ACTIVATION OF COMPLEMENT BY SERUM-RESISTANT *NEISSERIA GONORRHOEAE*

Assembly of the Membrane Attack Complex without Subsequent Cell Death*

BY G. R. HARRIMAN,‡ E. R. PODACK, A. I. BRAUDE, L. C. CORBEIL, A. F. ESSER, AND J. G. CURD

From the Departments of Molecular Immunology and Clinical Research, Scripps Clinic and Research Foundation, La Jolla, California 92037; and the Departments of Medicine and Pathology University Hospital, University of California, San Diego, California 92103

Some strains of *Neisseria gonorrhoeae* are sensitive to the bactericidal action of fresh human serum, whereas others are resistant. The majority of *N. gonorrhoeae* strains causing disseminated gonococcal infection (DGI)[1] are resistant to the bactericidal action of fresh human serum (1–4). Strains causing DGI, however, appear to be a heterogeneous group. Strains that give rise to tenosynovitis, skin lesions, and bacteremia are more resistant to normal human serum (NHS) or autologous convalescent serum than strains causing suppurative arthritis (5).

The mechanisms of serum resistance in *N. gonorrhoeae* are incompletely understood. Two types of serum resistance have been identified: stable and unstable (6–8). The stable form of serum resistance persists even after prolonged culture of the bacteria on agar, whereas the unstable form is rapidly lost during passage on agar in the absence of serum. Strains of *N. gonorrhoeae* causing DGI demonstrate stable serum resistance. Some studies have suggested that serum resistance in other gram-negative bacteria is related to the presence of blocking antibody in human serum (9–12). Moreover, antibody of the IgG class blocked killing of serum-resistant *N. gonorrhoeae* by rabbit serum (13). This blocking antibody also blocked IgM-mediated killing of serum-sensitive as well as serum-resistant *N. gonorrhoeae* by NHS or human immune serum, respectively (14).

Because the complement system is required for serum killing of serum-sensitive *N. gonorrhoeae*, an understanding of the interaction between complement and *N. gonorrhoeae* may clarify the mechanism of serum-resistance. Previous reports have described decreases in hemolytic activity in serum incubated with resistant *N. gonorrhoeae*, suggesting either specific complement activation or nonspecific consumption (15, 16).

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[1] *Abbreviations used in this paper:* CFU, colony-forming unit; CH_{50}, hemolytic complement activity in serum; DGI, disseminated gonococcal infection; E_R, rabbit erythrocytes; GVB, veronal buffered saline containing 0.5 mM Mg^{++}, 0.15 mM Ca^{++}, and 0.1% gelatin; HAGG, human aggregated IgG; MAC, membrane attack complex of complement; NHS, normal human serum; ΔNHS, heat-inactivated NHS; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; VB^{++}, veronal buffered saline containing 0.5 mM Mg^{++} and 0.15 mM Ca^{++}.

Studies of serum-resistant *Salmonella minnesota* have suggested that resistance in this bacterium is related to failure of the complement membrane attack complex (MAC) to insert into hydrophobic domains of the outer membrane (17, 18). We undertook the following studies to assess the interaction of the complement system with resistant and sensitive *N. gonorrhoeae* to characterize the relationship of the complement system to serum resistance.

Materials and Methods

Serum. Pooled NHS was prepared by centrifugation (2,000 *g* at 4°C) of freshly clotted blood (30–60 min at room temperature) obtained from three or more normal volunteers who had no history of gonococcal infection. This pooled NHS was used in all experiments except as indicated otherwise. Heat-inactivated NHS (ΔNHS) was prepared by heating NHS at 56°C for 30 min.

Bacteria. Serum-resistant strains (Willis and Davis) were isolated from blood or joint fluids of patients with DGI. One serum-sensitive strain (N24) was obtained from the San Diego Public Health Laboratories and another (F62) from Dr. Jerry Brown (Center for Disease Control, Atlanta, GA). Bacteria were maintained at −70°C in citrated rabbit blood until use. When needed, bacteria were grown on GC agar plates supplemented with 1% IsoVitaleX (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, MD) at 37°C in 5% CO_2 and subcultured daily by selecting multiple colonies of typical T2 colonial morphology (19). For cloning experiments, single colonies were selected, plated, and grown at 37°C in 5% CO_2.

Bactericidal Assays. Gonococci grown 18–24 h were examined to ensure that >99% were T2 colony type, suspended in Dulbecco's minimal essential media (MEM) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) by vigorous pipetting for uniform dispersion, and centrifuged (64 *g*) for 30 s to sediment agar and large clumps of bacteria. The suspension was adjusted by addition of MEM to an absorbance 610 nm (A_{610}) corresponding to the desired concentration of bacteria. The correlation between A_{610} and bacterial concentration had been determined previously by plate counts. Equal amounts of bacterial suspension and NHS or ΔNHS were mixed and incubated at 37°C in 5% CO_2 for 1 h. After incubation, bacterial viability was determined as previously described (20). The bactericidal capacity of NHS was expressed either as the difference between the number of colony-forming units (CFU) surviving in ΔNHS and in NHS or, in the case of $\log_{10}$ killing, as the difference between the $\log_{10}$ of the number of CFU in ΔNHS and in NHS.

Complement Depletion Assays. Suspensions of bacteria varying from 10^7/ml to 10^9/ml were prepared in MEM, mixed with equal amounts of NHS, and incubated at 37°C in 5% CO_2 for 1 h. For determination of the amount of complement in serum not exposed to bacteria, equal amounts of NHS and MEM were mixed and incubated. After incubation, an aliquot from each sample was removed for a bactericidal assay, and the remaining sample was centrifuged (15,000 *g*) for 10 min in a microfuge to pellet the bacteria. The supernatant was removed and used for complement determinations or frozen at −70°C if determinations were not done immediately.

The total hemolytic activity (CH_{50}), C4 hemolytic activity, and C2 hemolytic activity were determined by standard procedures (21). C7 hemolytic activity was determined as follows. C7-depleted serum (C7d) was prepared by affinity chromatography (22). A 1:20 dilution of the test sample was prepared, and 2–20 μl aliquots were added to mixtures of 0.1 ml antibody-sensitized sheep erythrocytes (5×10^8/ml), 0.3 ml veronal-buffered saline with gelatin (GVB), and 0.02 ml C7d serum on ice and then incubated at 37°C for 30 min. After incubation, 1.0 ml cold GVB was added, each mixture centrifuged (500 *g*) for 3 min, and the A_{412} of the supernatant determined. The C7 hemolytic activity expressed as z/ml was calculated.

C1s Activation Assay. Activation of C1s was determined as previously described (23). In brief, purified C1s, kindly provided by Dr. Robert Ziccardi of Scripps Clinic and Research Foundation, was labeled with ^{125}I (New England Nuclear, Boston, MA) by the solid-phase lactoperoxidase method to a specific activity of 0.5 μCi/μg. ^{125}I-C1s at 5% of the concentration of C1s in normal serum was added to NHS and equilibrated for 60 min at 37°C. Bacterial pellets were prepared by adding suspensions containing 10^8 or 10^9 bacteria to microfuge tubes, centrifuging

(10,000 *g*) for 5 min and removing the supernatants. Next, 0.025 ml of NHS containing ^{125}I-C1s was added to the bacterial pellets, vortexed vigorously to suspend the bacteria, and incubated at 37°C for 30 min. To assess spontaneous activation of C1s, 0.025 ml NHS containing ^{125}I-C1s was added to 0.025 ml MEM in the absence of bacteria. C1s activation was also assessed by adding 0.25 ml NHS containing ^{125}I-C1s to 100 μg human aggregated IgG (HAGG). (The HAGG was prepared by heating the IgG [10 mg/ml] at 63°C for 20 min and centrifuging [10,000 *g*] for 3 min to remove large complexes.) After incubation at 37°C for 30 min, EDTA (10 mM final concentration) was added to the mixtures, vortexed, allowed to stand for 5 min, and then the bacteria were pelleted. The ^{125}I-C1s in the supernatant was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in tube gels (3% stacking gel, 7% separating gel) under reducing conditions according to Laemmli (24). The gels were cut into 1.5-mm segments, and each slice was counted in a gamma scintillation spectrometer. C1s activation was determined as the fraction of total counts (in percent) migrating as activated ^{125}I-C1s. Specific C1s activation was determined by subtracting the percent C1s activated in NHS incubated with MEM from the percent C1s activation produced by HAGG or bacteria samples.

C5 Activation and Binding Assay. C5 was purified as described (25) except as a final step, affinity chromatography on concanavalin A-Sepharose 4B was used, and then labeled with ^{125}I by the iodogen method (26), to a specific activity of 0.15-0.3 μCi/μg. Freshly centrifuged ^{125}I-C5 (10,000 *g* for 5 min) was added to NHS or ΔNHS to give 2×10^6 cpm/ml serum. Pellets containing 10^9 bacteria were prepared in duplicate in microfuge tubes, and aliquots (0.05 ml or 0.2 ml) of NHS or ΔNHS containing ^{125}I-C5 were added, and the mixture vortexed and incubated at 37°C in 5% CO_2 for 1 h. Samples containing rabbit erythrocytes (E_R) (1×10^8) and zymosan (250 μg) were also prepared to assess C5 activation and binding in this assay. One set of the duplicate samples was then centrifuged (10,000 *g*) for 5 min, and aliquots of the supernatants and pellets were analyzed for ^{125}I-C5 activation on a SDS-PAGE slab gel (3% stacking gel, 7% separating gel) under reducing conditions according to Laemmli (24). The gel was dried and an autoradiograph was prepared to assess conversion of C5 to C5b. The second set of samples was analyzed for ^{125}I-C5 binding to the bacteria by vortexing, layering the samples on top of 0.3 ml of 10% (wt/vol) sucrose in veronal-buffered saline with Ca^{++} and Mg^{++} (VB^{++}) containing 0.1 mg ovalbumin (Calbiochem-Behring Corp., La Jolla, CA) per ml in 0.4 ml microfuge tubes, and centrifuging (10,000 *g*) for 3 min. The supernatants and pellets were separated by removing the bottoms of the tubes with a razor blade and counting in a gamma scintillation spectrometer. Molecules of C5 bound per cell were determined as follows. Molecules of C5 in the NHS containing ^{125}I-C5 were calculated based on normal serum concentration of C5 (70 μg/ml). The functional activity of ^{125}I-C5 used was estimated to be 33% based on maximum incorporation of ^{125}I-C5 into SC5b-9 in NHS upon activation by zymosan (10 mg/ml) (27). The amount of ^{125}I-C5 bound was corrected for functional activity of ^{125}I-C5 to calculate total C5 binding. Finally, the total C5 bound was divided by the number of bacteria (10^9) in the assay.

Specific binding of ^{125}I-C5 to the bacteria was determined according to the following formula:

$$\text{percent specific binding} = \left(\frac{\text{cpm pellet (NHS)}}{\text{cpm total (NHS)}} - \frac{\text{cpm pellet (}\Delta\text{NHS)}}{\text{cpm total (}\Delta\text{NHS)}}\right) \times 100.$$

^{125}I-C5 and ^{131}I-C9 Binding Assay. Purified C5 and C9 (28) were prepared and differentially labeled with ^{125}I and ^{131}I (ICN Nutritional Biochemicals, Cleveland, OH) respectively, by the iodogen method. The specific activity of ^{131}I-C9 was 0.4 μCi/μg. Freshly centrifuged ^{131}I-C9 (10,000 *g* for 5 min) was added to NHS and ΔNHS to give 2×10^6 cpm/ml serum. The specific activity of ^{125}I-C5 and amount added to NHS and ΔNHS were as described for the C5 activation and binding assay. To bacterial pellets containing 10^8 bacteria prepared in microfuge tubes as described above, increasing amounts of NHS containing ^{125}I-C5 and ^{131}I-C9 were added. The mixtures were vortexed and incubated at 37°C in 5% CO_2 for 1 h. ΔNHS (0.5 ml) containing ^{125}I-C5 and ^{131}I-C9 was incubated with bacterial pellets to determine nonspecific binding. After incubation, MEM was added as necessary to a final volume of 0.5 ml. An aliquot (0.1 ml) was removed from each tube and layered on 0.3 ml of 10% (wt/vol) sucrose in VB^{++}

containing 1.0 mg ovalbumin/ml in a 0.4 ml microfuge tube. The tubes were then centrifuged, after which supernatants and pellets were separated and counted for both ^{125}I-C5 and ^{131}I-C9. The ^{125}I counts were corrected for crossover from ^{131}I. The molecules of C5 and C9 bound per cell were calculated as described above. Calculations were based on 33% ^{131}I-C9 functional activity. The C9/C5 ratio was calculated from the molecules per cell data.

Assay of SC5b-9 Formation. Aliquots of the supernatants obtained from the C5 activation and binding assay were layered on top of a 4.5-ml 10%–40% sucrose gradient in VB^{++} with a 0.5 ml 66% sucrose cushion. The samples were centrifuged in a Beckman ultracentrifuge (L5-65; Beckman Instruments, Inc., Fullerton, CA) with a SW50.1 rotor at 36,000 rpm, 4°C for 16 h. The samples were then fractionated and counted in a gamma scintillation spectrometer for ^{125}I-C5. The location of radiolabeled SC5b-9 within the gradient was determined from zymosan-treated samples, and the percent of ^{125}I-C5 incorporated into SC5b-9 was calculated.

Electron Microscopy. Bacteria were incubated with MEM, NHS, or ΔNHS as described for the bactericidal assays. After incubation, they were centrifuged (10,000 *g*) for 5 min, washed twice with 0.2 ml of 0.5 M phosphate buffer (pH 8.0), and resuspended in 0.2 ml VB^{++}. To each sample, 20 μg of trypsin and 20 μg of chymotrypsin (Worthington Biochemical Corp., Freehold, NJ) were added before overnight incubation at room temperature. The samples were then washed twice in 0.2 ml VB^{++} and resuspended in 0.05 ml VB^{++}, after which 5 μl was placed on parlodion and carbon-coated 400-mesh copper grids, stained with 1–2% uranyl formate, and examined in a Hitachi 12A electron microscope operated at 75 kv accelerating voltage and a direct magnification of 40,000.

Results

Effect of NHS on Serum-resistant and Serum-sensitive N. gonorrhoeae. To determine the bactericidal capacity of NHS against resistant and sensitive *N. gonorrhoeae*, we determined the bacteria surviving as CFU when increasing numbers of bacteria were added to a constant volume of NHS. Fig. 1 shows that essentially all bacteria of the serum-sensitive strain N24 were killed when 10^7 CFU were added to 1 ml of NHS. As the concentration of N24 incubated with NHS was increased, more bacteria (CFU) were killed, but a smaller percentage of the total number was killed (decreasing $\log_{10}$ killed). In contrast, there was no significant killing (<0.1 log) of the serum-resistant strain Willis at bacterial concentrations of 10^7–10^8/ml.

Because subsequent experiments (see below) showed that complement was activated

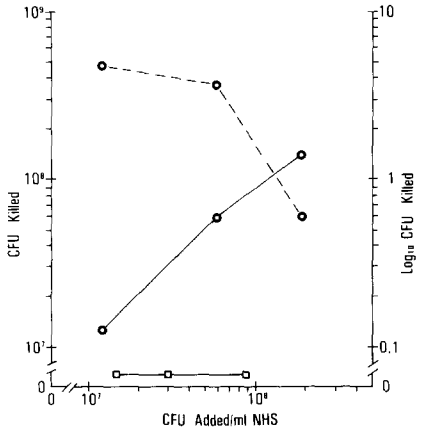


FIG. 1. Killing of serum-resistant and -sensitive *N. gonorrhoeae* by NHS. The numbers of CFU killed (——) and $\log_{10}$ CFU killed (-----) by NHS are shown for a serum-resistant strain, Willis (□), and a serum-sensitive strain, N24 (○) as increasing numbers of CFU were incubated with 1 ml of NHS.

by serum-resistant *N. gonorrhoeae*, it became important to determine if any resistant bacteria could be killed by NHS. Because our standard bactericidal assay does not detect killing of small numbers of bacteria when high bacterial concentrations (10^8/ml) are used, additional experiments in which 10^4 bacteria/ml had been added to NHS were performed. Results of five identical experiments showed that the decrease in CFU incubated in NHS compared with ΔNHS was 29% ± 17% for the resistant Willis strain and 19% ± 21% for the resistant Davis strain. To exclude the possibility that a subpopulation of relatively serum-sensitive clones existed within the population of the serum-resistant strain, 10 separate clones of Willis were also tested in bactericidal assays at a concentration of 10^8 bacteria/ml of NHS. None of the clones exhibited more than a 0.2 log decrease in the number of CFU in NHS compared with ΔNHS.

Depletion of Complement Components. In a series of experiments, the CH_{50}, C4, C2, and C7 activities were determined in NHS after incubation with resistant and sensitive *N. gonorrhoeae* (Table I). The serum-resistant strain Willis depleted 25% of the CH_{50} activity and 33% of the C2 activity in NHS. The two serum-sensitive strains F62 and N24 depleted ~33% of the CH_{50} activity and 50% of the C2 activity in NHS. Neither C4 nor C7 activity was depleted in detectable amounts by either resistant or sensitive

TABLE I

Complement Component Activity Remaining in NHS Incubated with Resistant and Sensitive N. Gonorrhoeae

Bacterial strain	Concentration organisms/ml	CH_{50} (three experiments)	C4 (one experiment)	C2 (two experiments)	C7 (one experiment)
			z/ml	*z/ml*	*z/ml*
No bacteria	0	77 ± 10.6	20,960 ± 2,028	116 ± 16.8	4,306 ± 442
Willis	10^8	58 ± 4.9 (25%)*	19,418	78 ± 20.6 (33%)	4,398
F62	10^8	52 ± 4.7 (32%)	18,182	60 ± 24.2 (48%)	4,186
N24	10^8	51 ± 10.0 (34%)	21,200	60 ± 23.6 (48%)	4,258

* Numbers in parentheses are percent decrease compared to controls with no bacteria.

TABLE II

Total Hemolytic Activity (CH_{50}) Remaining in NHS after Incubation with Resistant and Sensitive N. gonorrhoeae

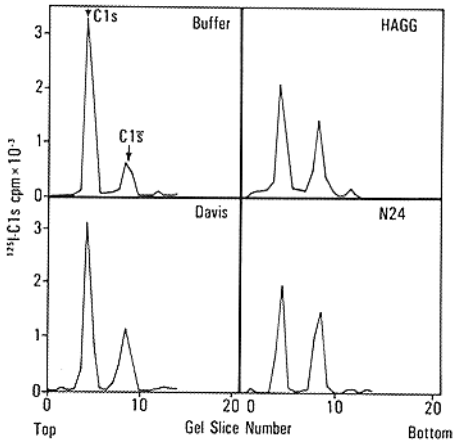
Bacterial strain	Concentration organisms/ml	CH_{50} units	Percent decrease (CH_{50})
No bacteria	0	63.4 ± 7.2	0
Willis	10^7	46	27
	10^8	42	33
	10^9	<28	>55
Davis	10^7	52	18
	10^8	44	30
	10^9	<28	>55
F62	10^7	47	25
	10^8	40	37
	10^9	<28	>55
N24	10^7	48	25
	10^8	38	40
	10^9	<28	>55

TABLE III

Activation of ^{125}I-C1s in NHS by Resistant and Sensitive N. gonorrhoeae

Assay constituents	Percent ^{125}I-C1s Conversion	
	10^8 Bacteria	10^9 Bacteria
HAGG + 0.025 ml ^{125}I-C1s	36.3	23.7
Willis + 0.025 ml ^{125}I-C1s	0*	21.4
Davis + 0.025 ml ^{125}I-C1s	0*	19.0
F62 + 0.025 ml ^{125}I-C1s	0*	18.1
N24 + 0.025 ml ^{125}I-C1s	0*	32.3

* No measurable conversion.



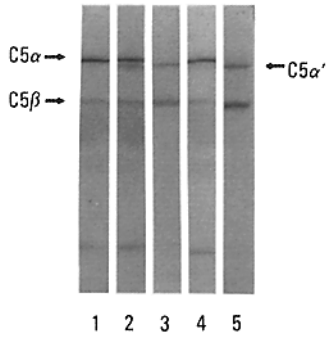


FIG. 2. (Left) SDS-PAGE tube gel analysis of ^{125}I-C1s activation in NHS incubated with serum-resistant and -sensitive *N. gonorrhoeae*. Positions of the unactivated heavy chain of ^{125}I-C1s (C1s) and the activated (cleaved) heavy chain of ^{125}I-C1s (C1s̄) are indicated in the upper left panel. Activation of ^{125}I-C1s in NHS by buffer, HAGG, serum-resistant (Davis) and -sensitive (N24) *N. gonorrhoeae* is shown.

FIG. 3. (Right) Autoradiogram of an SDS-PAGE slab gel demonstrating ^{125}I-C5 activation in NHS by serum-resistant and -sensitive *N. gonorrhoeae*. The positions of the C5α, C5α′, and C5β chains of C5 are indicated by arrows. The following samples were applied to the SDS-PAGE slab gel: (1) supernatant from ΔNHS containing ^{125}I-C5 incubated with a serum-resistant strain (Willis); (2) supernatant from NHS containing ^{125}I-C5 incubated with Willis; (3) bacterial pellet from incubation of Willis in NHS containing ^{125}I-C5; (4) supernatant from NHS containing ^{125}I-C5 incubated with a serum-sensitive strain (N24); (5) bacterial pellet from incubation of N24 in NHS containing ^{125}I-C5.

strains when 10^8 bacteria were incubated with 1 ml of NHS.

To evaluate the dose-response relationships between the number of bacteria incubated in NHS and consumption of complement, different amounts (10^7–10^9) of bacteria were added to 1 ml of NHS. As seen in Table II, both serum-resistant strains (Willis and Davis) and -sensitive strains (F62 and N24) depleted comparable amounts of complement. In addition, the CH_{50} depletion increased in all cases as more bacteria were added to NHS.

Activation of C1. To evaluate activation of the classical pathway, 10^8 bacteria were incubated with 0.025 ml of NHS containing ^{125}I-C1s. No C1s activation was detectable with either resistant or sensitive strains (Table III). However, when 10^9 bacteria were added to 0.025 ml of NHS containing ^{125}I-C1s, both resistant strains, Willis and Davis,

TABLE IV

Binding of ^{125}I-C5 in NHS to Resistant and Sensitive N. gonorrhoeae

Assay constituents*	Molecules C5 per cell‡	Percent ^{125}I-C5 binding	Percent specific binding
E_R + 0.05 ml NHS	48,000	15.2	14.3
E_R + 0.05 ml ΔNHS	2,800	0.9	
Willis + 0.05 ml NHS	3,700	11.6	8.0
Willis + 0.05 ml ΔNHS	1,100	3.6	
Willis + 0.2 ml NHS	9,500	7.5	6.2
Willis + 0.2 ml ΔNHS	1,600	1.3	
N24 + 0.05 ml NHS	3,350	10.6	8.8
N24 + 0.05 ml ΔNHS	600	1.8	
N24 + 0.2 ml NHS	9,500	7.5	6.2
N24 + 0.2 ml ΔNHS	1,600	1.3	

* The amounts of E_R and *N. gonorrhoeae* used in these assays were 1×10^8 and 1×10^9 per assay, respectively.

‡ For calculations of estimated molecules per cell see Materials and Methods.

TABLE V

Binding of ^{125}I-C5 and ^{131}I-C9 in NHS to Resistant and Sensitive N. gonorrhoeae

Assay constituents*	Molecules per cell‡		Percent binding		C9/C5 Ratio§
	C5	C9	^{125}I-C5	^{131}I-C9	
Willis + 0.05 ml NHS	24,000	127,000	7.7	18.5	5.2
Willis + 0.1 ml NHS	48,000	337,000	7.6	24.6	7.0
Willis + 0.25 ml NHS	100,000	818,000	6.3	23.9	8.2
Willis + 0.5 ml NHS	186,000	1,241,000	5.9	18.1	6.7
Willis + 0.5 ml ΔNHS	28,000	59,000	0.9	0.9	2.1
N24 + 0.05 ml NHS	16,000	96,000	5.0	14.0	6.1
N24 + 0.2 ml NHS	30,000	154,000	4.7	11.2	5.2
N24 + 0.25 ml NHS	61,000	418,000	3.8	12.2	6.8
N24 + 0.5 ml NHS	129,000	757,000	4.1	11.0	5.6
N24 + 0.5 ml ΔNHS	58,000	94,000	1.8	1.4	1.6

* The amounts of *N. gonorrhoeae* used in these assays were 1×10^8 per assay.

‡ For calculations of molecules per cell see Materials and Methods.

§ For calculations of C9/C5 ratio see Materials and Methods.

as well as one sensitive strain, F62, activated ~20% of the C1s, whereas the other sensitive strain, N24, activated a little more than 30% of the C1s (Table III, Fig. 2).

Activation and Binding of C5. When NHS containing ^{125}I-C5 was incubated with resistant and sensitive strains, both strains activated C5 as shown by the presence of C5α' in the pellets (Fig. 3). Bacteria incubated with ΔNHS did not activate C5. In addition to activating C5, both resistant and sensitive strains bound comparable amounts of ^{125}I-C5 to their surfaces at the end of 1 h incubation in NHS (Table IV). Far less C5 bound to the bacteria in ΔNHS than in NHS. Specific binding was 8.0% for Willis and 8.8% for N24 after incubation with 0.05 ml NHS. When a larger volume of NHS (0.2 ml) containing ^{125}I-C5 was incubated with the bacteria, more molecules of C5 were bound to the bacteria, but once again the amounts bound were

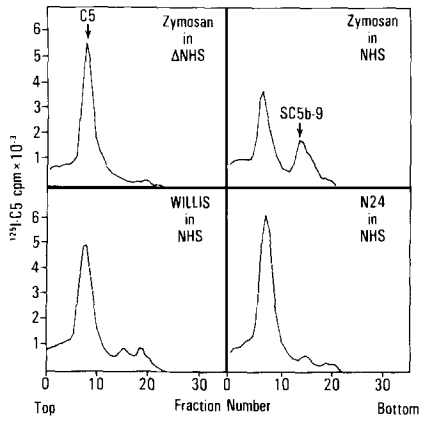


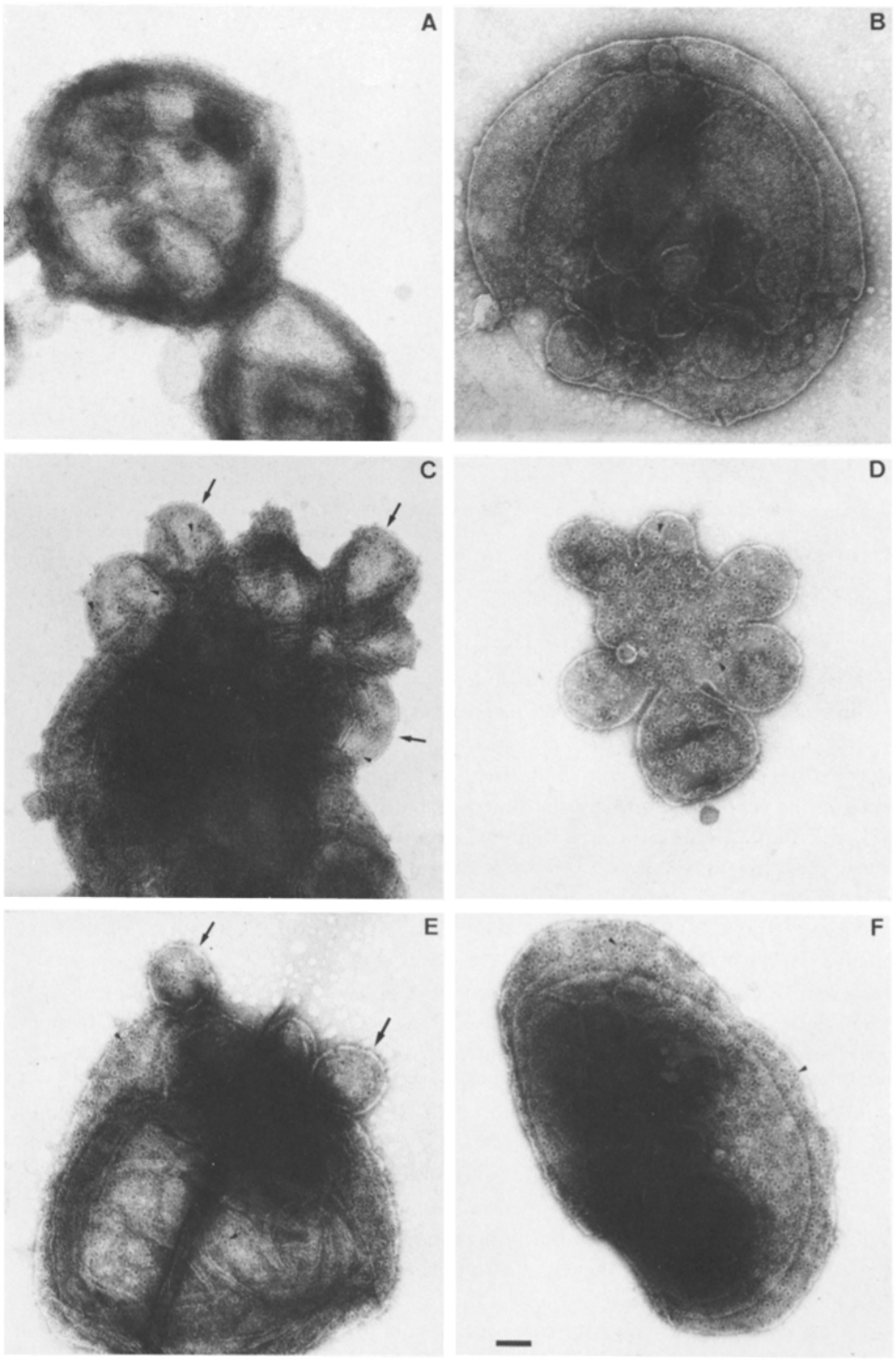
FIG. 4. Sucrose density gradient ultracentrifugation analysis of SC5b-9 formation in NHS containing ^{125}I-C5 incubated with serum-resistant and -sensitive *N. gonorrhoeae*. The sedimentation of ^{125}I-C5 (C5) and ^{125}I-SC5b-9 (SC5b-9) in the sucrose density gradient is indicated by arrows. Incorporation of ^{125}I-C5 in SC5b-9 is shown for ΔNHS incubated with zymosan, and NHS incubated with zymosan, serum-resistant strain Willis, or sensitive strain N24.

similar for both resistant and sensitive strains. Nonspecific binding in this larger amount of ΔNHS was equal for both strains (1.3%) as was specific binding (6.2%).

^{125}I-C5 and ^{131}I-C9 Binding to Resistant and Sensitive N. gonorrhoeae. The simultaneous binding of ^{125}I-C5 and ^{131}I-C9 was measured to assess formation of the MAC on the bacterial surfaces. Both the resistant strain Willis and the sensitive strain N24 specifically bound radiolabeled C5 and C9 (Table V). In these experiments, 10^8 bacteria were incubated with increasing amounts of NHS to which radiolabeled C5 and C9 had been added. The number of C5 and C9 molecules bound to both resistant and sensitive strains increased as the bacteria were incubated with increasing amounts of NHS. The binding of C9 to the bacteria was greater than that of C5 at higher serum concentration, resulting in a C9/C5 ratio with a maximum of 8.2 for Willis and 6.8 for N24. The nonspecific binding of ^{125}I-C5 and ^{131}I-C9 to both strains in ΔNHS was very low compared with binding in NHS, and the ratio of C9/C5 bound to the bacteria in ΔNHS was much lower than in NHS.

^{125}I-C5 and ^{131}I-C9 Binding to Resistant and Sensitive N. gonorrhoeae. The simultaneous binding of ^{125}I-C5 and ^{131}I-C9 was measured to assess formation of the MAC on the bacterial surfaces. Both the resistant strain Willis and the sensitive strain N24 specifically bound radiolabeled C5 and C9 (Table V). In these experiments, 10^8 bacteria were incubated with increasing amounts of NHS to which radiolabeled C5 and C9 had been added. The number of C5 and C9 molecules bound to both resistant and sensitive strains increased as the bacteria were incubated with increasing amounts of NHS. The binding of C9 to the bacteria was greater than that of C5 at higher serum concentration, resulting in a C9/C5 ratio with a maximum of 8.2 for Willis

FIG. 5. MAC formation on the surfaces of serum-resistant (Willis) and -sensitive (N24) *N. gonorrhoeae*. Electron micrographs of sensitive strain N24 incubated in buffer (A), in NHS (B), resistant strain Willis in NHS (C), fused membrane vesicles from Willis in NHS (D), and N24 in NHS (E and F). Arrows in panel C denote membrane blebs on surface of Willis and arrowheads indicate MAC. In panel D, fused membrane vesicles from Willis contain MAC (arrowheads). In panels E and F, arrows point to membrane blebbing on surface of N24 and arrowheads point to MAC. Bar = 100 nm.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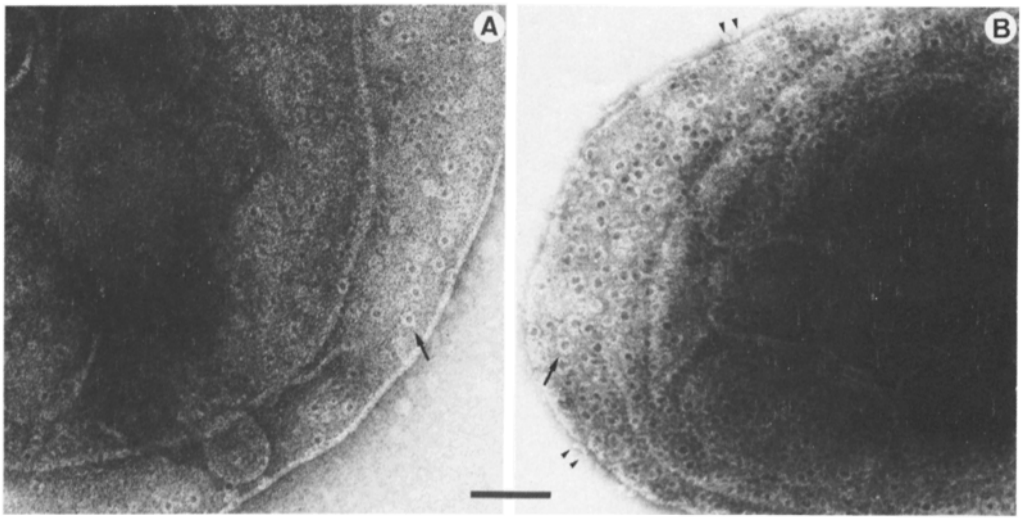

FIG. 6. Identification of bacterial surface structures and MAC on *N. gonorrhoeae*. Panel A demonstrates ring-like structures on surface of serum-sensitive bacteria (inner diameter, 8 nm) not exposed to NHS (arrow). Panel B shows serum-sensitive strain (N24) incubated with NHS. In panel B MAC are seen on bacterial surface in two views, from above (arrow) and side view (double arrowheads) (inner diameter of MAC, 11 nm). Bar = 100 nm.

and 6.8 for N24. The nonspecific binding of ^{125}I-C5 and ^{131}I-C9 to both strains in ΔNHS was very low compared with binding in NHS, and the ratio of C9/C5 bound to the bacteria in ΔNHS was much lower than in NHS.

SC5b-9 Formation by Resistant and Sensitive N. gonorrhoeae. NHS containing ^{125}I-C5 was incubated with bacteria, and then the radioactivity in the supernatant was analyzed by sucrose density gradient ultracentrifugation. Small amounts of ^{125}I-C5 in NHS incubated with either resistant or sensitive bacteria sedimented in a position identical to that of SC5b-9 formed upon treatment of NHS with zymosan (Fig. 4). The amount of ^{125}I-C5 at this position in NHS incubated with the resistant strain Willis varied from 10.9% to 16.7% of the total ^{125}I-C5 in the supernatant, whereas the sensitive strain N24 had 11.5%–11.9% ^{125}I-C5 at this position.

Electron Microscopy of Resistant and Sensitive N. gonorrhoeae. After incubation with NHS, both resistant and sensitive strains of *N. gonorrhoeae* had typical complement lesions on their surfaces (Figs. 5 and 6). In addition to complement lesions, ring-like structures (inner diameter, 8 nm) can be seen on the surface of the bacteria incubated in NHS or ΔNHS (Fig. 5 B). In contrast to the MAC, these complement-independent ring structures do not extend above the surface of the bacterial membrane as do the MAC (Fig. 6 B). Serum-resistant strains exhibited the same 8-nm ring structures on their surface (not shown). After incubation with NHS, the serum-resistant strain Willis and -sensitive strain N24 both had numerous MAC deposited on their surface (Fig. 5 C–F), which were clearly distinguishable from the 8-nm ring structures. Another prominent feature was the blebbing of the outer membrane of both resistant and sensitive bacteria. In some cases, fused membrane blebs, with MAC on their surface, could be seen (Fig. 5 D). This blebbing phenomenon appeared somewhat more prominent in the resistant strain.

Discussion

Our new finding regarding the interaction of human complement with *N. gonorrhoeae* is that the classical pathway of complement is specifically activated by resistant as well as sensitive *N. gonorrhoeae*. Complement activation by both types of bacteria is qualitatively and quantitatively similar and leads to MAC formation and stable deposition on the surface of both resistant and sensitive *N. gonorrhoeae*.

More specifically, we found that essentially 100% of the organisms of a serum-sensitive strain, N24, were killed in NHS at concentrations that did not completely deplete the complement in NHS. With higher concentrations of organisms, the $\log_{10}$ killed diminished significantly. This probably occurred because the bactericidal capacity of NHS was approached at the highest concentration of bacteria, as evidenced by loss of measurable CH_{50} activity in NHS incubated with 10^9 bacteria/ml. There was a clear dose-response relationship between the initial number of bacteria incubated with NHS and the number of sensitive bacteria killed, as well as the amount of CH_{50} activity depleted by both resistant and sensitive bacteria.

Although resistant strains at various concentrations consumed comparable CH_{50} to that of sensitive strains, they were not killed. At low concentrations (10^4/ml), a few of the resistant bacteria (20%–30%) seemed to be killed; however, because of errors inherent in the bactericidal assay and certain characteristics of the gonococcus (e.g., clumping), it is difficult to assess small amounts of killing. For these reasons, a significant amount of killing is usually defined as >1 log (29). If, in fact, a small percent of resistant bacteria are killed by NHS, the cause is not a sensitive subpopulation, as shown by our cloning experiments. In addition, the killing of such a small percentage of bacteria would be unlikely to account for comparable amounts of CH_{50} depletion by resistant and sensitive *N. gonorrhoeae*.

Stable serum resistance in *N. gonorrhoeae* does not result from lack of complement activation. Resistant as well as sensitive strains activate C1s and consume C2. Thus, both strains can activate the classical pathway. Because C2 represents the limiting component of the classical pathway, the decreased CH_{50} probably represents C2 depletion, whereas the absence of detectable C4 or C7 depletion indicates the relative excess of these components in serum. The large number of bacteria required to detect C1s activation is also consistent with the presence of an excess of C1 relative to C2 in serum.

The activation of C5 in NHS by both resistant and sensitive bacteria demonstrates that a C5 convertase had formed. After activation, C5b is bound to both resistant and sensitive strains as shown by equal deposition of C5b at the end of a 1-h incubation. As the amount of serum added was increased, additional molecules of C5b bound. C9 was also shown to remain bound to both resistant and sensitive bacteria. Previous reports have suggested that the ratio of C9 molecules to C5 molecules incorporated into the MAC, as measured by radiolabeled components, is ≥6:1. The ratio of C9/C5 bound to both resistant and sensitive strains in these experiments suggests that these components were incorporated into the MAC. Although some SC5b-9 was formed by both resistant and sensitive bacteria, there was no marked difference in the amount formed by either strain.

Both resistant and sensitive strains had MAC deposited on their surfaces as seen in the electron microscope. Also apparent were inherent ring-like structures clearly distinguishable from MAC. These ring-like structures have been reported previously

for the gonococcus (30) and meningococcus (31). Another notable feature was blebbing of the membrane with release of vesicles from bacterial membranes. Although blebbing appeared somewhat more prominent in the resistant bacteria than in the sensitive strain, the significance is uncertain. Blebbing of membranes has also been reported previously for the gonococcus (32).

Previous studies of serum-resistant and sensitive *S. minnesota* showed that both the resistant and sensitive strain activated complement (15, 16). After complement activation, C5b-7 bound stably to the resistant *Salmonella* strain, but upon the addition of C8 and C9, the C5b-7 disassociated from the bacteria in the form of SC5b-9. This dissociation did not occur in the sensitive strain, rather the terminal components remained bound to the bacteria, presumably in the form of the MAC, and were capable of killing the bacteria.

The interactions of complement with resistant strains of *S. minnesota* and *N. gonorrhoeae* differ in several respects, suggesting different mechanisms of resistance. Although both activate the complement system, resistant *S. minnesota* consumes more terminal complement components than the sensitive strain (15, 16), whereas resistant *N. gonorrhoeae* does not. Unlike resistant *S. minnesota*, the resistant *N. gonorrhoeae* have C5 and C9 bound to their surfaces at the end of 1 h of incubation, and the amounts of bound C5 and C9 are approximately equal to that of the sensitive strain. The ratios of C9/C5 bound to resistant and sensitive *N. gonorrhoeae* are comparable, and suggest that C5 and C9 are incorporated into MAC, unlike the decreased C9/C5 ratios observed on resistant *S. minnesota* (15, 16). The amounts of SC5b-9 formed by both resistant and sensitive *N. gonorrhoeae* are also comparable, indicating no difference in release of terminal component complexes from bacterial surfaces. Finally, electron microscopy revealed MAC bound to both resistant and sensitive *N. gonorrhoeae*. Therefore, MAC is formed and remains on the surfaces of both resistant and sensitive *N. gonorrhoeae* after activation of complement.

Although MAC is formed and remains on the surface of resistant *N. gonorrhoeae*, the precise location of the MAC in the membrane is unknown. Our electron microscopy studies suggest that MAC becomes inserted in the outer membranes of some resistant bacteria, but the possible relationship between MAC location and viability remains to be determined. Possibly, a critical region on the bacterial surface determines killing. Complement-mediated killing of *Escherichia coli* has been shown to occur with simultaneous release of markers from periplasmic and intracellular spaces (33, 34). Some studies suggest the existence of regions in *E. coli* where inner and outer membranes join to form adhesion zones (35). It is possible that MAC must insert at these critical regions of the bacterial surface to effect killing. Blocking antibodies are known to play a role in *N. gonorrhoeae* serum resistance, and blocking antibody may prevent the insertion of MAC at these critical regions.

The precise events at a molecular level that determine serum-resistance in certain *N. gonorrhoeae* remain to be determined. Regardless of what the mechanism of resistance is, it will have to take account of the fact that serum-resistant *N. gonorrhoeae* activate complement and bind MAC.

Summary

Interaction of the human complement system in normal human serum (NHS) with serum-resistant and -sensitive *Neisseria gonorrhoeae* was evaluated to better understand

the mechanism of serum-resistance. Complement activity (CH_{50}) was depleted from NHS in a dose-dependent fashion by both serum-resistant and -sensitive *N. gonorrhoeae*. No detectable CH_{50} remained in NHS incubated with 10^9 colony-forming units (CFU)/ml serum of either resistant or sensitive strains. When smaller numbers of bacteria were incubated with NHS, lesser, yet comparable, amounts of CH_{50} were depleted by both resistant and sensitive strains. Hemolytic C2 activity was diminished by 33% in the case of resistant *N. gonorrhoeae* (10^8 CFU/ml serum) and by 48% in the case of a sensitive strain. No detectable decreases in hemolytic C4 or C7 activities were found with either sensitive or resistant strains at this concentration.

Both resistant and sensitive strains activated C1s in NHS. Resistant strains specifically activated 19–21% of radiolabeled C1s in NHS, whereas sensitive strains activated 18–32%. Both resistant and sensitive strains also activated C5 in NHS.

In binding assays using radiolabeled C5 and C9 in NHS, resistant and sensitive strains bound comparable amounts of C5 and C9. The number of bound C5 and C9 molecules varied according to the number of bacteria or amount of serum used in the assay. The ratio of C9/C5 bound to a sensitive strain was 6.8, and to a resistant strain was 8.2, suggesting that C5 and C9 were incorporated into membrane attack complexes (MAC). Electron microscopic examination of resistant and sensitive strains incubated with NHS revealed that MAC is bound to the surfaces of the resistant strain as well as the sensitive strain.

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