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) 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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Abbreviations used in this paper: CFU, colony-forming unit; CHs0, hemolytic complement activity in serum; DGI, disseminated gonococcal infection; Er, 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; ANHS, 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% CO₂ 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% CO₂.

**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 (A₆₁₀) corresponding to the desired concentration of bacteria. The correlation between A₆₁₀ 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% CO₂ 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 ANHS and in NHS or, in the case of log₁₀ killing, as the difference between the log₁₀ of the number of CFU in ANHS and in NHS.

**Complement Depletion Assays.** Suspensions of bacteria varying from 10⁷/ml to 10⁹/ml were prepared in MEM, mixed with equal amounts of NHS, and incubated at 37°C in 5% CO₂ 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 (CH₅₀), 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 × 10⁶/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 A₄₁₂ of the supernatant determined. The C7 hemolytic activity expressed as z/ml was calculated.

**Cl₁ Activation Assay.** Activation of Cl₁ was determined as previously described (23). In brief, purified Cl₁, kindly provided by Dr. Robert Ziccardi of Scripps Clinic and Research Foundation, was labeled with ¹²⁵I (New England Nuclear, Boston, MA) by the solid-phase lactoperoxidase method to a specific activity of 0.5 μCi/μg. ¹²⁵I-Cl₁ at 5% of the concentration of Cl₁ in normal serum was added to NHS and equilibrated for 60 min at 37°C. Bacterial pellets were prepared by adding suspensions containing 10⁸ or 10⁹ bacteria to microfuge tubes, centrifuging
(10,000 g) for 5 min and removing the supernatants. Next, 0.025 ml of NHS containing $^{125}$I-Cls 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 Cls, 0.025 ml NHS containing $^{125}$I-Cls was added to 0.025 ml MEM in the absence of bacteria. Cls activation was also assessed by adding 0.25 ml NHS containing $^{125}$I-Cls 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-Cls 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. Cls activation was determined as the fraction of total counts (in percent) migrating as activated $^{125}$I-Cls. Specific Cls activation was determined by subtracting the percent Cls activated in NHS incubated with MEM from the percent Cls 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 $2 \times 10^6$ cpm/ml serum. Pellets containing 10⁹ 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 mixtures vortexed and incubated at 37°C in 5% CO₂ for 1 h. Samples containing rabbit erythrocytes (ER) (1 × 10⁹) 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 (3-7% 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 5 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 (10⁹) in the assay.

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

$$\text{percent specific binding} = \left( \frac{\text{cpm pellet (NHS)}}{\text{cpm total (NHS)}} \right) - \left( \frac{\text{cpm pellet (ΔNHS)}}{\text{cpm total (ΔNHS)}} \right) \times 100.$$
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](image)
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 (10⁸/ml) are used, additional experiments in which 10⁴ 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 10⁴ 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 CH₅₀, 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 CH₅₀ activity and 33% of the C2 activity in NHS. The two serum-sensitive strains F62 and N24 depleted ~33% of the CH₅₀ 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 | CH₅₀ (three experiments) | C4 (one experiment) | C2 (two experiments) | C7 (one experiment) |
|-----------------|-----------------|----------------|----------------|----------------|----------------|
|                 |                 | z/ml           | z/ml           | z/ml           | z/ml           |
| No bacteria     | 0               | 77 ± 10.6      | 20,960 ± 2,028| 116 ± 16.8     | 4,306 ± 442    |
| Willis          | 10⁴             | 58 ± 4.9 (25%)*| 19,418         | 78 ± 20.6 (33%)| 4,398          |
| F62             | 10⁴             | 52 ± 4.7 (32%) | 18,182         | 60 ± 24.2 (48%)| 4,186          |
| N24             | 10⁴             | 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 (CH₅₀) Remaining in NHS after Incubation with Resistant and Sensitive N. Gonorrhoeae**

| Bacterial strain | Concentration organisms/ml | CH₅₀ units | Percent decrease (CH₅₀) |
|-----------------|-----------------|------------|------------------------|
| No bacteria     | 0               | 63.4 ± 7.2 | 0                      |
| Willis          | 10⁵             | 46         | 27                     |
|                 | 10⁴             | 42         | 33                     |
|                 | 10³             | <28        | >55                    |
| Davis           | 10⁵             | 52         | 18                     |
|                 | 10⁴             | 44         | 30                     |
|                 | 10³             | <28        | >55                    |
| F62             | 10⁵             | 47         | 25                     |
|                 | 10⁴             | 40         | 37                     |
|                 | 10³             | <28        | >55                    |
| N24             | 10⁵             | 48         | 25                     |
|                 | 10⁴             | 38         | 40                     |
|                 | 10³             | <28        | >55                    |
TABLE III
Activation of \(^{125}\text{I}-\text{C1s}\) in NHS by Resistant and Sensitive \(N.\) gonorrhoeae

| Assay constituents | \(10^9\) Bacteria | \(10^9\) Bacteria |
|------------------|------------------|------------------|
| HAGG + 0.025 ml \(^{125}\text{I}-\text{C1s}\) | 36.3 | 23.7 |
| Willis + 0.025 ml \(^{125}\text{I}-\text{C1s}\) | 0* | 21.4 |
| Davis + 0.025 ml \(^{125}\text{I}-\text{C1s}\) | 0* | 19.0 |
| F62 + 0.025 ml \(^{125}\text{I}-\text{C1s}\) | 0* | 18.1 |
| N24 + 0.025 ml \(^{125}\text{I}-\text{C1s}\) | 0* | 32.3 |

* No measurable conversion.

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 \(\text{CH}_{30}\) 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 \(^{125}\text{I}-\text{C1s}\). No C1s activation was detectable with either resistant or sensitive strains (Table III). However, when \(10^8\) bacteria were added to 0.025 ml of NHS containing \(^{125}\text{I}-\text{C1s}\), both resistant strains, Willis and Davis,
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 C5a' 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

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**TABLE IV**

| 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 $1 \times 10^8$ and $1 \times 10^9$ per assay, respectively.

† For calculations of estimated molecules per cell see Materials and Methods.
125I-C5 and 131I-C9 Binding to Resistant and Sensitive N. gonorrhoeae. The simultaneous binding of 125I-C5 and 131I-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 125I-C5 and 131I-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.

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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 \(\Delta\text{NHS}\) (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 log₁₀ 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 CH₅₀ activity in NHS incubated with 10⁹ 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 CH₅₀ activity depleted by both resistant and sensitive bacteria.

Although resistant strains at various concentrations consumed comparable CH₅₀ to that of sensitive strains, they were not killed. At low concentrations (10⁴/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 CH₅₀ depletion by resistant and sensitive bacteria.

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 CH₅₀ 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 (CHS0) was depleted from
NHS in a dose-dependent fashion by both serum-resistant and -sensitive N. gonorrhoeae.
No detectable CHS0 remained in NHS incubated with 10⁹ 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 CHS0 were
depleted by both resistant and sensitive strains. Hemolytic C2 activity was diminished
by 33% in the case of resistant N. gonorrhoeae (10⁹ 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 specif-
ically 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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