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Gonococcal Infection in a Nonhuman Host Is Determined by Human Complement C1q

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Human C1q displayed a dose-dependent protection of gonococcal cells (GC) from the bactericidal effect of newborn rat serum. All rat pups injected with C1q-preincubated GC developed bacteremia, while none of the animals injected with GC only were infected. After clearance of bacteremia at day 6, live GC could still be recovered from tested organs, including the liver. Preincubation of GC with higher concentrations of C1q was associated with increased morbidity. In contrast to human serum as a source of C1q, rat, rabbit, and mouse sera did not increase the in vivo virulence of *Neisseria gonorrhoeae*. C1q-deficient human serum, heat-inactivated C1q or human serum, type IV collagen, and complement C3 were inefficient in inducing infection. Experimental infection by C1q-preincubated GC was inhibited by anti-C1q antibodies in a dose-dependent fashion, demonstrating a causal effect of C1q function. This report demonstrates the novel finding that human C1q, a component of the human immune system with a general function for elimination of infection, may increase GC virulence and result in the development of disseminated infection in a nonhuman host.

Gonorrhea is one of the most frequently reported sexually transmitted diseases in the United States (3). Studies on gonococcal virulence and vaccines have been hampered because there is no acceptable simple model of the disease other than that in male volunteers (28, 29, 31). In humans, infection develops as a result of the pathogen's virulence, including its survival in host fluids. *Neisseria gonorrhoeae* is an obligate human pathogen that is unable to survive in nonhuman mammals (15, 16). The contact of gonococcal cells (GC) with serum proteins that occur during menstruation is clinically associated with the development of pelvic inflammatory disease (PID) and septicemic dissemination (70% of cases) (8, 30). Clinical observations implied that serum components, instead of killing, may enhance GC virulence (20, 30). Identifying such a factor(s) would create an approach to the development of an experimental model, in which a human factor(s) would allow GC virulence by promoting GC survival.

We provide evidence that C1q is a human factor that promotes the capacity of GC to survive in newborn rat serum. C1q (and no other factor) enhanced the virulence of GC in vivo and resulted in the development of experimental bacteremia in newborn rats. This shows that *N. gonorrhoeae*, an obligate human pathogen, adapted to a nonhuman host in the presence of C1q.

MATERIALS AND METHODS

GC strains, culture conditions, and reagents. *N. gonorrhoeae* JC1 was isolated from a patient with disseminated gonococcal infection at the Veteran's Administration Hospital, Houston, Tex. *N. gonorrhoeae* JC1 has been characterized as being resistant to normal human serum (NHS) (21). *N. gonorrhoeae* SN1, SN2, SN3, and SN4 were isolated from patients with complicated gonorrhea admitted to obstetrics and gynecology clinics at the University of Texas Medical Branch at Galveston. All gonococcal strains as well as *Neisseria cinerea* were grown for 18 to 24 h on chocolate agar (Remel, Richardson, Tex.) or modified Thayer-Martin (Remel) plates in 5% CO_2 in air at 37°C. Bacterial suspensions were prepared from piliated (P^+) and opaque (OP^+) colonies (90% P^+ OP^+). Suspensions with concentrations of 5×10^6/ml were preincubated with 40 μg of C1q per ml or with bovine serum albumin (BSA) as a control.

Human C1q was isolated from human serum (26) or was purchased (catalog no. C0660; Sigma, St. Louis, Mo.). The C1q was shown to be pure by sodium dodecyl sulfate–5.6% polyacrylamide gel electrophoresis with or without dithiothreitol and by double immunodiffusion against a goat anti-C1q monospecific antiserum (Cytotech, San Diego, Calif.) (25). Other reagents, such as BSA (catalog no. A3156), human C1q-deficient serum (catalog no. C0410), normal rabbit serum (catalog no. S2632), NHS (catalog no. S1764), normal mouse serum (catalog no. S3269), nonimmune goat serum (catalog no. S2007), complement C3 (catalog no. C2910), and collagen IV (catalog no. C7521) were purchased from Sigma. Normal newborn rat serum (NNRS) was pooled from 5-day-old newborn Sprague-Dawley rats. Serum was collected from clotted whole blood and stored in −70°C. Phosphate-buffered saline (PBS) (pH 7.4) was prepared by the culture facility in the Department of Microbiology and Immunology at the University of Texas Medical Branch at Galveston.

Serum bactericidal assay. The resistances of strains JC1, SN1, SN2, SN3, and SN4 and of *N. cinerea* to complement-mediated killing of NNRS were determined by using a modified version of the method described by McShan et al. (21). Gonococci were removed from an overnight chocolate plate culture with a sterile Dacron swab and suspended in buffered saline (optical density of 0.2 at 595 nm). A 1:500 dilution of GC was supplemented with C1q, or with BSA as a control, at increasing concentrations starting with 2 μg/ml and was incubated at 37°C for 15 min. After incubation, NNRS was added to a final concentration of 25%, and incubation was continued for an additional 30 min at 37°C. Dilutions of 1:10 and 1:100 were made and subcultured on chocolate agar plates in triplicate. The plates were incubated for 24 h at 37°C in 5% CO_2, and the number of serum-resistant CFU per milliliter was counted. In control experiments, NNRS was replaced by an equal volume of buffered saline (pH 7.4).

Experimental model. Newborn Sprague-Dawley rats ($n = 100$) were used for experiments. Three-day-old newborn rats from one litter were divided into two groups. One group was inoculated intraperitoneally (i.p.) with GC pretreated with C1q (4 μg of C1q per animal), while the other group was inoculated with GC pretreated with BSA at the same concentration. A volume of 0.1 ml with 5×10^6 CFU of GC was used for inoculation. Five animals from each group were sacrificed and tested every 2 h for 12 h. At 24 h and at every following 24 hours for 6 days, five animals from each group were tested and sacrificed. A total of 705 newborn rats were used for the experiments. Twenty-microliter blood samples were collected from heart punctures without anticoagulant, dilutions were made immediately and subcultured on modified Thayer-Martin agar plates, and the number of CFU per milliliter was calculated. In the same set of experiments, 1 to 5 μl of cerebrospinal fluid was collected, and dilutions were made and immediately subcultured and incubated as described above. The liver, heart, and uterus were separated aseptically, fragmented, and washed three times in PBS (pH 7.4) to remove contaminating blood. Tissues were homogenized in 0.5 ml of PBS. Dilutions of homogenized organ in a volume of 100 μl were then plated on

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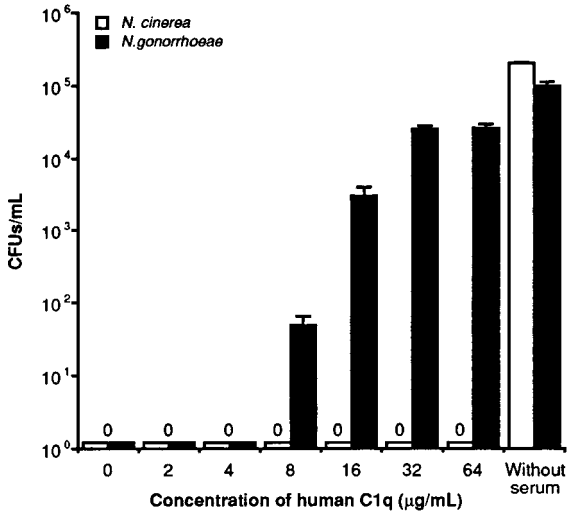

FIG. 1. C1q protection of *N. gonorrhoeae* JC1 from the bactericidal effect of NNRS (in vitro). The results are shown as numbers of CFU per milliliter of GC suspension after preincubation with different concentrations of C1q and a subsequent 30-min incubation in either 25% NNRS or GC broth (control). Each bar represents the mean value from three to five independent experiments and the standard error for each group of data.

modified Thayer-Martin agar in triplicate. The plates were incubated for 48 h at 37°C in 5% CO_2, and the number of CFU per milliliter was calculated. Colonies of *N. gonorrhoeae* were characterized by colonial morphology and by oxidase test, Gram stain, and sugar fermentation reactions. The conditions in the experiments testing the protective capacity of anti-C1q immunoglobulin G were identical to those described above, except that 1.5 h before inoculation of C1q-treated GC, pups received an i.p. injection of 50 μl of monospecific goat anti-C1q IgG or 50 μl of nonimmune goat IgG as a control. Blood samples were collected 24 h after GC inoculation. Animals were treated according to a protocol approved by the Animal Care and Use Committee of the University of Texas Medical Branch at Galveston.

Statistical methods. Statistical analysis was performed by calculating standard deviations and chi-square values; a P value of less than or equal to 0.05 was considered statistically significant.

RESULTS

Effect of C1q on GC killing by NNRS (in vitro). We first investigated whether *N. gonorrhoeae* incubated in human complement C1q resists the bactericidal effect of NNRS. The ability of GC to survive in NNRS in the presence and absence of human C1q was tested. Figure 1 shows the effect of increasing concentrations of human C1q on the survival of *N. gonorrhoeae* JC1 in NNRS. C1q displayed a dose-dependent protection of JC1 and four other clinical strains, SN1, SN2, SN3, and SN4, but not of *N. cinerea*. Alternative treatments in which C1q was directly added to NNRS protected GC but with less efficiency. The survival of GC decreased to 70% of the level observed in the experiment in which GC were preincubated with C1q in the excess of fluid-phase C1q. Washing of the fluid-phase C1q also resulted in protection, but the survival rate decreased to 50% of that in the control experiment shown in Fig. 1.

The serum bactericidal assay was also used to test both overnight agar-grown GC and broth-grown GC (mid-log phase, 4 h of incubation). Independently of the growth conditions, C1q displayed a protective effect in an NNRS bactericidal assay.

Effect of C1q on development of gonococcal infection in experimental animals. Because C1q-mediated survival of GC in NNRS was observed in vitro, we further investigated whether GC preincubated with human C1q (40 μg/ml) were able to infect the nonhuman host in vivo. The results show that 100% of the rat pups injected with C1q-preincubated GC developed bacteremia as a result of the i.p. injection. None of the animals inoculated with GC without C1q were infected (Fig. 2).

Organ and cerebrospinal fluid cultures were positive only in animals inoculated with *N. gonorrhoeae* preincubated with C1q. Quantitative cultures on organs of tested newborn rats were performed (Fig. 3). Organ (liver and heart) infection was found in 100%, peritonitis was found in 80%, and meningitis was found in 30% of the newborn rats, and hemorrhage and skin lesions were detected in some pups. After a significant

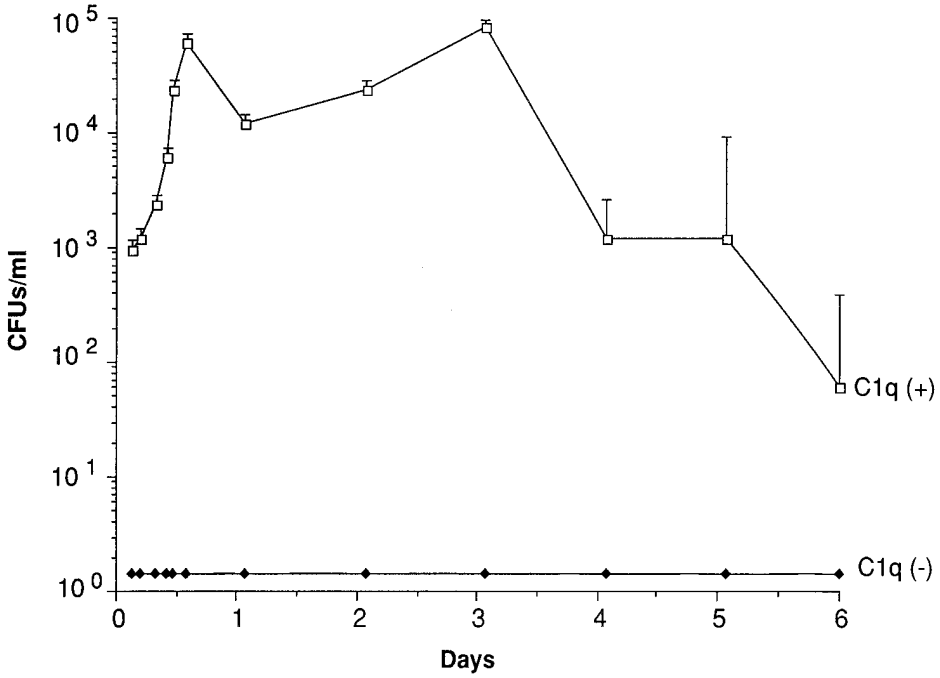

FIG. 2. Kinetics of *N. gonorrhoeae* JC1 bacteremia in newborn rats. □, kinetics of infection due to i.p. inoculation of GC preincubated with C1q; ◆, control experiments with GC only. The average numbers of pups tested at single time point were five with and five without C1q. Three independent kinetic experiments with 120 newborn rats each were performed (total of 360 pups). The average counts from the tested animals and standard error values are shown.

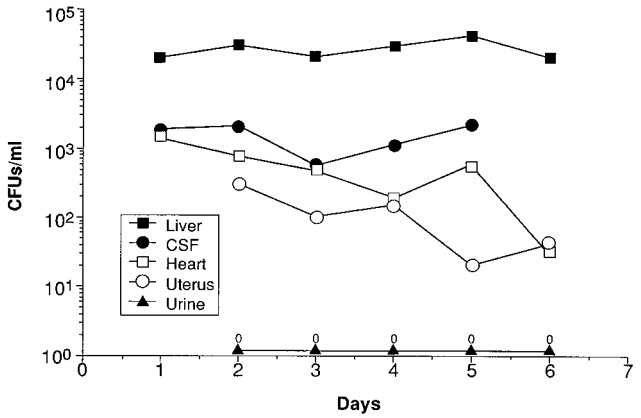

FIG. 3. Numbers of gonococci in liver and other organs and fluids after i.p. inoculation of *N. gonorrhoeae* JC1 preincubated with C1q. Three independent kinetic experiments with 54 pups (total) were performed. The results given are the average counts from three pups in each group. CSF, cerebrospinal fluid.

reduction of bacteremia to 10^2 CFU/ml at day 6, live GC (10^4 CFU/g) were recovered from the liver.

We then tested the effect of a higher concentration of C1q on the morbidity of newborn rats. In this experiment a total of 40 newborn rats were tested. Pretreatment of GC with a higher concentration of C1q (100 μg/ml) was associated with increased morbidity and resulted in a 10% mortality rate. Symptoms developed by infected rats included poor color and tone, poor temperature regulation, and decreased feeding and activity. Control pups inoculated with a 10-times-greater density of GC (5×10^7/ml) (not preincubated with C1q) were not bacteremic and did not have any symptoms or complications associated with potential biological effects of lipooligosaccharide. Pups inoculated with 100 μg of C1q per ml only (without bacteria) were healthy and did not have any complications (data not shown). *N. gonorrhoeae* JC1 pretreated with lower concentrations of C1q, including 10, 8, 4, and 2 μg/ml, was capable of causing bacteremia in rat pups.

Inoculations of GC pretreated with C1q but washed with liquid-phase C1q resulted in bacteremia in newborn rats. Injection of C1q prior to the inoculation of untreated GC also resulted in the development of bacteremia. The rate of survival, tested at 12 h, was reduced to 50 and 90%, respectively, compared with the data presented in Fig. 2.

To investigate whether in vivo infection with other GC strains was possible, experiments were performed with four additional strains of *N. gonorrhoeae*, SN1, SN2, SN3, and SN4, isolated from four different patients with complicated gonococcal infections who were admitted to obstetrics and gynecology clinics at the University of Texas Medical Branch at Galveston. The results with these strains were comparable to those with *N. gonorrhoeae* JC1. All four strains were virulent and caused infections in newborn rats in the presence of human C1q. These strains were not able to cause infections without C1q.

In a set of control experiments, the effect of C1q on nonpathogenic *N. cinerea* was tested. The conditions of the in vivo experiments were identical to those described for GC. *N. cinerea*, with or without C1q, was not able to cause bacteremia in newborn rats, and viable *N. cinerea* was not recovered from these animals (data not shown).

Effect of human and animal sera on GC infectivity in vivo. Human serum, a source of C1q, is similar to purified C1q in that it allows GC infection in pups. To estimate whether the sera of other species may also provide GC with the capacity to survive in the pups, fresh rat, mouse, and rabbit sera were used. The concentration of C1q in human serum is estimated to be approximately 70 μg/ml. The concentration of C1q in rat serum is lower and is estimated to be approximately 40 μg/ml (18). In contrast to NHS, sera from other species (normal rat serum, normal rabbit serum, and normal mouse serum) did not increase the virulence of *N. gonorrhoeae* in vivo. Only human serum allowed *N. gonorrhoeae* infection to develop.

Effect of C1q-deficient serum and other proteins on GC virulence in vivo. To further determine whether C1q is the specific protein in the human serum allowing for the development of GC experimental bacteremia, C1q-deficient human serum, heat-inactivated serum, or C1q-related molecules, such as type IV collagen and complement factor C3, were used and compared with NHS. Type IV collagen was used because of its similarity to the C1q collagen-like structure (27). Complement factor C3 was used to test whether any complement components other than the C1q would display a similar effect (18). The C1q activity of the C1q-deficient serum was less than 5% of that of NHS, as assayed with a modified version of a method described by Kolb et al. (17). In experiments with C1q-deficient serum with inactivated human serum, C1q heated at 56°C for 30 min, or type IV collagen, the number of gonococcal CFU per milliliter recovered from blood of inoculated animals was significantly lower (0.7×10^2 CFU/ml) than that in the positive control experiment, in which NHS or purified C1q was used. GC preincubated with C1q resulted in gonococcal bacteremia with 2×10^5 CFU/ml of blood of infected animals (Fig. 4). Only 0.07% of the gonococcal CFU in the positive control were recovered, indicating that 99.93% of GC in the blood were killed in experimental animals in which heated C1q, type IV collagen, or C1q-deficient serum was present. When denatured C1q (boiled in 100°C for 30 min) or purified active C3 was used for experiments, not a single colony was recovered. Similarly, boiled human serum was inefficient in inducing infection (Fig. 4). The results of these experiments support the hypothesis that C1q is a human factor of NHS that increases the virulence of *N. gonorrhoeae* and is a necessary factor for the development of gonococcal bacteremia in experimental animals.

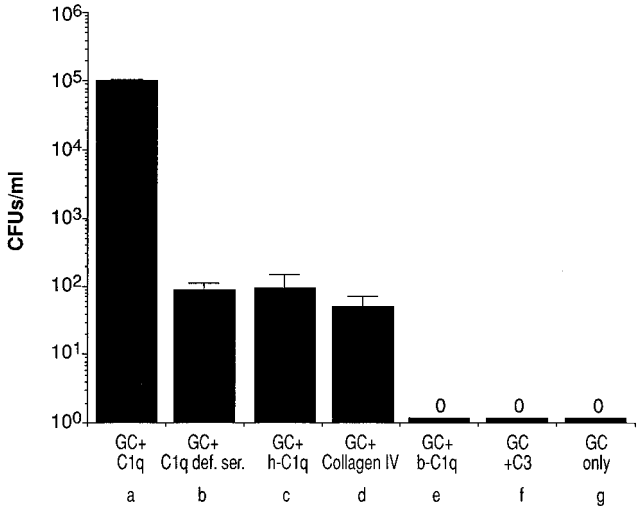

FIG. 4. Effects of C1q-deficient serum, C1q inactivation, type IV collagen, and C3 on *N. gonorrhoeae* JC1 bacteremia in newborn rats. a, GC plus C1q (70 μg/ml); b, GC plus C1q-deficient human serum (5% activity of C1q) (C1q def. ser.); c, GC plus heated C1q (56°C, 30 min) (h-C1q); d, GC preincubated with collagen (70 μg/ml); e, GC plus boiled C1q (100°C, 30 min) (b-C1q); f, GC preincubated with C3 (70 μg/ml); g, control without C1q treatment. Five newborn rats in each group were sacrificed and bled at a given time point. Each bar represents the mean value from three independent experiments with standard errors for each group of data.

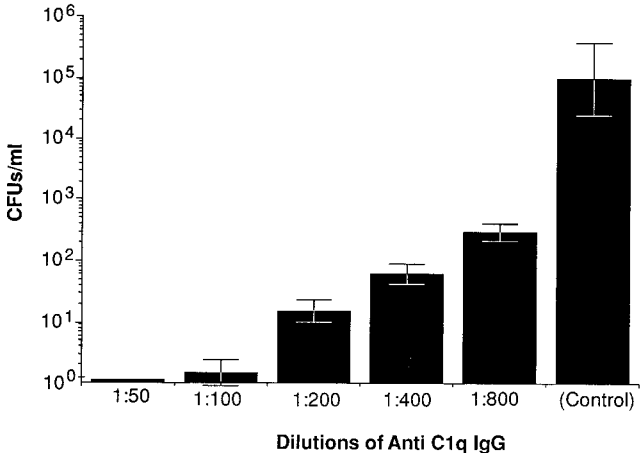

FIG. 5. Inhibitory effect of anti-C1q immunoglobulin G on the development of gonococcal bacteremia. The inhibition observed at the 1:800 dilution was statistically significant ($P \leq 0.05$). For the control, newborn rats received goat preimmune immunoglobulin G at a dilution of 1:100. Three independent experiments with 30 newborn rats each were performed (total of 90 newborn rats).

Experimental infection in animals pretreated with anti-C1q antibodies. We investigated the possibility that anti-C1q antibodies reduce the virulence of GC preincubated with C1q. Anti-human C1q antibodies used in dilutions (1:800 to 1:50) reduced the number of GC recovered from the blood to 10^3 and 0 CFU/ml, respectively; blood from control animals without antibody treatment showed 10^5 CFU/ml (Fig. 5). Anti-C1q antibodies protected animals from gonococcal infection (by GC preincubated with C1q) in a dose-dependent fashion, demonstrating the causal effect of C1q function.

DISCUSSION

This study demonstrated that human C1q, a component of the human immune system with a general function for elimination of infection, may increase GC virulence and result in the development of a disseminated infection in a nonhuman host. The bacteremia and peritonitis in the rat pups resembled bacteremia in human disseminated gonococcal infection and peritonitis observed in PID patients.

The development of an animal model of gonorrhea has been hampered for 50 years because only humans develop gonococcal infection (12). Gonococcal urethritis has been produced experimentally in primates, but only 30% of male chimpanzees developed infection (4). Attempts to infect other species, including monkeys and rodents (15, 16), have been unsuccessful. Models including implanted chambers were proposed but practically limited; for example, survival of GC was observed in the artificial chamber but not in the animal tissues (1, 2, 7, 10, 15).

JC1 isolated from patients with gonococcal bacteremia is resistant to the bactericidal effect of human serum but is killed by NNRS. Preincubation of JC1 with NHS protected JC1 from being killed by NNRS. This suggests that NHS may contain a factor that allows JC1 to survive in both NHS and NNRS. Recent studies indicate that C1q may bind to *N. gonorrhoeae* JC1 in an antibody-independent manner (24). We also observed the association of C1q with the GC surface in direct smears from patients with gonococcal infection (25). Both observations supported our attempts to investigate the role of C1q, the first component of complement, in gonococcal virulence.

Purified C1q was found to protect JC1 from the bactericidal effects of NNRS. Pretreatment of GC with C1q in the presence of free liquid-phase C1q or removal of fluid-phase C1q protected GC from being killed by NNRS. Also, prior addition of C1q to NNRS protected the GC from the bactericidal effect of the serum. Allowing the survival of GC by preincubation in the presence of an excess amount of the free C1q was the most effective way of creating and maintaining bacteremia in newborn rats. We propose that two factors could contribute to efficient GC survival in NNRS: (i) precoating GC with C1q before exposure to the bactericidal NNRS and (ii) the interference of free human C1q with the rat complement cascade and the subsequent assembly of a nonfunctional membrane attack complex without resultant cell death (11).

Our experiments suggest that GC may use alternative strategies for survival in the host. This report illuminates the mechanism by which C1q-mediated GC resisted the bactericidal effect of rat serum, resulting in GC bacteremia. The i.p. injection of GC with the excess C1q allowed the microorganism to multiply and establish an infection that lasted several days. The infecting dose of GC consisted of 40 μg of C1q per ml. It has been reported that in rats, the half-life for ^{125}I-labeled human C1q in vivo was 12.4 h (5). This suggests that the C1q concentration was reduced from 40 to 0.08 μg/ml during the 4.5-day experimental period. Further reduction in the C1q concentration was associated with decreases in the numbers of GC CFU per milliliter on days 5 and 6 of approximately 100 and 1,000 times, respectively, which agreed with the above estimation.

One of the relevant questions we pursued was whether C1q of nonhuman species would increase GC virulence. It is highly unlikely that rat C1q would protect GC, for the following reason: both in vitro and in vivo experiments indicated that pretreatment of GC with rat serum, which on average contains about 40 μg of C1q per ml, resulted in killing of the microorganism. In contrast, pretreatment with human serum as a source of C1q or with human purified C1q protected GC from the killing effect of NNRS in vitro and in vivo, even at a concentration of 2 μg/ml. Despite the high level of homology between the human and rat C1q, the antibody to human C1q does not recognize rat C1q. In our experiments, the use of an anti-human C1q antibody resulted in a dose-dependent abolition of the virulence of the C1q-treated GC and protected rats from bacteremia. This suggests that the reduction of the virulence function was associated with the neutralization of human, but not rat, C1q.

Several other experiments documented that C1q was the human factor increasing GC virulence. The dissemination of GC either did not occur or was substantially reduced in pups inoculated with GC after preincubation with human C1q-deficient serum, heat-inactivated purified C1q, and collagen type IV, which is structurally related to C1q. Finally, the ineffectiveness of purified C3 clearly demonstrated that the crucial component of human serum involved in the bacteremic spread of GC was C1q, not C3.

We propose that the mechanism of C1q-dependent infection included GC resistance to the bactericidal activity of the NNRS. This was indicated by the survival of GC both in serum (in vitro) and in blood (in vivo). The lack of a bactericidal effect of NNRS on GC preincubated with C1q resembles the survival of GC that may occur in human serum (6, 11) and correlates with the occurrence of bacteremia in both rat and human disseminated gonococcal infection.

The mechanism of C1q-dependent virulence may include C1q binding to GC. Human C1q has been shown to bind to *N. gonorrhoeae* JC1 in a dose-dependent manner (24). The C1q concentration that saturated binding sites on JC1 was about 8 μg/ml. Our unpublished data indicated that four GC peptides may bind C1q directly on a Western blot (immunoblot) assay. Which of these peptides contributes to the C1q-mediated virulence of GC remains to be elucidated.

Previous reports indicated that human C1q efficiently activates rat complement (13). We propose that the interaction of GC with human C1q may activate and deposit newborn rat complement in a configuration that has no lytic effect on GC. A related phenomenon was proposed to occur in human serum, in which GC activated complement in a configuration that was not able to kill the microorganism (11). The alternate hypothesis is that human C1q may prevent GC contact with the newborn rat complement by shielding its surface as a "capsule." Therefore, rat complement would be less likely to reach lipooligosaccharide and other structures of bacteria where receptors for bactericidal antibody are documented to be expressed.

In addition to disseminated gonococcal infection, bacteremic events are suspected to occur in other clinical forms of gonococcal infections (9). The symptoms of PID may include fever, chills, hepatitis, and peritonitis, suggesting the occurrence of bacteremia. We have recently studied a few patients with gonococcal PID. *N. gonorrhoeae* was found in peritoneal fluid and blood samples of four PID patients. In direct peritoneal fluid smears, the GC found were heavily coated with C1q (25). This strongly suggests that C1q-GC-mediated peritonitis and bacteremia in the experimental infection may be related to the human infection.

Experiments showed that disseminated infection in rat pups was established with five GC isolates. Further studies to characterize this phenomenon for strains of different clinical and biological characteristics are in progress. The finding that C1q may act as a virulence factor is very intriguing and may represent an example of bacterial parasitism that utilizes human factors in addition to microbial antigens (14, 19, 22, 23). The practical aspect of our experimental model is that it provides a simple option for studying gonococcal infection. The existence of several days of lasting bacteremia also offers novel, exciting opportunities to investigate preventive and therapeutic approaches. Studies documenting applications of experimental newborn rat infection and the role of C1q in the pathology of gonococcal infection are in progress.

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