CHAPTER III

RESULTS

1. Purification of the Gonococcal Pili

Thirty one strains of gonococci from fresh clinical isolates were grown on the GCBS medium for colonial morphological typing and electron microscopic examination. There were 13 strains showing T_1 or T_2 colony type, each of which possess surface pili, as shown in Fig.12 and Table 5. These piliated strains of gonococci were then selected for pili purification. Gonococci were grown on 200 plates of GCBS medium for 20 to 24 hours at 37°C in candle jars, harvested, and surface pili sheared off by blending at highest speed in a Sorvall Omnimixer in icebath for 2 min. The sheared pili were suspended in Tris buffer pH 9.5. Depiliated cells, as evidenced by electron microscopy (Fig.13), debris and large amount of outer membrane proteins were removed by sequential centrifugation at 12,000 xg for 10 min, followed by 48,000 xg for 60 min (Fig. 14c,e). Pili and a small amount of contaminants remained in the supernate from which pili were later precipitated by addition of saturated ammonium sulfate to a final 10% concentration. This crude pilus preparation (first cycle) containing some outer membrane proteins (Fig. 14h) was further purified by disaggregation of crude

pili at high pH of tris buffer, followed by precipitation with ammonium sulfate, at which impurities were removed by centrifugation. The final product after two cycles of reaggregation and disaggregation consisted predominantly of pili (Fig.14j). The preparation of pili in each strain had to be redone several time owing to insufficient produce.

Consequently, because of the time consumed, only twelve strains were processed for pili purification. Of the 12 freshly isolated strains, only five produced enough purified pili demonstrable by SDS-PAGE (Fig. 15). From each preparation of 200 culture plates (9 cm in diameter), approximately 7g of bacteria (wet weight) were obtained and the yield of pili preparations varied from 0.22 to 1.03 mg protein per 10 g of bacteria (wet weight), as shown in table 6.

2. Assessment of Purity

The purity of the pili preparations were demonstrated by SDS-PAGE and electron microscopy. In Fig. 15, the slab SDS-polyacrylamide gel patterns of pili preparations from strains J 070229, S 280229, S 200329, S 040629, and S 160829 were presented. Majority of the pilin from each strain migrated as a single band except for S 280229 which gave 2 bands migrated closely. In one pili preparation, S 160829, there is a minor contamination of a 31,000 dalton protein.

As shown in Fig. 16, the electron micrograph of negatively stained purified gonococcal pili shows abundant pili filaments and neither the outer membrane vesicles, recognizable cells, membrane and cell wall fragments could be observed.

Purified pili from strain S 040629 which gave the highest yield were selected for the production of anti-pili antibody.

3. Morphology and Subunit Molecular Weight of Gonococcal Pili

The gonococcal pili preparations examined under electron microscopy were morphologically indistinguishable among different strains as shown in Fig.16. Moreover, purified pili retained its morphology as long continuous strand with a diameter of 7 nm even after the purification process. The pili preparations were also similar both in morphology and in diameter to that previously illustrated by other investigators (39,65,83,107,110,114,135,136) and are morphologically indistinguishable from that of E. coli (167,168).

As shown in Fig.15, when analysed by SDS-PAGE, each pili migrated at different relative mobility. The subunit molecular weight of pili from each strain varied slightly as

illustrated in Fig.17. The apparent subunit molecular weights of gonococcal pili from each strain were approximately 18,000 (J 070229), 18,000 (S 160829), 18,750 (S 200329), 21,500 (S 040629), and 22,500 and 19,500 (S 280229) (Table 6).

4. Rabbit Antiserum against Purified Gonococcal Pili

The pili from strain S 040629 giving the highest yield was used to immunize rabbits according to the method of Buchanan and Pearce (65). Anti-pili antibody was demonstrated in two rabbits with a titre of 1:2 by immunodiffusion (Fig.18) and 1:10240 by indirect hemagglutination test (IHA)(Fig.19). Thus, IHA was more sensitive and was chosen for further determination of anti-pili antibody. The IHA titer of antiserum was notably increased to 1:81920 after booster (Fig.19).

5. Analysis of Rabbit Anti-pili by Coagglutination Test

The specificity of anti-gonococcal pili antiserum was demonstrated by coagglutination with homologous, heterologous N.gonorrhoeae, and other bacteria. Staphylococcal protein A sensitized with anti-pili S 040629 showed strongly positive reaction with homologous gonococci, as shown in Fig.20, within 30 seconds observed by naked eye. However, the antiserum did not cross react with other 102 isolates of gonococci,nor with six strains of N.meningitidis, four of

N.sicca, three of N.mucosa,and one each of B.catarrhalis, M.osloensis, P.aeruginosa, E.coli and S.aureus.

6. Preparation of Rabbit Anti-pili IgG

IgG of rabbit anti-pili antibody was isolated by passage of 4 ml of dialysed whole rabbit serum through a column of DE-52 anion exchange chromatogragphy equilibrated with 0.01 M phosphate buffer pH 8.0. IgG was eluted in the first single peak as shown in Fig.21. The purity of IgG fraction was indicated by a single precipitin line in immunodiffusion and immunoelectrophoresis against anti rabbit serum and anti rabbit IgG as demonstrated in Fig. 22,23. The yield of rabbit IgG was about 13 mg/four ml of rabbit antiserum and estimated to 21% of total rabbit serum IgG. The purified rabbit IgG was further used for enzyme conjugation in ELISA methods.

7. Setting Up of an ELISA for the Determination of Pili Antigen

7.1. Determination of Suitable Levels of Anti-pili IgG for Coating the ELISA Microplate

The optimal concentration of anti-pili IgG for coating was determined by checkerboard titration with varying concentrations of antipili IgG, ranging from 0.1 to

10 ug/ml. As shown in Fig.24, there was only slightly increased in absorbance value beyond an antibody level of 5 ug/ml. Consequently, for practical and economic purposes, antipili IgG at 5 ug/ml was then selected as the optimal concentration for antibody coating.

7.2. Determination of Working Dilution of Rabbit Anti pili IgG Conjugated with Alkaline Phosphatase

The suitable dilution of the conjugate was determined in a similar manner by varying dilutions of the conjugate, ranging from 1:200 to 1:800. As depicted in Fig. 25, the dilution of 1:300 of anti-pili IgG conjugate was chosen to economize on reagent, and the enzyme substrate incubation time at 37°C for 60 min was also selected.

7.3. Determination of the Optimal Period for Antigen and Conjugate Incubation

The effect of time for antigen antibody reaction was investigated at 37°C for 30, 45, 60, and 120 min, respectively. As shown in Fig. 26, the highest absorbance reading was obtained when the incubation period approached 45 min. Hence, antigen incubation at 37°C for 45 min was chosen in practice.

The optimal time for conjugate incubation was also determined in a simialar manner. As shown in Fig.27, for rapid and practical purposes, the 45 min period of incubation was selected for subsequent tests.

7.4. Sensitivity and Specificity of ELISA

Using the optimal conditions outlined above, graded amount of reference pili S 040629 antigen was assayed. To determine the sensitivity of this ELISA system, absorbance of 0.2 (twice the mean absorbance of negative control) was chosen as the cut off point. A typical curve obtained from 3 experiments with pili antigen varied from 0.01 to 5 ug/ml was shown in Fig.28. The minimum concentration of pili antigen demonstrable by ELISA was 0.5 ug/ml. When sonicated and intact whole cells of homologous gonococci were used as antigen, the minimum number of organisms of which pili antigen were detectable corresponded to $5x10^{8}$ and $5x10^{7}$ CFU/ml, respectively (Fig.29). Thus, the sensitivity of the assay decreased approximately 10 times when unsonicated cells were employed. The whole cell lysate corresponded to 10^{8} CFU/ml was used to ensure sufficient amount of antigen in subsequent ELISA tests.

Other closely related organisms were tested in this ELISA system including 6 strains of N.meningitidis. four of N.sicca,three of N.mucosa,one each of B.catarrhalis,

M.osloensis, P.aeruginosa, E.coli and S.aureus,no reactivity has ever been identified.

8. Determination of Pili Antigen by ELISA

Pili preparation from homologous and heterologous strains were tested by double antibody sandwich ELISA. As shown in Fig.30, rabbit anti-pili antibody reacted strongly with homologous pili S 040629 and one heterologous pili S 280229. It did not react with pili from strains S 200329, S 160829, and J 070229. Thus, our anti-pili IgG showed little cross reactivity among pili of three different strains. The reactivity of pili S 280229 with anti-pili (S 040629) antibody indicated that there may be some antigenic similarity between pili of the 2 strains, which are antigenically distinguishable from those of the other three.

Whole cell lysates of gonococci were then tested for pili antigen in the ELISA. As shown in Table 7, of the 102 clinical isolated gonococci, 5 were reactive with anti S 040629 pili antibody (4.9%). When these 102 gonococcal isolates were examined under the electron microscope, 35 presented surface pili of which only four showed positive results for ELISA (11.4%,Table 8). Of the 67 pilus-lacking gonococci, 66 were negative by ELISA whereas one strain exhibited reactivity indicating the presence of pili antigen.