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Neisseria gonorrhoeae and ABO Isohemagglutinins

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Depending on the ABO blood group, gonorrhea may affect the titers of isohemagglutinins compared with those of uninfected controls. The isohemagglutinin titers in group O patients were significantly increased ($P < 0.001$) against erythrocytes A, B, and AB. In group A patients, only the titer against AB erythrocytes was significantly increased. In group B patients, the titer against AB erythrocytes was significantly lower ($P < 0.001$) as compared with that in sera of healthy persons. In six of eight volunteers, an increase in isohemagglutinin titer was observed after an injection of small doses of killed gonococci. However, guinea pigs, rabbits, or small monkeys, i.e., species for which gonococci are not pathogenic, when immunized with gonococci either did not form ABO hemagglutinins or did so with very low titers. In white gonorrhea patients, there was a significantly higher frequency of group B individuals over those with group A, AB, or O. No such correlation was found in black patients. Isohemagglutinins from human sera could be absorbed by cultured gonococci. The implications of these findings in the pathogenesis of gonococcus infection and the problems associated with vaccine development are briefly discussed.

In a previous paper we described that in human pus, degranulation in phagocytes containing clusters of gonococci appeared incomplete and may have been inhibited (9). The organelles of these phagocytes, presumably macrophages, surrounded the cocci very closely. A study of ultrathin sections suggested that some organelles of the phagocyte have a tendency to fuse with gonococci, and therefore a similarity of the surface of the gonococci and human cell membranes was considered a possible explanation.

In preliminary experiments using antisera prepared in rabbits, no antigenic cross-reactivity was demonstrated between human peripheral phagocytes and gonococci (manuscript in preparation). However, a study of five patients with gonococcal septicemia showed that their sera contained gonococcal antibodies as demonstrated by complement fixation, agglutination, immunofluorescence, and immune electron microscopy, and two of these had high titers of ABO isohemagglutinins (≥1:2,048). These results, together with the observation that the isohemagglutinins from sera could be absorbed by gonococci, stimulated a study of isohemagglutinin activity of the sera of patients and of persons voluntarily vaccinated with gonococci.

MATERIALS AND METHODS

Human sera. Human sera were obtained from: (i) 177 gonorrhea patients (124 males and 53 females, 17 to 42 years old) in whom the clinical diagnosis had been confirmed by the positive culture of gonococcus; (ii) 8 male volunteers immunized with an experimental gonococcal vaccine (P. D. Walker and D. S. Freestone, unpublished data) (this consisted of a formolized suspension of the prevalently T_1-T_2 colonial form of gonococci from a liquid medium, resuspended to a density of 10^9 colony-forming units and containing Alhydrogel [Superflos Export Co., Copenhagen; 0.7 mg of aluminium/ml]. The volunteers were injected with 0.03 to 0.1 ml of vaccine subcutaneously and boosted with 0.2 or 0.4 ml on one or several occasions [see Table 4]); and (iii) 130 adults 18 to 48 years old (92 males and 38 females), none of whom admitted to a history of gonorrhea, who were used as controls. Of the latter group, 104 were employees of The Wellcome Research Laboratories and were therefore more likely to be exposed to various bacterial, parasitic, and/or viral antigens than were the others. All had been immunized and boosted with Tetanus Vaccine Adsorbed (Wellcome), and their antitoxic levels were checked regularly. Nineteen of them had also been boosted every 3 years with typhoid vaccine (Wellcome). A further 26 sera were from car workers who had volunteered in a Tetanus Vaccine Adsorbed trial. Although tetanus immunization in various forms is supposed to be a

source of high titers of isohemagglutinins (7), no significant difference in the isohemagglutinin titers in their sera before and after the trial was seen, neither was there a significant difference between the laboratory and car workers. Tetanus Vaccine Adsorbed is widely used in Britain.

Animal sera. Animal sera were obtained from (i) rabbits (White Ranch) after immunization with gonococci (10), (ii) guinea pigs (Duncan Hartley) after immunization or after infection of subcutaneous chambers (16), or (iii) monkeys (*Erythrocebes patas*) before and 10 days after immunization with two intramuscular doses (2 ml) of alum-precipitated gonococci containing 10^9 colony-forming units/ml injected 2 weeks apart.

Hemagglutination. All sera were stored at −20°C until used. Sera were diluted with phosphate-buffered saline, pH 7.2, in twofold steps using Takatsy loops (Microtitrator) and plastic trays (U-shaped wells; IS-MRC-96, Linbro). To the dilutions were added equal volumes of 0.5% (vol/vol) suspensions of washed fresh human erythrocytes (RBC) of known ABO group. The trays were sealed with self-adhesive membranes and incubated for 1 h at 37°C and then overnight at 4°C, after which the results were read. All sera from controls and from patients having high titers of isohemagglutinins were repeatedly titrated with RBC of different donors during the gonococcal absorption experiments. Using RBC from different donors, the titers obtained varied within one step of the twofold dilution, and there was no difference when the donor was rhesus positive or negative. RBC of one AB, Rh+ donor out of seven AB cells tested, however, reacted with some sera of control as well as gonorrhea groups with exceptionally high titers compared with tests performed with RBC of other donors. These RBC were not used for the screening tests.

Blood groups. Blood groups were determined by slide agglutination of washed RBC using anti-A and anti-B blood-grouping sera obtained from the Blood Group Reference Laboratory, London.

Absorption of ABO antibodies by gonococci. A standard loopful of gonococci from GC plates (10) (approximately 3 mg, wet weight) was resuspended in a mixture of 0.1 to 0.15 ml of serum and an equal volume of phosphate-buffered saline. After 30 min at 37°C, the mixtures were centrifuged at 10,000 × *g* at 4°C for 30 min. The absorption of the remaining supernatant (less 0.025 to 0.05 ml necessary for titration) was repeated several times.

Strains of gonococci used for absorptions. These strains were isolated from patients cultured on GC plates (9) and were stored frozen as primary cultures (16). From these, subcultures were made when necessary. The T_4 colonial form of strain 2686 was kindly supplied by T. Buchanan (University of Seattle); in other strains the T_4 colonies were isolated on subcultures.

RESULTS

Agglutination of human RBC by sera of patients with gonorrhea. When collection of sera from patients with gonorrhea was initiated, the importance of the ABO blood group was not recognized, and therefore approximately two-thirds of the serum samples were from donors whose ABO group had not been determined directly. However, since serum isohemagglutination is dependent on the blood group (O donors agglutinate A, B, and AB cells; A donors agglutinate B and AB cells; B donors agglutinate A and AB cells; and AB individuals do not form ABO isohemagglutinins), it was possible to determine the blood group of the donor from the hemagglutinating activity of his serum. Using this indirect method, the distribution of blood groups of the control group was determined and found to be consistent with the distribution of blood groups in the population of Southern England (Table 1) except for those sera tabulated as "AB," in which ABO isohemagglutinin titers were too low to be grouped in this way but could be shown to be not all of blood group AB donors when checked by the direct blood-grouping method.

The frequencies of the isohemagglutinin titers of the sera in the control group and the

TABLE 1. *Distribution of blood groups as determined indirectly from the sera of patients with gonorrhea and those of a control group*

Blood group	Patients with gonorrhea			Controls (4)	Blood group distribution (%) in Southern England[a] (5)	Blood group distribution (%) of West Indians in England[b] (6)
	All (1)	White (2)	West Indians (3)			
A	59 (33.3)[c]	30 (41.7)	29 (27.6)	50 (38.5)	44.70 (A_1 + A_2)	23.80
B	35 (19.8)	12 (16.7)	23 (21.9)	9 (6.9)	8.59	18.55
"AB"[d]	8 (4.5)	5 (6.9)	3 (2.9)	9 (6.9)	3.26 (A_1B + A_2B)	4.67
0	75 (42.4)	25 (34.7)	50 (47.6)	62 (47.7)	43.45	52.98

[a] Race and Sanger (12).
[b] Mourant et al. (8).
[c] Numbers in parentheses indicate percentages.
[d] The group designated "AB" includes sera of individuals that did not form sufficient isohemagglutinins, but some of them were of other blood groups.

TABLE 2. *Comparison of the frequencies of isohemagglutinin titers in the sera of the gonorrhea group and a control group*

Blood group	Patient group	No.	Agglutinated RBC	No. of subjects with HA titers of:					Results of U tests
				0	2-8	16-64	128-512	1,024	
A	Gonorrhea	59	B	1	18	27	8	5	No significant difference
	Control	50		0	8	31	11	0	
	Gonorrhea	59	AB	0	20	20	13	6	Gonorrhea group had significantly higher titer ($P < 0.001$)
	Control	50		2	30	18	0	0	
B	Gonorrhea	35	A	0	3	17	10	5	No significant difference
	Control	9		0	0	5	4	0	
	Gonorrhea	35	AB	22	7	4	2	0	Control group had significantly higher titer ($P < 0.001$)
	Control	9		0	4	2	3	0	
O	Gonorrhea	75	A	1	2	13	38	21	
	Control	62		0	6	40	15	1	
	Gonorrhea	75	B	0	9	34	22	10	Gonorrhea group had significantly higher titers ($P < 0.001$) with RBC of groups A, B, and AB
	Control	62		0	21	36	5	0	
	Gonorrhea	75	AB	0	4	30	30	11	
	Control	62		0	22	31	8	1	

gonorrhea group are shown in Table 2. In general, the values obtained for control sera are comparable with the values resulting from natural sensitization described elsewhere (7), and only 1 of 130 sera had a titer ≥1,024, whereas in the gonorrhea group 32 of 177 sera (18%) had a titer ≥1,024; 93 (52.5%) had a titer of 128, whereas in the control group only 31 (23.8%) had a titer of 128.

The antibody titers of gonorrhea and control groups of similar blood groups were compared, and Mann-Whitney U tests were performed to evaluate the data. The following results were obtained (Table 2): (i) blood group O patients had significantly higher titers ($P < 0.001$) against RBC of groups A, B, and AB than did the controls; (ii) between patients and controls classified as blood group A there was no significant difference in the titer of antibodies against group B cells, whereas against group AB cells a significant difference ($P < 0.001$) was found, the gonorrhea group having higher titers than the controls; and (iii) between patients and controls classified as group B there was no significant difference with regard to antibodies against group A RBC. However, it was surprising that the majority of the sera in the gonorrhea group (22 of 35) did not agglutinate AB blood cells, whereas all 9 control sera had titers ≥2. Indeed, control sera had significantly higher titers ($P < 0.001$) than did sera of the gonorrhea group.

None of the sera agglutinated group O RBC. There was no difference in the isohemagglutinin titers between men and women and between whites and blacks.

No simple relationship could be established between the duration of the disease and the isohemagglutinin titer. Some patients had high titers within a few days of infection, whereas others had relatively low titers when infected for several weeks. Few sera from homosexuals with positive rectal cultures had titers ≥1:128. In one case of gonorrhea of the oropharynx the titer was 1:2,048. The kinetics of changes in the titer of ABO antibodies during the time course of the disease is under investigation.

It has been shown (1) that the occurrence of gonorrhea was significantly higher in black patients of group B than in those of group A, AB, or O. In this study the patients were both white and black. Since the control sera originated from a white population, comparisons were made between column 2 (white patients) and column 4 (controls) and between column 3 (black patients of West Indian origin) and column 6 (the normal distribution of blood groups in West Indians) in Table 1.

The number of group B subjects in white English gonorrhea patients was more frequent than in the control population. Indeed, using χ^2 and rearranging the data in Table 1 into B and non-B subjects, the difference appears also significant ($0.05 > P > 0.02$). Virtually identical significance was obtained using the more appropriate maximum-likelihood estimate (17), which is unaffected by the number of gonorrhea and control subjects. In black patients, on the

other hand, the overall distribution of blood groups was similar to the normal distribution of blood groups of West Indians living in Britain, group B not being significantly higher. Hence the argument that there is a significantly increased risk of gonorrhea in group B subjects appears to be weak, and the difference may be due to non-representative sampling.

Absorption of ABO antibodies from human sera. ABO isohemagglutinins in sera from both patients and controls could be absorbed by either RBC or gonococci taken from GC plates. There was no difference in the capacity for absorption using either T_1–T_2 (so-called virulent) or T_3–T_4 (avirulent) colonial forms of gonococci. There was also no difference when the infecting strain was used for the absorption (Table 3). It is apparent that with only one absorption the anti-A antibodies were considerably reduced, whereas repeated absorptions were necessary for anti-B antibodies. This difference in the absorbing capacity of gonococci was evident when sera containing similar titers of either anti-A or anti-B were compared. With sera containing both antibodies (anti-A,B from group O subjects) the difference was not as apparent; indeed, both anti-A and anti-B titers decreased to a comparable extent after repeated absorptions. Although all 10 gonococcal strains tested absorbed ABO antibodies, subcultures of strain 38 and the T_4 colonial form of strain 2686 appeared the most consistent in absorbing capacity. No strain was found that would preferentially absorb one or the other antibody.

Agglutination of human RBC by sera of volunteers immunized with gonococci. Three volunteers responded to the vaccine with high isohemagglutinin titers (Table 4, no. 5, 6, and 10). In three volunteers (no. 4, 7, and 11) the titer was low (up to 1:128). Two volunteers (no. 8 and 9; not shown in the table), although of blood group A, did not form isoagglutinins after two doses (0.1 and 0.4 ml, respectively), their titers remaining 1:8 or less. It is apparent from the table that booster doses generally increased titers. All volunteers had measurable amounts of gonococcal antibodies after vaccination.

Surprisingly, although the sera of group B gonorrhea patients agglutinated group A RBC, quite often to a high titer, the agglutination of AB cells was significantly lower than in the sera of controls of a similar blood group. This paradoxical behavior of "anti-A" antibody could be seen also in volunteer no. 11, whose post-vaccination serum did not agglutinate in a routinely visible titer AB RBC using RBC from several donors. Indeed, serum samples of volunteer no. 11 taken more than 2 years after the

TABLE 3. *Absorption of anti-A and anti-B serum antibodies by gonococci*

Serum	Absorbing antigen (gonococcal strain, colonial form)	Titer								
		Anti-A:A RBC system					Anti-B:B RBC system			
		Before absorption	After absorption				Before absorption	After absorption		
			1st	2nd	3rd	4th		1st	2nd	3rd	4th
Anti-A[a] blood grouping	102, T_1		0								
	104, T_2	128	0								
	38, T_2		0								
Anti-B[a] blood grouping	102, T_1							128	64	64	16
	104, T_2						128	128	128	32	16
	38, T_2							32	32	8	4
Control no. 112	38, T_2							1024	128		
	38, T_4						1024	256	256		
	2686, T_4							1024	64		
Patient no. 158	38, T_2		16	16	8						
	38, T_4	1,024	32	16	16						
	2686, T_4		64	16	8						
Patient no. 185	38, T_2		64	32	8			32	32	8	
	38, T_4	4,096	32	32	8		1,024	32	16	8	
	2,686, T_4		32	16	8			32	16	8	
Volunteer no. 5	38, T_2		16	16				8	0		
	38, T_4	512	8	8			64	4	0		
	2,686, T_4		32	16				4	0		
Patient no. 173	173, T_2						256	128	64		
Patient no. 174	174, T_2	256	64	64	32		64	16	4		

[a] These sera were adsorbed with identical cultures and tested the same session.

TABLE 4. *ABO isohemagglutinin titers in human volunteers after immunization with an experimental gonococcal vaccine*

Time in weeks	Titer with given RBC group[a]																	
	4; A; 0.03[b]			5; O; 0.03, 0.1			6; O; 0.1, 0.2, 0.2			7; A; 0.1, 0.2			10; A; 0.1, 0.4			11; B; 0.1, 0.4		
	A	B	AB	A	B	AB	A	B	AB	A	B	AB	A	B	AB	A	B	AB
0	0	0	0	0	4	16	128	32	32	0	16	16	0	8	4	4	0	0
1				8	32	16	2,048	256	512	0	32	32	0	4	4	64	0	0
2	0	2	8				1,024	128	512				0	128	128	128	0	0
3													0	256	64	32	0	0
4	0	2	32	512	64	128	1,024	128	64	0	128	64						
5				128	32	128				0	32	32						
6							>4,096[d]	256	1,024	0	16	16						
7										0	32	128	0	64	128	16	0	0
8													0	256	256	128	0	0
9				512	64	128												
10													0	>4,096	1,024	128	0	0
13							1,024	256	1,024				0	1,024	512	128	0	0
14				256	32	128												
17				512	128	256												
18				256	64	256												
20							>4,096	128	4,096									
21				32	64	128												

[a] Sera from individual volunteers were examined on the same day. Sera from volunteers 4 through 8 and 9 through 11, respectively, were examined with RBC of the same RBC donors.

[b] Volunteer number; blood group; vaccine dose (in milliliters).

[c] Day of the first vaccine injection.

[d] Underlined numbers are titers of sera taken 7 to 14 days after boosting.

first dose of vaccine still retained this paradoxical behavior, whereas the agglutination titer with group A cells remained at 1:128 to 1:256. The abnormality resided in the sera rather than in the RBC, since with the AB cells from the same donor, serum from volunteer no. 10 caused agglutination whereas serum from volunteer no. 11 did not (Table 4).

Failure to agglutinate human RBC by sera of immunized experimental animals. In contrast to humans, experimental animals responded to contact with gonococci in a different way. Eight guinea pigs and seven of nine rabbits did not form ABO-agglutinating antibodies. In the other two rabbits the titer was 1:8 to 1:16. All the animals had high titers of antibodies against gonococci as detected by complement fixation, agglutination, and immune electron microscopy (10). Of five monkeys (*E. patas*), only two that had low titers of natural antibodies to O, B, and AB RBC (1:8 to 1:16) showed a slight increase in titer against B and AB cells (1:16 to 1:32), but also a slight decrease from 1:8 to 1:2 with O cells after immunization with gonococci. In none of these species, therefore, did immunization with gonococci result in the induction of high hemagglutinin titers comparable to those observed in some patients with gonorrhea or in volunteers after vaccination. It should be stressed that all immunized animals received, on a body-weight basis, 200 to 1,400 times more antigen than did the humans.

DISCUSSION

It is known that human sera contain natural isohemagglutinins in low titers, which have resulted from previous sensitization by various antigens of microbial and animal origin (2, 14). The influence on isohemagglutinin titers of gonococcal infection or immunization with gonococci has to our knowledge not yet been described. According to the studies described here, *Neisseria gonorrhoeae* may be a powerful stimulus of isohemagglutinin production in humans. Several gram-negative bacteria are known to be inducers of isohemagglutinins, in most cases anti-B (14). In gonococci, the induction of isohemagglutinin titer depends on the blood group of the patient. However, there is a discrepancy in the hemagglutinin specificity induced by gonococci, which is apparent in subjects of blood groups A and B. For instance, sera agglutinating group A RBC failed to agglutinate group AB RBC (Table 4, volunteer 11), and there was a difference between A,AB and

B,AB agglutinability by sera of gonorrhea patients (Table 2). Although the available data do not explain this paradoxical behavior, two possibilities must be considered. It may be that another so-far unrecognized blood group-specific substance is also involved in the reaction or that the antigenic similarity in gonococci to RBC substances is incomplete but greater with the A substance than with B, involving also some sites of the substances that are common to both A and B. This latter explanation is based on the observation that gonococci absorb anti-A and anti-A,B antibodies from O sera more easily than anti-B antibodies from A sera, a phenomenon similar to that observed in the study of anti-A and anti-B cross-reacting antibodies (12). This is under further investigation.

The increase of isohemagglutinins after administration of the vaccine was dramatic in some volunteers considering that a small dose of the vaccine, 30 to 100 μl for the first dose and 100 to 400 μl for the second, was used. This corresponded to 3.10^7 and 10^8 to 4×10^8 cells, approximately 2 to 3 logs less than the number of cells in a single human dose of other vaccines such as *Bordetella pertussis* (20×10^9), and *Vibrio cholerae* (8×10^9) (according to World Health Organization requirements).

Although the isohemagglutinins induced by some parasitic infections are suggested as possible etiological agents of ABH erythroblastosis (3), this does not appear to be the case with isohemagglutinins induced by gonococci since these were sensitive to dithiothreitol treatment and hence were predominantly of the immunoglobulin M class, which does not penetrate the placental barrier. However, this may not be the case after a complete course of immunization, for which presumably much higher doses would be necessary considering that several or many immunotypes must be expected in gonococci (4, 10, 16).

Other unforeseen incompatibility problems could arise. For instance, anti-A and anti-B antibodies are present in cervical secretions of about 20% of women (12). It may be, therefore, that the cervical secretion of a woman immunized by a gonococcal vaccine could agglutinate incompatible sperm to such an extent that infertility induced by high titers of cross-reacting antibodies would develop (13). Such questions must be considered before a gonococcal vaccine is released for general use, and they certainly present problems for the producer. Could, for instance, such a vaccine remain effective if the isohemagglutinin-inducing antigen were removed?

It was surprising to find that the three animal species tested failed to respond to gonococcal antigens by the formation of antibodies agglutinating human RBC. This is not necessarily the rule for experimental animals in other circumstances. For instance, infection or immunization of selected rabbits by *Ascaris lumbricoides* results in the production of high titers of anti-A agglutinins (11). It appears that possible gonococcal vaccines must be tested on human volunteers to determine the presence and concentration of isoagglutinogens.

The suggested increased risk to gonorrhea in blood group B subjects (1) appeared to be attractive when a similar increase in the white British population was observed in this study. However, the results on West Indians did not confirm this hypothesis. Bearing in mind that the reference data used for West Indians were shown by the calculation of ABO gene frequencies to be a representative sample and that these British immigrants are of less mixed descent than, for instance, the North American Negroes (6), the heterogeneity of the West Indian patients may be rather low and may have a higher degree of statistical significance than the combined data for whites. Therefore, it appears evident that data must be carefully collected on well-defined ethnic groups over a wider range of samples before a definite conclusion on intrinsic susceptibility to gonorrheal infection based on blood group difference can be made.

Since gonococci are pathogens only in humans, it is tempting to speculate as to whether the ability to form isohemagglutinins by humans is a prerequisite for the development of clinically typical disease. A simple causal relationship seems unlikely in view of our finding that group AB gonorrheal patients do not form ABO isoagglutinins. In addition, some patients with blood groups O, A, and B, respectively, suffering from gonococcal septicemia or uncomplicated gonorrhea and also some volunteers injected with gonococcal vaccine do not respond with increased isohemagglutinin formation.

The distribution of the ABH substance, apart from blood cells, blood serum, endothelial cells, and some nonmucous glands, is limited to mucous membrane secretions, mucous acini, mucosa of the endocervix, fallopian tubes, prostate secretions, and seminal vesicles, urine, and the transitional epithelia of the lower urinary tract (2, 15). All the tissues for which the gonococcus has an affinity contain the ABH substance. It is possible, therefore, that both the glycolipid or glycoprotein form of ABH substance (2) and the corresponding antigenic determinant from gon-

ococci may share an affinity for certain tissues. One may also speculate as to whether the antigenic similarity of gonococci with the surrounding tissue is great enough to hamper immune recognition with consequent defects in antibody formation or to trigger defense mechanisms directed against targets unimportant for gonococcal survival. This would help to explain the apparent lack of immune resistance to gonococcal reinfection (5).

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