in anti-pilus antibodies during the course of infection, but these showed considerably lower cross-reactivity with heterologous pilus types. A similar difference in the cross-reactivity of antisera raised against purified pili from different strains has been observed by Brinton and his colleagues, who coined the term "senior" pili to denote those which produce high levels of cross-reacting antibody (2).

Previous studies (4, 13, 18) have suggested that anti-pilus antibodies are relatively unimportant in the protection of guinea pig chambers against infection with gonococci despite their protective effect against phagocytosis in vitro (7).

In one attempt to elicit protection with a gonococcal surface component antigen, a, now known to be pili, no protection of chambers was observed (13). Parsons et al. subsequently suggested that the lack of protection was due to the selection of a variant antigenic pilus type (14). It is of interest to note that antigen a appears, on the basis of its aggregation characteristics, to resemble the nonprotective β pili rather than the protective α pili. The current study suggests that the specificity of the antibodies raised and the ability of the gonococcus for antigenic variation must be considered in protection experiments. Immunization with β pili does not protect against infection since the antibodies formed show a low degree of cross-reactivity with the heterologous pilus types produced by variants of the same strain. In contrast, the antibodies raised by immunization with α pili have much greater cross-reactivity and, therefore, a significantly greater protective effect. It is also interesting to note that, after the subculture of surviving gonococci, in no case did we detect pili of the same type as that used for immunization. Organisms recovered from the chambers on day 9 were subcultured once for pilus purification and identification. The resulting mixture of variants isolated from the β-immunized group produced either α or δ pili but no β pili, whereas only β or γ pili could be detected in the low numbers of survivors in the α-immunized group. The factors influencing survival are likely to be complex and may depend on other host-gonococcus interactions, in addition to the levels of cross-reactive antibody present. For example, although anti-γ antibodies were higher in the α-immunized group, γ-pilated organisms were isolated from this group but not from the β-immunized group. However, such comparisons are difficult to interpret since the concentration of organisms in chambers of the α group were 100- to 1,000-fold lower than those of the β group so that the predominant variant present in the α group would be present in lower amounts than a minor variant which represented only 1% of the organisms present in the β group.

These results emphasize that the potential of the gonococcus for antigenic variation may play a significant role in the pathogenesis of human disease, including, for example, the reported inability of patients to develop effective immunity to reinfection with apparently the same strain (17). Indeed, we have recently demonstrated that consorts may be concurrently infected with variants of the same strain of gonococcus but bearing different pilus types (J. E. Heckels *in* D. Schlessinger, ed., *Microbiology—1982*, in press). Any attempt to utilize pili in a future vaccine must, therefore, consider the problems of antigenic heterogeneity, both of the pili present on different strains (2, 12) and of those present on variants of the same strain. The results obtained in this study indicate that the problem of intrastrain antigenic heterogeneity, like interstrain heterogeneity, might be overcome by the selection of a senior pilus type which induces high levels of cross-reactive antibody (2). Clearly, careful selection of the immunizing pilus type is likely to be crucial to the success of a pilus vaccine.

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