Fimbrial haemagglutination by *Neisseria gonorrhoeae*

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The presence of filamentous appendages distinct from flagella was first discovered in electron microscope preparations by Anderson (1949) and Houwink and van Itersen (1950). Duguid, Smith, Dempster, and Edmunds (1955) confirmed the existence of these structures and suggested the name 'fimbriae' for them; others have called them 'cilia' (Pershina and Vasilieva, 1960) and 'pili' (Brinton, 1959). Because of the points Duguid and Anderson (1967) put forward in favour of the term 'fimbriae' it is perhaps the more correct form.

Bacterial fimbriae are described as filamentous surface appendages having various diameters and lengths. They do not exhibit the sinuous forms characteristic of bacterial flagella, are devoid of any recognizable amplitude or wavelength, and are apparently not related to the motility of flagellated bacteria (Brinton, 1965; Duguid, Anderson, and Campbell, 1966); they can also be found in capsulated organisms, e.g. *Klebsiella* (Duguid, 1959). Fimbriae have been demonstrated in many of the enterobacteriaceae, and more recent studies have shown that other organisms also possess fimbriae, for example, *Pseudomonas* (Fuerst and Hayward, 1969); *Vibrio* (Tweedy, Park, and Hodgkiss, 1968); *Herella* (Swanson and Goldschneider, 1969) and *Neisseria* species (Wistreich and Baker, 1971; Jephcott, Reyn, and Birch-Andersen, 1971; Swanson, Kraus, and Gotschlich, 1971). Their occurrence is not limited to Gram-negative organisms, since they have been reported in various strains of streptococci (Swanson and McCarty, 1969) and *Corynebacterium renale* (Yanagawa and Otsuki, 1970).

Fimbriae are usually thinner than flagella and may differ from them in detailed fine structure (Duguid and others, 1955; Brinton, 1967). Duguid (1959) suggested, on the basis of electron micrographs, that there may be two types of fimbriae, one with a diameter of 100nm. and the other 70nm.; he suggested that such a difference in apparent sizes might be a

result of the preparative methods used for electron microscopy. Thornley and Horne (1962) confirmed these differences but found them to be 65–70nm. and 48nm. respectively. Fimbriated organisms readily became attached to a variety of animal cells, such as erythrocytes, leucocytes, intestinal epithelial cells, and plant cells, e.g. *Candida albicans*, giving rise to agglutination of the cells.

The presence of fimbriae can be detected directly by the electron microscope, and indirectly by employing their ability to attach to red blood cells and give rise to haemagglutination (Duguid and others, 1955; Brinton, 1959). The haemagglutination test alone, however, is not a reliable indicator since the haemagglutination activity can be attributed to non-fimbrial haemagglutination (Duguid and others, 1955, 1966). Moreover, some fimbriated bacteria fail to haemagglutinate red blood cells (Duguid and Gillies, 1958; Cruickshank, 1965). Fimbrial haemagglutination can be inhibited by D-mannose (0.7 per cent. w/v) and α methyl-mannoside (Duguid and Gillies, 1957), although in a few species of fimbriated organisms such inhibition was not observed (Duguid, 1959). These mannose resistant (MR) strains will weakly haemagglutinate red cells but adhere more readily to erythrocytes treated with tannic acid.

Cultural conditions greatly influence production of fimbriae. Broth cultures incubated at 37°C. for 48 hrs have given the best results for the enterobacteriaceae (Duguid and Gillies, 1958). Shedden (1962) studied the development of fimbriae on *Proteus hauseri* both in peptone water and on solid nutrient agar under anaerobic and aerobic conditions, and concluded that the lowering of oxygen tension improved the production of fimbriae. Swanson and others (1971) grew *Neisseria gonorrhoeae* in broth cultures incubated at 37°C. for 4 hrs on a rotary platform shaker revolving at 150 rpm and reported that fimbriae could be detected in Types 1 and 2 using the electron microscope, whereas Types 3 and 4 did not possess fimbriae. Jephcott and others (1971) also reported seeing fimbriae in Types 1 and 2

gonococci but not Types 3 and 4. Punsalang and Sawyer (1973) demonstrated that Kellogg Types 1 and 2 would haemagglutinate various mammalian red blood cells while the avirulent types would not. Thus it seemed possible that Types 1 and 2, which are considered to be virulent (Kellogg, Peacock, Brown, and Pirkle, 1963), could be differentiated from the avirulent types by their haemagglutinating activities. I have also studied the optimal conditions under which this haemagglutinating activity occurs.

Methods

CULTURAL METHOD

(i) *Solid medium* All organisms were cultured on Difco G.C. medium base plus 2 per cent. defined supplement and 1 per cent. vancomycin (500 µg./ml.) (White and Kellogg, 1965) incubated at 37°C. for 18 and 48 hrs in a 5 per cent. CO₂ atmosphere. The organisms were harvested by scraping off, suspending, and diluting in saline to a final concentration of 10⁸ gonococci/ml. as determined by the method of Miles, Misra, and Irwin (1938). This suspension was used in the test system.

(ii) *Liquid medium* Liquid cultures in Gerhardt's biphasic medium (Gerhardt and Hedén, 1960) plus 1 per cent. glucose and 1 per cent. vancomycin (500 µg./ml.) were incubated at 37°C. for 18, 36, and 48 hrs. The liquid phase, containing the organisms, was then centrifuged, washed once, and re-suspended in saline. A final concentration of 10⁸ gonococci/ml. (Miles and others, 1938) was used in the test system.

ORGANISMS

All the strains of *Neisseria gonorrhoeae* were isolated from patients attending the Sheffield V.D. clinics and initially cultured on 5 per cent. lysed horse blood agar with 1 per cent. vancomycin (500 µg./ml.). They were identified by Gram's stain, a positive oxidase reaction, and the fermentation of glucose, but not maltose or sucrose. Fermentation tests were performed using a modified carbohydrate medium containing Difco G.C. medium base with 1 per cent. sugar concentration (Flynn and Waitkins, 1972). After strains had been shown to be pure, they were suspended in 1 per cent. glycerol peptone and preserved by snap freezing in liquid nitrogen. By selective transfers on solid medium, colonial variants 1, 2, and 4 were isolated from these strains (Kellogg and others, 1963).

ANTISERA

(1) *Rabbit immune sera*

N. gonorrhoeae A New Zealand white rabbit was immunized by intravenous injection of a suspension of live gonococci (mixture of Types 1 and 2), standardized to a density of approximately 10⁸ organisms/ml. Three injections were given each week for 3 weeks, increasing the

dose from 0.1 to 1 ml. The animal was bled 7 days after the last injection and the serum Seitz-filtered and stored at -20°C. Before testing, the serum was thawed, heated at 56°C. for 30 min., and absorbed with the appropriate red blood cells. For this absorption, three parts of serum were added to one part of washed, packed erythrocytes, and the mixture was incubated for 15 min. at 37°C. The erythrocytes were then removed by centrifugation.

E. coli The method of preparing antiserum against *E. coli* was exactly as above.

(2) *Erythrocytes*

Human O Rh-negative and animal erythrocytes (fowl, guinea-pig, horse, mouse, rabbit, rat, and sheep) were obtained from whole blood collected in ACD-solution. The erythrocytes were washed three times with 6 to 10 volumes of phosphate buffered saline and packed by centrifugation at 1,000 rpm for 5 min. These were finally prepared as a 3 per cent. suspension in phosphate buffered saline.

Tanned fowl and human O Rh-negative erythrocytes were prepared by making a 3 per cent. suspension of erythrocytes in tannic acid diluted 1 in 40,000 in phosphate buffered saline (w/v). The mixture was kept at 37°C. for 15 min. The erythrocytes were washed twice and finally prepared as a 3 per cent. suspension in phosphate buffered saline.

(3) *Haemagglutination*

Gonococcal haemagglutination was studied using red blood cells from the eight animal species mentioned above. The sensitivity of this haemagglutination to heat, D-mannose, and both anti-gonococcal and anti-*E. coli* serum was further studied using fowl and human O Rh-negative erythrocytes, which were prepared as described above. The haemagglutination procedure was similar to that of Tweedy and others (1968), except that the erythrocytes, saline, and bacterial suspension were added in 0.02 ml. volumes to W.H.O. plates and incubated for 1 hr at 0°, 22°, and 37°C.

The effect of heating fimbriated gonococci was tested by heating the bacterial suspension (10⁸ gonococci/ml.) at 85°C. for 1 hr. and measuring the haemagglutinating activity using only the fowl and human O Rh-negative erythrocytes. Similarly, their sensitivity to D-mannose (final concentration 0.7 per cent. w/v) and to rabbit anti-gonococcal and anti-*E. coli* sera were examined (see Table I, overleaf).

ELECTRON MICROSCOPY

Bacterial suspensions were washed twice in saline and mounted in distilled water on copper grids. Specimens were shadowed with gold/palladium and evacuated at low pressure (10⁻⁴ torr) in an A.E.I. Vacuum Coating Unit. An A.E.I. E.M.6B electron microscope was used for examining the specimens.

TABLE I Amount of reagents (ml.) added to depressions in chilled WHO plates in haemagglutination tests of *N. gonorrhoeae* Type 2

Reagents	Depression numbers							
	1	2	3	4	5	6	7	8
(1) 3 per cent. (v/v) tanned fowl red blood cells	0.2	0.2	0.2	0.2				
(2) 3 per cent. (v/v) tanned human O Rh-negative red blood cells					0.2	0.2	0.2	0.2
(3) 2 per cent. (w/v) D-mannose		0.2				0.2		
(4) Saline	0.2				0.2			
(5) Anti-gonococcal serum			0.2				0.2	
(6) Anti- <i>E. coli</i> serum				0.2				0.2
(7) Gonococcal suspension (10^8 cells/ml.)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Results

Table II shows that maximum production of fimbriae, estimated by electron microscopy, occurred when the gonococcus was incubated for 18 hrs on a solid medium; however, increased yields were obtained if the organisms were incubated for a further 3 hrs in biphasic medium at 37°C. Fig. 1(a, b) illustrates fimbriae of various lengths radiating from a typical diplococcus of Type 1 (Fig. 1a) and Type 2 (Fig. 1b); note also the aggregation of these structures

to one another. Fig. 2 shows a Type 4 gonococcus devoid of fimbriae. These results are in agreement with those of Jephcott and others (1971). Having established that these fimbrial structures occurred in Types 1 and 2, but not in Type 4 gonococci, their haemagglutinating activities were examined using a variety of animal blood cells. As haemagglutination was very weak, the test was repeated with cells treated with tannic acid to give a better haemagglutination reaction (Table III).

TABLE II Effect of medium and incubation time on production of fimbriae by *N. gonorrhoeae* grown at 37°C.

Kellogg type <i>N. gonorrhoeae</i>	Solid medium			Biphasic medium		
	18 hrs	36 hrs	48 hrs	18 hrs	36 hrs	48 hrs
Type 1	+++	++	+	+	±	—
Type 2	+++	+++	+	++	±	—
Type 4	±	—	—	—	—	—

Percentage fimbriated organisms observed by electron microscope studies

+++ = 51 to 75 per cent.	} of organisms fimbriated
++ = 26 to 50 per cent.	
+ = 11 to 25 per cent.	
± = 5 to 10 per cent.	
— = <5 per cent.	

TABLE III Effect of variations in temperature on the haemagglutination of eight species of tanned red blood cells by three types of *N. gonorrhoeae*

Red blood cells	Fimbriated						Non-fimbriated		
	Type 1			Type 2			Type 4		
	4°C.	22°C.	37°C.	4°C.	22°C.	37°C.	4°C.	22°C.	37°C.
Fowl	(+)	(+)	(+)	(+)	(+)	(+)	—	—	—
Rat	—	—	—	—	—	—	—	—	—
Guinea-pig	—	—	—	—	—	—	—	—	—
Horse	—	—	—	—	—	—	—	—	—
Human O Rh-negative	+	+	(+)	+	+	(+)	—	—	—
Mouse	—	—	—	—	—	—	—	—	—
Rabbit	—	—	—	—	—	—	—	—	—
Sheep	—	—	—	—	—	—	—	—	—

+ = Haemagglutination (+) = Slight haemagglutination — = No haemagglutination

FIG. 1(a) Typical diplococcus of Type 1 with fimbriae of various lengths radiating from the organism. $\times 20,000$

Using tanned erythrocytes, both colonial Types 1 and 2 agglutinated human red cells and to a less extent fowl red cells, whereas Type 4 cultures would not agglutinate any of the red cells tested. Thus it is possible to differentiate between virulent and avirulent types according to their haemagglutinating activities. The best haemagglutination results were obtained after allowing the mixture to react for 1 hr at 4°C.

Table IV shows that treatment of fimbriated gonococci by heating to 85°C. for 1 hr completely destroys the haemagglutinating powers of the fimbriae; exposure to rabbit antisera containing antibodies to the gonococcus will similarly inhibit haemagglutination, presumably because of specific antibodies directed against the gonococcal fimbriae. However, if anti-*E. coli* serum is introduced into the last system, there is no interference with fimbrial

TABLE IV Effect of heat, D-mannose, rabbit anti-gonococcal, and anti-*E. coli* serum on haemagglutinating activity of Type 2 gonococci when mixture allowed to react for 1 hr at 4°C.

Tanned red blood cells	Control saline unheated	Heated	D-mannose	Rabbit normal serum	Rabbit anti-gonococcal serum	Rabbit anti- <i>E. coli</i> serum
Human	+	—	+	+	—	+
Fowl	+	—	+	+	—	+

+ = Haemagglutination — = No haemagglutination

FIG. 1(b) *Diplococcus* of Type 2 with fimbriae twisted around each other, forming an aggregation of these organelles into bundles. $\times 15,000$

haemagglutination, suggesting that this could be a specific test for fimbriated gonococci. It was also noted that gonococcal fimbriae were totally resistant to the action of D-mannose (final concentration 0.7 per cent. w/v).

Discussion

Earlier reports of fimbriae in the genus *Neisseria* were given by Swanson and others (1971), Jephcott and others (1971), and Wistreich and Baker (1971), all of whom demonstrated fimbriae in Types 1 and 2. Punsalang and Sawyer (1973) briefly mentioned their findings of gonococcal haemagglutination with various mammalian red cells and observed haemagglutination with rabbit as well as fowl cells. These findings differ slightly from mine, since I was able to haemagglutinate only fowl red cells. However, I noticed that if the erythrocytes were not pre-treated with tannic acid, their haemagglutination was difficult to assess. Since Punsalang and Sawyer did not use tanned red

cells, perhaps the discrepancy lies here. In the present study haemagglutination of both fowl and human erythrocytes was seen and these cells were subsequently used to test the optimal conditions required for fimbrial haemagglutination by the gonococcus. The methods found to give good production of fimbriae were similar to those employed by Swanson and others (1971), *i.e.* cultivation on solid agar followed by short incubation in a liquid medium. Growth in liquid culture tends to favour the rapid degeneration of Types 1 and 2 (virulent) to Types 3 and 4 (avirulent) (Jephcott, 1972). It is quite possible that in the present study Types 1 and 2 gonococci which were initially inoculated into the biphasic medium degenerated over the incubation times studied to Type 4; hence the lack of good production of fimbriae as seen under the electron microscope. The electron microscope examinations of fimbriated gonococci showed the same sort of variation in the length of the fimbriae as was reported by Jephcott and others (1971) and Swanson and

FIG. 2. Typical Type 4 organism completely devoid of all fimbriae. $\times 30,000$

others (1971). Likewise, the number of fimbriae per gonococcus also varied from a few per specimen (2 to 4) to large numbers (30 to 40) radiating in bundles from the organism.

The haemagglutinating activity of the gonococcus is much weaker than that of fimbriated enterobacteria, although even their haemagglutinating power varies directly with the degree of fimbriation of the organism (Duguid and Gillies, 1957). In the present study electron microscope evidence reveals that the gonococcus is not as well fimbriated as, for example, *E. coli*, and this would partially explain the weaker haemagglutination reactions observed. Moreover, the total number of fimbriated organisms per sample of organisms present was smaller.

In common with all other fimbriated species, the haemagglutinating activity of the gonococcus is completely destroyed by heating at 85°C. for 1 hr. Fimbriated gonococci are resistant to the action of D-mannose (final concentration 0.7 per cent. w/v) and it is therefore not surprising that they require tanned red blood cells to give good haemagglutination

reactions. Inhibition of fimbrial haemagglutination was observed when rabbit anti-GC serum was added but not when rabbit anti-*E. coli* serum was used; normal rabbit serum did not affect the reaction mixture. Thus, although antibodies were raised to whole gonococci, specific activity against gonococcal fimbriae was noted. It may be that gonococcal fimbriae may provide an antigen which could be used in a specific serological test for the gonococcus, providing cross-reaction with other *Neisseria* could be overcome by previous absorption of the sera.

Summary

The haemagglutinating activity of fimbriated *N. gonorrhoeae* Types 1 and 2 was investigated using a variety of red blood cells. Human O Rh-negative and fowl red blood cells were found to be haemagglutinated. This activity was completely destroyed by heating at 85°C. for 1 hr. but was not impeded by D-mannose. Apparent specific inhibition of haemagglutination by rabbit anti-gonococcal serum was also noted.

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References

- ANDERSON, T. F. (1949) 'On the mechanism of adsorption of bacteriophages on host cells' in 'The Nature of the Bacterial Surface': I. Symposium of The Society for General Microbiology, ed. A. A. Miles and N. W. Pirie, p. 76.
- BRINTON, C. C. (1959) *Nature (Lond.)*, **183**, 782.
- (1965) *Trans. N.Y. Acad. Sci.*, Series II, **27**, 1003.
- (1967) 'Contributions of pili to the specificity of the bacterial surface and a unitary hypothesis of conjugal infectious heredity' in 'The Specificity of Cell Surfaces', ed. B. D. Davis and L. Warren, pp. 37–70. Prentice Hall, New Jersey.
- CRUICKSHANK, R. (1965) 'Medical Microbiology', 11th ed. Williams and Wilkins, Baltimore.
- DUGUID, J. P. (1959) *J. gen. Microbiol.*, **21**, 271.
- (1966) *J. Path. Bact.*, **92**, 137.
- and ANDERSON, E. S. (1967) *Nature (Lond.)*, **215**, 89.
- , —, and CAMPBELL, I. (1966) *J. Path. Bact.*, **92**, 107.
- and GILLIES, R. R. (1957) *Ibid.*, **74**, 397.
- (1958) *Ibid.*, **75**, 519.
- , SMITH, I. W., DEMPSTER, G., and EDMUNDS, P. H. (1955) *Ibid.*, **70**, 335.
- FLYNN, J., and WAITKINS, S. A. (1972) *J. clin. Path.*, **25**, 525.
- FUERST, J. A., and HAYWARD, A. C. (1969) *J. gen. Microbiol.*, **58**, 227.
- GERHARDT, P., and HEDÉN, C. G. (1960) *Proc. Soc. exp. Biol. (N.Y.)*, **105**, 49.
- HOUWINK, A. L., and VAN ITERSOM, W. (1950) *Biochim. biophys. Acta*, **5**, 10.
- JEPHCOTT, A. E. (1972) *Brit. J. vener. Dis.*, **48**, 369.
- , REYN, A., and BIRCH-ANDERSEN, A. (1971) *Acta path. microbiol. scand.*, Sect. B, **79**, 437.
- KELLOGG, D. S., JR., PEACOCK, W. L., JR., DEACON, W. E., BROWN, L., and PIRKLE, C. I. (1963) *J. Bact.*, **85**, 1274.
- MILES, A. A., MISRA, S. S., and IRWIN, J. O. (1938) *J. Hyg. (Lond.)*, **38**, 732.
- PERSHINA, Z. G., and VASILIEVA, I. G. (1960) *Ž. Microbiol.*, **31**, no. 3, p. 14.
- PUNSALANG, A. P., and SAWYER, W. D. (1973) *Infect. and Immun.*, **8**, 255.
- SHEDDEN, W. I. H. (1962) *J. gen. Microbiol.*, **28**, 1.
- SWANSON, J., and GOLDSCHNEIDER, I. (1969) *J. exp. Med.*, **129**, 51.
- , KRAUS, S. J., and GOTSCHLICH, E. C. (1971) *Ibid.*, **134**, 886.
- and MCCARTY, M. (1969) *J. Bact.*, **100**, 505.
- THORNLEY, M. J., and HORNE, R. W. (1962) *J. gen. Microbiol.*, **28**, 51.
- TWEEDY, J. M., PARK, R. W. A., and HODGKISS, W. (1968) *Ibid.*, **51**, 235.
- WHITE, L. A., and KELLOGG, D. S., JR. (1965) *Appl. Microbiol.*, **13**, 171.
- WISTREICH, G. A., and BAKER, R. F. (1971) *J. gen. Microbiol.*, **65**, 167.
- YANAGAWA, R., and OTSUKI, K. (1970) *J. Bact.*, **101**, 1063.

Hémagglutination par *N. gonorrhoeae* filamenteux

SOMMAIRE

On a recherché l'activité sur l'hémagglutination de *N. gonorrhoeae* filamenteux, type 1 et 2, en utilisant diverses hématies. Les hématies humaines O Rh-négatives et les hématies de volailles présentèrent le phénomène d'hémagglutination. Cette activité fut complètement détruite par chauffage pendant 1 heure à 85° C., mais ne fut pas empêchée par la mannose D. On obtint également une inhibition de l'agglutination apparemment spécifique, par le sérum de lapin anti-gonocoques.