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Natural and Immune Human Antibodies Reactive with Antigens of Virulent *Neisseria gonorrhoeae*: Immunoglobulins G, M, and A

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Natural and immune human antibodies reactive with heat-labile and heat-stable antigens of virulent *Neisseria gonorrhoeae* were studied by use of an indirect fluorescent-antibody (IFA) procedure. The immunoglobulin class of the reactive antibodies was identified by using fluorescein-conjugated antisera specific for human IgG, IgA, or IgM in the IFA procedure. The effects of heat and mercaptoethanol on IFA reactivities were also studied. It appeared that antibodies of the IgG, IgM, and IgA classes present in the sera of both infected persons (immune antibodies) and normal persons with no history of gonococcal infection (natural antibodies) react with heat-stable somatic antigens. Immune IgG antibodies, however, were distinguishable from natural IgG antibodies by their ability to recognize heat-labile surface antigens. The distinction between natural and immune IgM antibodies was less obvious. IgM antibodies from both infected and normal persons appeared to react with heat-labile antigens. Some, but not all, infected persons had immune IgA antibodies to heat-labile as well as to heat-stable antigens. Treatment of sera with mercaptoethanol had no effect on IgG antibodies. The IFA activity of IgM antibodies was decreased, but not abolished. The effects of mercaptoethanol on IgA antibodies were variable. Some sera showed a decrease in IgA titer, and others showed an increase in IgA activity to certain antigens. Immune IgG antibodies were more resistant to heating than were natural IgG antibodies. Natural and immune IgM antibodies appeared equally sensitive to heating. IgA activity, on the other hand, was increased by heating sera at 60 C, but was decreased at higher temperatures. Thus, it appears that natural and immune human IgG antibodies to *N. gonorrhoeae* may be distinguished by their interactions with heat-labile antigens and by their resistance to heating.

Deacon and his colleagues (6), using direct fluorescent staining and agglutination procedures, have described heat-labile surface antigens and heat-stable somatic antigens of *Neisseria gonorrhoeae*. The surface antigens were preserved by treatment with Formalin and were destroyed by heating.

It was found in a recent study (3) that the sera of normal uninfected humans possess natural antibodies reactive with heat-stable antigens of gram-negative bacteria. These natural antibodies appeared to develop early in life and were found to be present in all three major immunoglobulin classes, IgG, IgM, and IgA (1).

In the present study, natural antibodies reactive with virulent *N. gonorrhoeae* were contrasted with immune antibodies, those found in the sera of infected humans. An indirect fluorescent-antibody (IFA) procedure employing fluorescent antisera specific for IgG, IgM, and IgA was used to identify the immunoglobulin class of antibodies reactive with the heat-labile and heat-stable antigens of virulent *N. gonorrhoeae*.

The effects of 2-mercaptoethanol and heat on the IFA reactivity of the immunoglobulins were also studied.

MATERIALS AND METHODS

Immune sera. Sera from patients with gonococcal arthritis, urethritis, and cervicitis were collected with the aid of T. F. Sellers, Jr., and J. Tatum at Grady Memorial Hospital, Atlanta, Ga.; T. E. Billings and P. Utley at the Fulton County Health Department,

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Atlanta, Ga.; and D. S. Kellogg, Jr., and J. D. Thayer of the Venereal Disease Research Laboratory, National Communicable Disease Center. Gonococcal infection was documented by culture of *N. gonorrhoeae* from the patients.

Normal sera. Sera from normal young children were supplied by the Serum Bank of the National Communicable Disease Center. Normal adult sera were drawn from professional colleagues who did not have a history of previous gonococcal infection.

Gonococcal antigens. The virulent colony type 1 (T1) of *N. gonorrhoeae* strain F26 (11; Kellogg et al., *in preparation*) was used in these studies. The virulence of this organism was confirmed by its ability to cause urethritis in human volunteers (Kellogg et al., *in preparation*; Cohen et al., *in preparation*). Selected bacteria were cultured on the surface of agar plates made with G C Medium Base (Difco) plus a defined supplement (Kellogg et al., *in preparation*). After 16 to 18 hr of incubation in candle jars at 36 to 37 C, bacteria were harvested into phosphate-buffered saline, *p*H 7.2 (PBS). The resulting bacterial suspension was adjusted to a concentration of approximately 2×10^9 to 4×10^9 bacterial units/ml by using a standard optical density reading on a Coleman Nepho-colorimeter.

Heat-labile (F) and heat-stable (121 C) antigens were prepared from standard bacterial suspensions for use in the IFA procedure and for absorptions.

Formalin-treated bacteria (F). A modification of the method of Hess et al. (9) was used to fix *N. gonorrhoeae* cells to glass slides. One drop (0.04 ml) of a 1:10 dilution of the standard suspension was dried at 37 C on a circumscribed area of a glass slide. The slide was immersed in 3% Formalin in PBS for 10 min, washed for three 5-min periods in fresh distilled water, and stored in a sealed container at 4 C until use. F cells for use in absorption studies were prepared by exposing the standard suspension to 3% Formalin for 10 min. The suspension was then centrifuged at 10,000 rev/min for 2 min, and the bacterial cells were resuspended to the original volume in fresh PBS.

Bacteria heated at 121 C (121 C). The heat-labile surface antigens were destroyed and the full reactivity of the heat-stable antigens was obtained by heating a standard bacterial suspension to 121 C for 2 hr in an autoclave. After heating, the bacteria were sedimented by centrifugation and were resuspended in fresh PBS to the original volume. The 121 C antigens were stored at 4 C for no more than 5 days before use. On the day of IFA testing, the suspension was diluted 1:10 in PBS and one drop was dried at 37 C on a circumscribed area of a glass slide. Figures 1 and 2 show the morphology and IFA staining of F and 121 C cells.

Fluorescent anti-immunoglobulin reagents. Fluorescein conjugated goat antisera specific for human IgG, IgM, and IgA were kindly supplied by E. Shanbrom and K. Lou of Hyland Laboratories, Los Angeles, Calif. The antisera were stored at −20 C in small amounts, which were thawed and diluted 1:40 in PBS just before each test. The specificity of these reagents in the IFA test system was confirmed by specific staining of purified 7*S* and 19*S* serum fractions and by cross-absorptions with such fractions. In addition, the reaction of these antisera in gel-precipitation tests was comparable to that of reference antisera to human immunoglobulins generously supplied by J. L. Fahey, J. H. Vaughan, F. A. Wollheim, and R. W. Williams, Jr. Fractionation of sera by gel-filtration through Sephadex G-200 was kindly performed by Alfredo J. Julian of the National Communicable Disease Center. A detailed report of these specificity studies is in preparation (Julian and Norins).

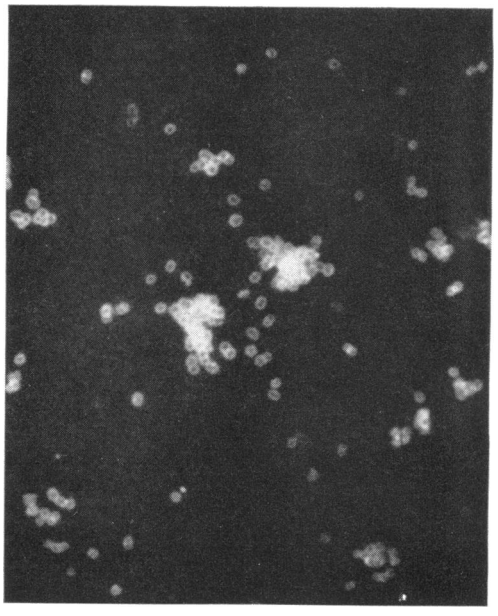

FIG. 1. *Indirect fluorescent staining of Formalin-treated (F) cells of virulent T1 Neisseria gonorrhoeae. Note that these cells stain predominantly at their periphery.*

IFA procedure. The IFA test was performed in the following manner. One drop of test serum diluted in PBS was placed over treated cells fixed to glass slides (see above) and incubated at 37 C for 30 min. The slides were washed, and the immunoglobulin class of antibodies binding to the treated *N. gonorrhoeae* cells was determined by incubating the antibody-bacterium complexes with the fluorescein-conjugated anti-immunoglobulin reagents. After further washing and drying, cover slips were fixed to the slides with buffered glycerol mounting fluid (*p*H 9), and the slides were coded. A fluorescence microscope (American Optical, Osram HBO-200 lamp, Corning 5113 and Schott GG 9 filters, 54 × oil immersion lens) with a dark-field condenser was used to examine the coded and unknown slides within 2 hr of preparation. The last dilution of serum showing fluorescence scored as 2+ (on a 1 to 4+ scale) was considered to be the titer of antibodies of the particular immunoglobulin class.

RESULTS

Reactivity of natural and immune immunoglobulins with heat-labile (F) and heat-stable (121

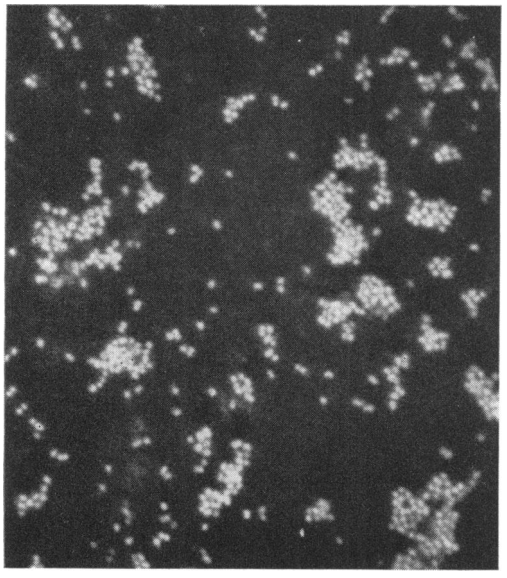

FIG. 2. *Indirect fluorescent staining of heat-treated (121 C) cells of virulent T1 Neisseria gonorrhoeae. Note that the heated cells appear smaller than the Formalin-treated cells and lack the capsule-like rim seen in the latter (Fig. 1).*

C) antigens. Table 1 includes the mean titers of natural antibodies reactive with heat-labile (F) and heat-stable (121 C) antigens in the sera of 10 normal adults and 10 children. The children were between the ages of 5 months and 2 years. The 10 adults were chosen because they had a reliable history of freedom from gonococcal infection. Sera sampled from the general adult population of a maternity ward and an orthopedic hospital demonstrated IgG, IgM, and IgA titers similar to those recorded for these representative normal sera.

It was found that natural antibodies of all three classes were reactive with antigens of *N. gonorrhoeae*. However, in addition to differences in titer, there was a difference in the relative reactivity of natural IgG, IgM, and IgA antibodies with heat-labile (F) as compared to heat-stable (121 C) antigens. Natural IgG antibodies in each person's serum were two to six times more reactive with heat-stable than with heat-labile gonococcal antigens. The mean IgG titer to the heat-stable (121 C) antigens was 1:64, and the mean IgG titer to the heat-labile (F) antigens was 1:16. The ratio of natural IgG titer with F to natural IgG titer with 121 C (F/121 C) was less than 1 in all normal sera. Natural IgM antibodies, on the other hand, reacted with both heat-labile (F) and heat-stable (121 C) antigens to about the same titers in each individual serum. The IgM F/121 C ratio, except for one juvenile serum, was at least 1 in all sera. The titers of IgG and IgM antibodies to the heat-labile (F) antigens were approximately equal in each serum. However, the titers of IgG antibodies to the heat-stable (121 C) antigens were generally found to be fourfold higher than the titers of IgM antibodies to the heat-stable (121 C) antigens.

IgA antibodies, except for one adult serum, reacted at titers of 1:2 with heat-stable (121 C) antigens only.

The reactivities of immune antibodies with F and 121 C antigens are also illustrated in Table 1. The immunoglobulin titers of five patients with gonococcal arthritis and six females who were chronically infected are shown. The antibody reactivities found in the sera of these patients were typical of those found in 10 other patients with arthritis and 20 other chronically infected females.

Immune antibodies were found to differ from natural antibodies in several aspects. In addition to an increase in titer of some immune antibodies, there was a significant difference between the patterns of immune and natural antibody reactivities with heat-labile and heat-stable antigens (F/121 C). This was most striking in regard to IgG antibodies. The IgG F/121 C values of the immune sera ranged from 2 to 16, whereas the IgG F/121 C values found in normal sera ranged from 0.06 to 0.50 (Table 1). In other words, the predominant serum IgG reactivity of infected persons was with heat-labile surface antigens (F). Natural IgG antibodies in normal sera, on the other hand, were primarily reactive with heat-stable somatic antigens (121 C) of *N. gonorrhoeae*.

It is noteworthy that, despite the increased titers of immune IgG antibodies to heat-labile (F) antigens, immune IgG antibody titers to heat-stable (121 C) antigens were approximately equal to titers found in the sera of normal persons (Table 1).

The reactivity of immune IgM antibody was less consistently elevated above normal than was immune IgG antibody. However, some patients did show increased IgM reactivity to heat-labile (F) antigens (Table 1).

IgA antibodies reactive with heat-stable (121 C), as well as heat-labile (F), antigens were present in immune sera at higher titers than in normal sera. In this respect, immune IgA appeared to differ from immune IgG and IgM antibodies (Table 1).

Specificity of antibodies reactive with heat-labile (F) and heat-stable (121 C) antigens. More detailed studies were carried out with the use of a representative normal serum (I.R.C.) and a representative immune serum (L.W.). To study the specificity of immunoglobulins reactive with

TABLE 1. *Natural and immune antibodies reactive with heat-labile (F) and heat-stable (121 C) antigens[a] of Neisseria gonorrhoeae*

Serum	Immunoglobulins (reciprocal titer)								
	IgG			IgM			IgA		
	Antigens			Antigens			Antigens		
	F	121 C	F/121 C[b]	F	121 C	F/121 C	F	121 C	F/121 C
10 normal adults, mean titer	16 (4–32)[c]	64 (16–128)	0.25 (0.06–0.50)	16 (8–32)	16 (8–32)	1 (1–2)	N (N–2)	2 (N-2)	0
10 normal children, mean titer	8 (4–32)	32 (16–128)	0.31 (0.06–0.50)	8 (4–32)	8 (4–16)	1 (0.5–2)	N (N)	2 (2)	0
Patients with arthritis									
L. W.	1,024	64	16	256	32	8	16	16	1
J. H.	512	64	8	64	8	8	N[d]	2	0
Y. L.	1,024	256	4	256	128	2	8	16	0.5
V. P.	256	64	4	256	16	16	2	8	0.25
G. M.	256	64	4	256	128	2	32	16	2
Chronically infected females									
J.	256	64	4	16	16	1	N	16	0
P.	512	64	8	32	16	2	4	8	0.5
96	128	64	2	16	16	1	N	4	0
144	128	32	4	16	16	1	2	4	0.5
34	256	64	4	128	64	2	2	8	0.25
37	256	64	4	64	64	1	N	8	0

[a] See Materials and Methods.
[b] F/121 C = the ratio of IFA titer with F antigen to IFA titer with 121 C antigen.
[c] Parentheses indicate range.
[d] N serum diluted 1:2 failed to show a 2+ reaction with the anti-IgA reagent.

heat-labile (F) and heat-stable (121 C) antigens of *N. gonorrhoeae*, I.R.C. and L.W. sera were absorbed with F and 121 C cells. The residual IgG and IgM reactivities of the sera with F and 121 C antigens were measured by the IFA method.

The results obtained by absorbing immune L.W. serum are shown in Table 2. Immune IgG and IgM activities with the heat-labile (F) antigens were removed by absorbing with F cells. Absorption with F cells also lowered the reactivities of immune IgG and IgM antibodies with heat-stable (121 C) antigens. This finding suggested that some heat-stable (121 C) antigens may be exposed on the surface of Formalin-treated (F) cells. Therefore, absorption with F cells removed antibodies reactive with both F and 121 C antigens.

Absorption of L.W. serum with 121 C treated cells completely removed IgG and IgM IFA reactivities with heat-stable (121 C) antigens without apparently decreasing the reactivities of these antibodies with heat-labile (F) antigens. This indicated that the role of exposed heat-stable antigens was relatively small in the overall reactivity of immune antibodies with the Formalin-treated (F) gonococcal surface.

In contrast, natural antibodies, particularly IgG, appeared to react with Formalin-treated (F) cells by binding primarily to heat-stable (121 C) antigens exposed on the Formalin-treated (F) surface. This is demonstrated in Table 3, which describes the results of absorbing normal serum with 121 C or F cells. Absorption of normal serum (I.R.C.) with 121 C cells removed IgG reactivities to both 121 C and F antigens. This finding indicated that the reactivity of natural IgG antibodies with Formalin-treated (F) cells depended largely on the binding of natural IgG to exposed heat-stable (121 C) antigenic sites. The ability of F cells to remove partially anti-F, but not anti-121 C, reactivity suggested, in addition, that I.R.C. serum could react with some heat-labile components of the F surface. The reactivity of this natural IgG with the F surface was found to be relatively minor compared with that demonstrated by immune IgG antibodies (Table 2). Related

TABLE 2. *Cross-absorption of immune antibodies (L.W.) with heat-labile (F) and heat-stable (121 C) antigens of Neisseria gonorrhoeae*

Serum[a]	IFA antigen	IFA reactivity	
		IgG	IgM
Unabsorbed	F	4+	2+
	121 C	3+	1+
Absorbed with F	F	—	—
	121 C	2+	—
Absorbed with 121 C	F	4+	2+
	121 C	—	—

[a] Approximately 2×10^{10} F or 121 C cells were incubated for 60 min at 37 C with 1.0 ml of L.W. serum diluted 1:50 and 1:16. The cells were removed by centrifugation; the absorbed serum diluted 1:50 was tested with the anti-IgG reagent and the serum diluted 1:16 was tested with the anti-IgM reagent, because of the lower titer of IgM antibody.

TABLE 3. *Cross-absorption of natural antibodies (I.R.C.) with heat-labile (F) and heat-stable (121 C) antigens of Neisseria gonorrhoeae*

Serum[a]	IFA antigen	IFA reactivity	
		IgG	IgM
Unabsorbed	F	2+	2+
	121 C	4+	2+
Absorbed with F	F	+	—
	121 C	4+	2+
Absorbed with 121 C	F	—	2+
	121 C	+	—

[a] I.R.C. serum was diluted 1:5 and absorbed in the manner described in footnote to Table 2.

studies (Cohen, *in preparation*) of a large number of sera from infected and normal persons appeared to indicate that immune IgG antibodies were able to recognize heat-labile sites on the F surface with which natural IgG antibodies were not reactive.

Natural IgM antibodies reactive with F, unlike natural IgG antibodies, were not removed by absorption of I.R.C. serum with 121 C cells (Table 3). It appeared that natural IgM antibodies may react to a greater degree with heat-labile (F) antigens than do natural IgG antibodies. The relatively greater reactivity of natural IgM antibodies for heat-labile (F) antigens was also indicated by the differences in F/121 C ratios between IgM and IgG antibodies observed in normal sera (Table 1).

Effect of 2-mercaptoethanol on IFA reactivity. Treatment of sera with 2-mercaptoethanol has been reported to influence the reactivity of IgM but not IgG antibodies in agglutination tests (7, 8, 12). To study the effects of this reducing agent on IFA reactivity, a number of normal and immune sera were each incubated at 37 C for 60 min with an equal volume of 0.2 M 2-mercaptoethanol. Table 4 shows the results of treating an immune serum (G.M.) with 2-mercaptoethanol. This treatment reduced, but did not abolish, the reactivity of IgM in the IFA test. IgG antibody activities were not significantly influenced. The effects of 2-mercaptoethanol on IgA activity were found to vary. Some sera showed decreased IgA titers and some showed increased titers after treatment. It was found that the titer of IgA antibodies in G.M. serum reactive with heat-stable (121 C) antigens was decreased, whereas the titer of IgA antibodies reactive with heat-labile (F) antigens was increased by treatment with 2-mercaptoethanol (Table 4).

Effect of heating on the IFA reactivities of sera. It has been reported that heating serum can destroy antibody reactivity and that natural antibodies are more heat-labile than immune antibodies (13). To study the heat lability of antibodies, in the IFA test, a number of normal and immune sera were exposed to graded temperatures,

TABLE 4. *Effects of 2-mercaptoethanol on the IgG, IgM, and IgA reactivity of an immune serum (G.M.)*

Immunoglobulin	Antigen	Serum dilution	Treatment[a]	
			PBS	2-Mercaptoethanol
IgG	121 C	1:32	3+	3+
		1:64	2+	2+
	F	1:128	3+	3+
		1:256	2+	2+
IgA	121 C	1:2	3+	+
		1:4	2+	+
	F	1:4	+	2+
		1:8	—	+
IgM	121 C	1.4	2+	2+
		1:8	3+	+
		1:16	2+	+
	F	1:32	4+	+
		1:64	3+	+
		1:128	2+	+

[a] G.M. serum was incubated for 60 min at 37 C with an equal amount of PBS (phosphate-buffered saline) or 0.2 M 2-mercaptoethanol. The serum was then further diluted in PBS or 0.1 M 2-mercaptoethanol and tested with 121 C or F treated *Neisseria gonorrhoeae* in the IFA procedure.

and their immunoglobulin reactivities to *N. gonorrhoeae* were studied.

Table 5 illustrates the results of heating I.R.C. and L.W. sera at 37, 60, 65, 70, and 75 C for 30 min. It was found that natural IgG antibodies (I.R.C.) reactive with *N. gonorrhoeae* were destroyed by heating serum to 70 C. Immune IgG antibodies (L.W.), however, were still active after serum was heated to 70 C. To ascertain the effects of antibody concentration on resistance to heating, L.W. serum was diluted 1:60 before heating. This lowered, but did not destroy, the IgG activity of L.W. serum at 70 C (Table 5). It is therefore unlikely that increased antibody concentration alone was responsible for the increased heat resistance of immune IgG.

There were no significant differences in heat resistance observed between the IgM antibody activities of normal and immune sera (Table 5). IgM antibody activities of both I.R.C. and L.W. sera were reduced after heating at 65 C. IgA antibodies, unlike IgG and IgM antibodies, showed increased IFA activity after heating sera at 60 C (Table 5). I.R.C. serum (1:2) had no detectable IgA activity when it was tested unheated or after heating at 37 C. After heating at 60 C, however, IgA antibody activity with F cells was clearly demonstrable. L.W. serum, which initially demonstrated IgA antibody, showed an increase in IgA reactivity after heating at 60 C. At 65 C, the IgA reactivity returned to preheating levels, and at 70 C IgA reactivity was destroyed.

TABLE 5. *Effect of heating on serum reactivity in the IFA test*

Serum	Temp[a]	IFA reactivity to F antigens		
		IgG	IgM	IgA
	C			
I.R.C. (1:2) natural antibody	37	3+	3+	—
	60	3+	3+	2+
	65	3+	+	—
	70	—	—	—
L.W. (1:4) immune antibody	37	4+	2+	2+
	60	4+	2+	3+
	65	4+	+	2+
	70	4+	+	—
L.W. (1:60)	65	3+		
	70	2+		
	75	—		

[a] Sera were diluted in buffered saline and heated for 30 min in water baths at the indicated temperature. The IFA test was done with the F preparation of *Neisseria gonorrhoeae*.

DISCUSSION

IFA procedures, with the use of complex antigens such as bacterial cells, measure an undefined number of antigen-antibody interactions. There are many antigenic sites present on a bacterium such as *N. gonorrhoeae* (5), and a sufficient quantity of antibody molecules binding to any or all of them can cause a positive fluorescent reaction. The interactions of individual antibody-antigen systems thus can be obscured in an unmodified IFA reaction. Several qualifications therefore were made in the IFA procedure used in this study to identify some of the components taking part in the overall interaction between gonococcal antigens and human antibodies. Fluorescent antisera specific for immunoglobulins G, A, and M were used to identify the immunoglobulin class of the reactive antibodies, and the participating antigens were separated into heat-labile and heat-stable components by treating gonococcal cells with Formalin (F) or heat (121 C). In addition, immune and normal sera were absorbed with treated gonococcal cells and tested for residual antibody activities. In this way, immune and natural antibodies reactive with *N. gonorrhoeae* could be characterized in a more defined manner than that obtained by merely comparing quantity of reaction or titer.

Although reactive antibodies of all three major immunoglobulin classes were found in both normal and immune sera, different patterns of antibody reactivity appeared to characterize normal and immune sera. Natural IgG antibodies were found to react primarily with heat-stable somatic antigens of *N. gonorrhoeae*. Immune IgG antibodies also reacted with heat-stable gonococcal antigens but, in addition, were able to recognize heat-labile surface antigens with which natural IgG antibodies had relatively little or no reactivtiy.

It is noteworthy that the reactivities of immune and natural IgG antibodies with heat-stable somatic antigens were of equal magnitude. The apparent absence of an increased IgG response to heat-stable gonococcal antigens in infection is paradoxical. This observation was confirmed in a study of the immune response of humans in experimental gonorrhea (Cohen et al., *in preparation*). It is possible that the heat-stable somatic antigens are buried beneath heat-labile capsular antigens so that they cannot effectively stimulate the host during infection. The elevated titers of immune IgA to heat-stable antigens, however, argues against this possibility by indicating that these antigens do stimulate antibodies (IgA) during infection.

In contrast to IgG antibodies, the differences

between the reactivities of natural and immune IgM antibodies were found to be less clear-cut. Both natural IgM antibodies and immune IgM antibodies reacted with heat-labile as well as with heat-stable antigens. In addition, the titers of IgM antibodies in a number of sera from infected persons appeared comparable to titers in normal sera, whereas other immune sera showed elevated titers.

IgA antibodies in normal sera were found to be reactive primarily with heat-stable antigens. Immune IgA antibodies differed from natural IgA antibodies by demonstrating increased reactivity with heat-stable antigens and by being able to interact with heat-labile antigens.

These findings demonstrate the complexity of the reactions of human immunoglobulins with bacterial antigens. The competition between, and the effectiveness of, the antibodies themselves has been considered in an earlier publication (4).

It appears that antibodies belonging to different immunoglobulin classes may demonstrate affinities for distinct classes of antigens and may also recognize certain antigens in common. It is possible that the antibody response to a particular bacterial antigen is conditioned by the presence of other antigens on the same organism, as well as by the physicochemical nature of the antigen itself. Further studies are needed to characterize these complex relationships.

The effects of mercaptoethanol and heat on the reactivities of IgG, IgA, and IgM antibodies in the IFA procedure should be interpreted with caution. The detection of antibodies by IFA methods depends on two basic reactions: (i) the reaction of the antibody with the bacterium, and (ii) the reaction of the fluorescent "anti-antibody" with the antibody itself. Therefore, the antigenicity of the antibody, as well as the specificity of its antigen-combining site, can influence the results of the IFA procedure. Thus, if heat or mercaptoethanol were to destroy the antigenic site of the antibody molecule, the fluorescent reagents would fail to detect the antibody even though it might be firmly bound to the bacterium by an intact combining site.

It has been shown that treatment of antibodies with mercaptoethanol affects the agglutinating activity of IgM, but not IgG, antibodies (7, 8, 12). Recently, a number of studies (2, 10, 15) have indicated that mercaptoethanol-treated IgM and IgA molecules maintain intact combining sites and can bind to antigens. The results of the IFA procedure used in this study paralleled the findings demonstrated by these other methods. IgG antibodies completely resisted mercaptoethanol treatment. The persistent, but reduced, activity of IgM antibodies after mercaptoethanol treatment was consistent with the findings of these workers (2, 10, 15) that part of the antigen-combining activity of IgM remains after mercaptoethanol reduction.

The effects of mercaptoethanol on IgA antibodies were found to be variable. Some sera showed increased IgA activity and some showed decreased IgA activity after treatment. The immune serum of G.M. was found to have both increased and decreased IgA reactivities to different gonococcal antigens (Table 4). Further studies are needed to explain these unexpected findings.

Michael et al. (14) have confirmed the observation that immune antibodies are more resistant to heating than natural antibodies. Michael and Rosen have also suggested (13) that differences between natural and immune antibodies to gram-negative bacteria result from the physicochemical properties of two different classes of immune globulin. However, it was found in the studies reported here and elsewhere (3) that natural antibodies to gram-negative bacteria were represented in all three major classes of immunoglobulin. In addition, the present study demonstrated that immune IgG antibodies were more resistant to heating than natural IgG antibodies. This suggests that antibodies belonging to the same class of immune globulin (IgG) may undergo changes during immunization which result in increased heat stability. This was confirmed in a number of instances in which the heat stability of IgG was observed to increase in the course of experimental gonorrhea (Cohen et al., *in preparation*).

Reactive antibodies for which we know of no obvious stimulus are defined as natural antibodies (14). The ubiquity of antigens cross-reactive with those of gram-negative bacteria and the development of natural antibodies in germ-free aniamls (16) suggest that at least some natural antibodies, like immune antibodies, are the result of antigenic stimulation.

As demonstrated in this detailed study of a few sera, and confirmed in related studies (Cohen, *in preparation*) of a large number of sera, immune antibodies (IgG) could react with heat-labile antigens on the gonococcal surface with which natural antibodies could not react. These heat-labile antigens would appear to be less cross-reactive with other antigens in the environment and hence more specific for *N. gonorrhoeae*. Further studies have shown that these heat-labile antigens may be characteristic of virulent clones and are deficient in avirulent clones of the same strain of *N. gonorrhoeae* (Cohen, *in preparation*). Persons infected with virulent *N. gonorrhoeae* would therefore be stimulated to produce "immune" antibodies (IgG) to the specific gonococcal heat-labile antigens. "Natural" antibodies, on the

other hand, could result from the exposure of normal persons to substances antigenically similar to the heat-stable antigens found on *N. gonorrhoeae*. Although both natural and immune antibodies react with the bacterial cell, some of the antigens to which they are directed may be distinct.

It is possible that problems regarding the differentiation of natural and immune antibodies to other gram-negative bacteria might also be resolved if more defined antigen-antibody systems are studied.

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