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Bacterial Hemagglutination by *Neisseria gonorrhoeae*

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Direct bacterial hemagglutination was investigated with 20 clinical isolates of *Neisseria gonorrhoeae*. The hemagglutination tests were performed by both a macrotechnique with glass slides and a microtechnique with autotrays. Only organisms from type 1 or type 2 colonies caused hemagglutination. There was no statistical difference at a 10% or higher level in hemagglutination powers of type 1 and type 2 organisms, of male urethral and female cervical isolates, and of the eight major human blood types (ABO-Rh). Of seven erythrocyte species tested, only human cells were agglutinated. D-Mannose did not prevent the agglutination. Rabbit antigonococcal serum and high-titer antigonococcal human sera inhibited the hemagglutination. The results suggest that pili are the mediators of hemagglutination and that their specific agglutination of human erythrocytes may be a correlate of their adherence to human mucosal cells in natural infection. Also, although the procedure is presently insensitive, it is possible to detect human antigonococcal antibody by inhibition of direct bacterial hemagglutination.

Direct bacterial hemagglutination was first described in 1902 by Kraus and Ludwig (12), who observed coarse clumping of erythrocytes (RBC) with isolates of vibrios and staphylococci. The mechanism of this reaction was not elucidated until pili (fimbriae) were demonstrated with electron microscopy by Anderson (1), Houwink (6), and Houwink and Van Iterson (7). In 1955, Duguid et al. (5), working with *Escherichia coli*, demonstrated the absence of pili in non-hemagglutinating strains and the presence of pili in hemagglutinating strains. However, three strains without pili produced weak hemagglutination reactions that varied according to the source of the RBC. The inhibition of shigella hemagglutination by antisera specific for piliated shigella provided further evidence that pili are the mediators of hemagglutination (4). Brinton (2) demonstrated hemagglutination activity with a concentration of isolated pili as small as 0.5 μg/ml. Other cell constituents did not produce hemagglutination. In 1966, Duguid et al. (3) discussed the seven types of pili on the basis of appearance, species of RBC agglutinated, presence of mannose-sensitive or -resistant hemagglutinin, and ability to absorb male-specific bacteriophages (F pilus).

Wistreich and Baker (20) used electron microscopy to demonstrate pili in *Neisseria catarrhalis*, *N. perflava*, and *N. subflava*. Although hemagglutination was related to the presence of pili, the various species reacted differently. Recent investigations demonstrated the presence of pili on type 1 and type 2 organisms of *N. gonorrhoeae* and the absence of pili on organisms of types 3, 4, or 5 (9, 17). Since gonococci from colony types 1 and 2 are virulent and those from types 3 and 4 are avirulent (10), pathogenicity may be directly related to the presence of pili. One way in which pili could exert their effort is by promoting the adhesion of gonococci to mucosal cells. As was stated above, in other organisms pili appear to be the mediators of hemagglutination. Bacteria-mediated hemagglutination and bacterial attachment to mucosal cells may involve similar mechanisms. Therefore we decided to investigate the ability of the various colony types of *N. gonorrhoeae* isolates to produce hemagglutination.

MATERIALS AND METHODS

Organisms. Ten female cervical and ten male urethral isolates of *N. gonorrhoeae* were obtained from the DeKalb County Health Department clinic. CDC stock strain 2686 was also used. Colony type 5 of strain K243562 was obtained from the Neisseria Department, Statens Seruminstitut, Copenhagen (8).

Culture methods. Specimens were obtained directly from patients and cultured on GC base medium (Difco) with IsoVitaleX (BBL) and VCN (vancomycin-colistin-nystatin). These isolates and the stock strain of *N. gonorrhoeae* were repeatedly subcultured to isolate the four major colony types as described by Kellogg et al. (11). Colony type 5 of K243562 was cultured on a similar medium. Bacteria used for hemagglutination were from plates that were at least 99% pure for a particular colony type. Each isolate was characterized by colony morphology, Gram staining, oxidase reactivity, and sugar fermentation. To simplify terminology, we shall refer to cells from a given colony type, e.g., type 1, as T_1 cells.

Hemagglutination. RBC from freshly drawn citrated or heparinized blood were centrifuged ($800 \times g$ for 10 min) and washed three times with phosphate-buffered saline (PBS), pH 7.2, adjusted to a 3% (vol/vol) suspension, and refrigerated until used. The 3% suspension was used for the glass slide hemagglutination technique, and a 1:6 dilution in PBS (0.5%) was used for the microhemagglutination technique. Bacterial suspensions were prepared in PBS, and the optical density was determined turbidimetrically at 540 nm, 1-cm light path. It was determined that an optical density of 0.3 corresponded to 10^8 colony-forming units.

Glass slide hemagglutination was performed by mixing equal volumes (0.1 ml) of RBC and bacterial suspensions or PBS (control) in depressions on a glass slide and manually rotating the slide gently. Usually within seconds, hemagglutination with coarse clumping was readily visible to the naked eye (Fig. 1), but occasionally it took as long as 5 min. If glass slide hemagglutination results were positive for a particular isolate, the organism was tested by a microhemagglutination technique. This technique was performed in U-bottom clear autotrays (Canalco). Equal volumes (0.025 ml) of serially diluted (1:2) bacterial suspensions and RBC were mixed in each well. After 15 min, the autotray was centrifuged at $30 \times g$ for 3 min. The highest dilution with hemagglutination was recorded.

Each colony type of the 20 clinical isolates and the stock strain and of colony type 5 of strain K243562 was used for hemagglutination with human Rh^+ RBC. Two bacterial isolates were used with RBC of sheep, guinea pigs, mice, rabbits, chimpanzees, and goats. In addition, two isolates were used with the eight major human ABO and Rh blood types. The hemagglutination of three isolates was tested for mannose sensitivity with D-mannose (final concentration 0.75% [wt/vol] in PBS) (4).

The hemagglutination power (HP) of an isolate was calculated according to the method of Duguid and Gillies (4) as 10^{11} divided by the minimal concentration of bacteria producing hemagglutination. The minimal concentration of bacteria was computed by dividing the original bacterial suspension concentration by the titer from microhemagglutination.

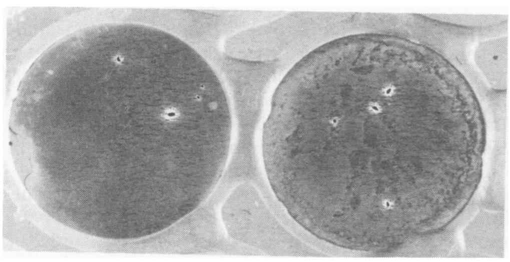

FIG. 1. Well on the right (photographed 30 s after gentle mixing) contains equal volumes of 3% RBC and a suspension of type 1 organisms. The well on the left is the control. Coarse clumping, which is characteristic of hemagglutination, is present.

Hemagglutination inhibition. The ability of rabbit and human antigonococcal sera to inhibit hemagglutination produced by T_1 cells from isolate 37 (HP = 200) was investigated. Rabbit antiserum was prepared by intramusclar injection of New Zealand white rabbits with 5×10^9 live T_2 cells per ml in Freund incomplete adjuvant followed in 1 month by an intramuscular injection of 10^{10} T_2 cells per ml. This latter injection was repeated every 2 weeks for 1 month. The rabbits were bled 1 week after the final injection, and the serum was absorbed with human RBC (ORh^+) to eliminate heterophile antibody. This antiserum was then used to test for hemagglutination inhibition. Human serum was obtained from patients with active gonococcal infection, and the antigonococcal titer was determined by complement fixation (CF) (14).

Equal volumes of a suspension of T_1 cells (10^9 cells/ml in PBS) and serial 1:2 dilutions of human sera were gently mixed on a glass slide. Rabbit antiserum was not diluted for the reaction. Saline was used in place of antiserum for a control. After 5 min, a 3% suspension of RBC was added, the slide was rotated manually, and the presence or inhibition of hemagglutination was recorded.

RESULTS

Hemagglutination occurred only between T_1 and T_2 cells and human RBC. The T_3, T_4, and T_5 cells did not produce hemagglutination. There were no reactions with sheep, guinea pig, mouse, rabbit, chimpanzee, and goat RBC. T_1 and T_2 cells of strain 2686, known from previous studies to be piliated (14), agglutinated human RBC; nonpiliated T_3 and T_4 cells failed to agglutinate human RBC. Hemagglutination with RBC of the eight human blood types (ABO-Rh) failed to reveal significant differences.

Table 1 contains the HP values of the 20 clinical isolates. The range of HP values was from 300 to 30,000. No significant differences (male isolates: $P = 0.365$; female isolates: $P = 0.368$) were observed between the HPs of T_1 and T_2 cells, whether derived from male urethral isolates or female cervical isolates. Although their mean HP was higher, type 2 colonies did not differ significantly (combined isolates: $P = 0.308$) from type 1 colonies in their power to induce hemagglutination. D-Mannose at a final concentration of 0.7% did not inhibit the hemagglutination reaction.

Table 2 contains the results of hemagglutination inhibition by rabbit antiserum and seven human sera. Rabbit antiserum as well as two human sera with high CF titers, 1:2,000 and 1:128, inhibited the reaction. Diluting these sera 1:4 and 1:2, respectively, continued to

TABLE 1. *Hemagglutination powers (HP) of T_1 and T_2 organisms from 20 clinical isolates*

Source	Isolate no.	HP[a]	
		T_1	T_2
Male urethral	45	500	1,500
	50	10,000	10,000
	26	2,000	1,500
	16	2,500	1,300
	24	5,000	20,000
	45	2,000	1,300
	8	10,000	1,000
	60	1,000	2,000
	63	500	1,000
	10	1,000	1,300
		3,450	*4,090*
Female cervical	49	1,500	2,000
	20	1,000	500
	28	500	300
	23	10,000	30,000
	35	5,000	1,000
	17	2,000	2,500
	19	5,000	10,000
	33	10,000	1,000
	14	2,500	500
	12	3,000	1,300
		4,050	*4,910*

[a] Italicized numbers are mean values.

TABLE 2. *Hemagglutination inhibition of T_1 cells by immunized rabbit sera and sera from patients with gonococcal infection*

Serum	CF titer	Hemagglutination (titer of inhibiting serum)
Rabbit		+[a]
Human #AG	1:2,000	+ (1:4)
Human #O15	1:100	−
Human #WJ-2	1:128	+ (1:2)
Human #1315	Negative	−
Human #1498	1:32	−
Human #1462	Negative	−
Human #1465	1:32	−

[a] +, Inhibition of hemagglutination; −, no inhibition of hemagglutination.

inhibit hemagglutination, but further dilutions did not. The control sera with negative CF titers did not inhibit hemagglutination.

DISCUSSION

Considerable evidence suggests that pili are the mediators of bacterial hemagglutination (2–5, 20). Our results support this contention. T_1 and T_2 cells of *N. gonorrhoeae* induce hemagglutination, whereas T_3, T_4, and T_5 cells do not. The only known ultrastructural difference between the cells in these colony types is the presence of pili on T_1 and T_2 cells and their absence on T_3, T_4, and T_5 cells (9, 17). Strain 2686, shown by electron microscopy to possess pili only on T_1 and T_2 cells, demonstrated hemagglutination only with these cell types. Jephcott et al. (9) found that pili were more abundant in T_2 than in T_1 cells. In our study, T_2 cells of clinical isolates did have a higher mean HP than T_1 cells. However, this was statistically insignificant because of the wide variance in the data. Zones of adhesion which correlate with gonococcal autoagglutination are probably not the mediators of this reaction because of their abundance in T_3 cells and the absence of hemagglutination with these organisms (17). In our study, D-mannose had no effect on hemagglutination with any of the isolates tested. D-Mannose was also not a factor in hemagglutination with other piliated species of *Neisseria* (20).

The microtechnique of direct bacterial hemagglutination may be a sensitive method of defining differences between isolates in their power to induce hemagglutination. Autotray contents were spun down after 15 min of reaction in order to prevent autoagglutination of bacteria. The duration of centrifugation at a given relative centrifugal force was critical in obtaining reproducible results. Type 2 organisms were especially difficult to work with because of their tendency to agglutinate rapidly. This may be one reason why type 2 organisms did not have a significantly higher HP than type 1 organisms, although they are thought to have more pili (9).

Since only piliated *N. gonorrhoeae* infect human volunteers (10), pili may be related to pathogenicity. Perhaps pili enhance the organisms' ability to adhere to mucosal surfaces. thereby making it difficult for them to be washed away by secretions and urine flow. Adhesion may also facilitate intracellular localization of gonococci within epithelial cells. The adherence to and agglutination of RBC by *N. gonorrhoeae* may be a correlate of its adherence to mucosal surfaces.

In 1973, Punsalong and Sawyer (15) investigated hemagglutination with a clinical isolate obtained from our laboratory at the Center for Disease Control. After 16 h, they examined settling patterns for the presence of hemagglutination. They also concluded that type 1 and type 2 gonococci produced hemagglutination but type 4 gonococci did not. Type 3 cells were not used. However, they observed abnormal settling patterns with rabbit, human, guinea pig, sheep, and chicken RBC types. By means of the macrotechnique used by numerous investigators to demonstrate hemagglutination in the past (3–5, 12, 16, 18, 20), we demonstrated hemagglutination only with human RBC. The reasons for the discrepancy between the two studies are not readily apparent. However, using our microtechnique, we found that it was necessary to centrifuge the autotrays within minutes to prevent autoagglutination of the gonococci, which may influence the settling patterns. When the contents of the autotrays were left to settle overnight and were not spun down, settling patterns indicating hemagglutination were present even with type 3 and type 4 organisms.

Recently, Waitkins (19) demonstrated hemagglutination by T_1 or T_2 cells of *N. gonorrhoeae* by using tanned human and fowl RBC. In this study guinea pig, horse, mouse, rabbit, rat, or sheep RBC did not agglutinate with T_1 or T_2 cells. Also, rabbit antigonococcal serum inhibited the hemagglutination. These results are similar to ours.

Human sera with high antigonococcal CF titers from patients with active gonococcal infection inhibited hemagglutination by *N. gonorrhoeae*. A minimal concentration of organisms (10^9 cells/ml in isolate 37 with an HP of 200) was used in order to increase sensitivity. Nevertheless, only sera with high titers of antigonococcal antibody inhibited the reaction. Since pili are mediators of hemagglutination, it is probable that the anti-pili antibody of these human sera specifically inhibits hemagglutination. In the future, it may be possible to increase the sensitivity of this system by using small concentrations of purified gonococcal pili. Increased sensitivity and an understanding of the specificity of the reaction could make bacterial hemagglutination inhibition a useful technique to detect antigonococcal antibodies.

With our technique, only human RBC were agglutinated when combined with piliated *N. gonorrhoeae*. Since man is the only natural reservoir for *N. gonorrhoeae*, it is tempting to postulate mechanisms of pathogenesis. However, type 1 and type 2 organisms did not agglutinate RBC from a chimpanzee, an animal that can be infected experimentally even though it is not a natural reservoir for gonorrhea (13). There may be some subtle differences between chimpanzee and human experimental infections. Kraus et al. (J. Clin. Invest., in press) studied two *N. gonorrhoeae* isolates whose type 1 and type 2 organisms were unable to infect chimpanzees. Factors other than pili may prove to be important for the infection of this animal with *N. gonorrhoeae*.

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