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In Vitro Kinetics of Phagocytosis and Intracellular Killing of Gonococci by Peritoneal Macrophages from Mice Deficient in Complement Component 5

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Unstimulated resident peritoneal macrophages were harvested from complement-sufficient ($C5^+$) and complement-deficient ($C5^-$) mice by peritoneal lavage and cultured for 14 h. Adherence to cover slips was determined, and the monolayer was infected with transparent T_1 gonococci. At various times after infection, the macrophages were observed for both attachment and phagocytosis of the gonococci by scanning and transmission electron microscopy. This analysis indicated that $C5^+$ macrophages were capable of immediate phagocytosis of gonococci, with maximal phagocytosis occurring by 60 to 90 min. In contrast, $C5^-$ macrophages had a greater lag time before initiation of phagocytosis; this event was started by 30 min and completed by 90 min. The intracellular gonococci which were phagocytized by either $C5^+$ or $C5^-$ mice were completely killed after 30 min of incubation. It appears that $C5^-$ mice are at a disadvantage in the early kinetics of the phagocytosis of gonococci, but that this does not affect the ultimate intracellular destruction of gonococci.

The importance of the polymorphonuclear neutrophils (PMN) in gonococcal infection has been described (7, 8, 14) with regard to adherence and intracellular killing. However, the role of macrophages in gonococcal infection is not well defined. Drutz (8), on the other hand, has shown that human monocytes and macrophages kill intracellular gonococci without difficulty. Blake and Swanson (2) and Jones and Buchanan (11) both showed an association of gonococci with mouse peritoneal macrophages. Ota et al. (15) also reported phagocytosis by murine macrophages. In these reports, there is no information concerning the intracellular fate of the ingested gonococci.

It has been reported that mouse strains which lack the component 5 of complement ($C5^-$) harbor more bacteria than mouse strains having normal C5 ($C5^+$) (3, 9, 16, 17). In these infections, which involve *Streptococcus pneumoniae* and *Listeria*, *Corynebacterium*, and *Staphylococcus* species, there was a difference in susceptibility to infection between a $C5^+$ and a $C5^-$ strain. These studies suggested that C5 played a role in promoting phagocytosis. Since B10.D2 old and new strain mice, as far as is known, differ only at the *Hc* locus, which is the determinant for the presence or absence of C5, they were chosen for these studies.

In the present study, resident peritoneal macrophages from both $C5^+$ and $C5^-$ mice were examined for their ability to ingest and kill intracellular gonococci.

MATERIALS AND METHODS

Organisms. *Neisseria gonorrhoeae* strain 12094 was used throughout the investigations. These organisms were of colony type T_1 and grew as transparent colonies according to the classification scheme described by Swanson (18). These organisms were maintained on GC agar base (Difco Laboratories, Detroit, Mich.) supplemented with IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.). When used for antigen production, gonococci were grown in liquid media as previously described (4).

Animals. Female B10.D2/OSN ($C5^-$) and B10.D2/NSN ($C5^+$) mice weighing approximately 18 to 25 g were obtained from Jackson Laboratories, Bar Harbor, Maine. The B10.D2/OSN and B10.D2/NSN mice are congenic, resistant mice possessing the same $H\text{-}2^d$ locus and differing from each other only at the *Hc* locus.

Preparation of immunogens. The ribosomal preparations were prepared by a modification of the method of Youmans and Youmans (21) as previously described (4). Gonococcal outer membranes were isolated by modification of the method of Wolf-Watz et al. (20) as described previously (4). Lipopolysaccharide (LPS) was extracted from whole cells (50 g [wet weight]) by the hot phenol method of Westphal and Jann (19).

Isolation of macrophages. Unstimulated resident macrophages were harvested from either a B10.D2/OSN or B10.D2/NSN mouse by using a peritoneal lavage of 3 ml of medium 199 (GIBCO, Grand Island,

N.Y.) containing a 0.5% streptomycin-penicillin solution (GIBCO), 0.01% bovine serum albumin (fraction V) (Miles Laboratories, Inc., Kankakee, Ill.), and 0.5 U of sterile heparin per ml. Mixing of the medium and peritoneal fluid was accomplished by gentle manipulation of the abdomen followed by removal via a cannula (18 gauge by 2.25 in. [5.7 cm]; intravenous catheter, C. R. Bard, Murray Hill, N.J.). The exudate cells were washed in medium 199 at 2,500 rpm for 10 min at 4°C. The cells were suspended gently, and the cell concentration was adjusted to 2×10^6 cells per ml. A 1-ml amount of this cell suspension was added to plastic Leighton tubes (Costar, Cambridge, Mass.), and the cells were allowed to adhere for 1 h at 37°C in an atmosphere of 5% CO_2. After 1 h, the medium was removed, 1 ml of fresh medium 199 supplemented with 20% heat-inactivated horse serum (HIHS) (GIBCO) was added, and the cultures were incubated at 37°C in an atmosphere of 5% CO_2. After overnight incubation, the medium was discarded, and 1 ml of gonococci (8×10^6 to 4×10^7) colony-forming units [CFU]) suspended in medium 199 with 20% HIHS was added to the monolayers. Phagocytosis was allowed to take place for various time intervals. Control cover slips consisting of macrophages alone were stained with Giemsa and examined microscopically for morphology and enumeration. Cover slips containing the macrophage monolayers and gonococci were fixed and either processed for scanning electron microscopy (SEM) or transmission electron microscopy (TEM) or used in determination of intracellular killing.

Monolayer preparation for SEM. Cover slips were prepared for electron microscopy by using the critical-point drying technique. First, cover slips were fixed in Karnovsky fixative (12) overnight, washed with 0.2 M NaCaC, and postfixed with 1.0% OsO_4 in 1.0 M NaCaC buffer. Dehydration was achieved by passing the cover slips through 30, 50, 70, 85, and 95% ethyl alcohol for 5 min each and finally 100% ethyl alcohol for 10 min. The macrophages were dried in a critical-point drying apparatus. After being dried, the cover slips were mounted on SEM stubs, and the macrophages were coated with approximately 10 to 11 ± 1.5 nm of gold and palladium in a vacuum evaporator. Specimens were observed in a Hitachi S-500 scanning electron microscope.

Monolayer preparation for TEM. The macrophage monolayer was washed three times with saline and then covered with 2.5% glutaraldehyde in 0.1 M NaCaC buffer for 5 min. The glutaraldehyde solution was discarded and replaced with a cocktail mix of 1 part 2.5% glutaraldehyde and 2 parts 1.5% OsO_4 in 0.1 M NaCaC for 20 min (10). After being rinsed with saline three times, the macrophage monolayer was covered with 0.25% uranyl acetate in 0.1 M acetate buffer (pH 6.3) for 30 min. The monolayers were rinsed with saline three times and dehydrated in 30, 50, 70, 85, 95, and 100% ethanol for 5 min each. After this, the macrophage monolayers were infiltrated in 1 part Spurr to 1 part ethanol for 30 min, 2 parts Spurr to 1 part ethanol for 30 min, straight Spurr for 30 min, and then a straight Spurr for 60 min. The monolayer side of the cover slip was then placed on top of a small beam capsule overfilled with Spurr. This was heated in a 65°C oven for 2 days. The cover slip was dipped in liquid nitrogen and then into hot water to remove the cover slip. Individual beams were sectioned with an LKB 8800. Sections were visualized in a Philips 201 microscope.

Determination of intracellular killing. Gonococci (8×10^6 to 4×10^7 CFU) of the transparent type 1 were inoculated into Leighton tubes containing monolayers of 10^6 macrophages or into Leighton tubes alone as a control for nonspecific adherence to the cover slips. The gonococci were suspended in medium 199 supplemented with 20% HIHS. Phagocytosis was allowed to take place for 0, 15, 30, 60, 90, and 120 min on a gyratory platform at 75 rpm at 37°C in a 5% CO_2 atmosphere. At appropriate time intervals, the monolayers were rinsed with medium 199, and the rinse was plated onto GC agar for quantitation of unphagocytized and unattached gonococci. The monolayers were pulsed with 30% fresh rat serum in medium 199 for 30 min at 37°C in a 5% CO_2 atmosphere to kill both unattached and attached gonococci. The monolayers were rinsed again and placed in sterile distilled water for 3 min for osmotic disruption of the macrophages. The lysate was quantitatively plated onto GC agar and placed into a CO_2 incubator at 37°C for enumeration of viable gonococci. As a control for osmotic stability, gonococci were suspended in sterile distilled water to check for viability. After 30 min of incubation in water at 37°C, the gonococci were 99% viable. Macrophages were suspended in distilled water for 1, 3, 5, and 10 min and examined for intact morphology by Giemsa staining. After 3 min of incubation, the macrophages were totally disrupted.

RESULTS

Attachment and phagocytosis of gonococci by immune and nonimmune macrophages. The phagocytosis and attachment of transparent gonococci by murine peritoneal macrophages are shown in Fig. 1. The kinetics of normal peritoneal macrophage monolayers being infected with gonococci in a 20:1 ratio (4×10^7 gonococci to 2×10^6 macrophages) are depicted in Fig. 1A. In general, the $C5^+$ macrophages had low numbers of attached gonococci in the early times of infection. The phagocytic events associated with these macrophages commenced immediately and were completed in approximately 60 to 90 min. However, the $C5^-$ macrophages had a high degree of attachment during the first 30 min, with a correspondingly low incidence of phagocytosis. A typical example of the early phagocytic events seen in nonimmune $C5^-$ macrophages by SEM at 5 min postinfection is shown in Fig. 2. This micrograph corresponds to the data shown in the 5-min period for $C5^-$ macrophages (Fig. 1A). The $C5^-$ macrophages appeared to be at a disadvantage when compared kinetically with $C5^+$ macrophages. However, by 90 min postinfection, both groups of macrophages had ingested at least 20 gonococci per cell. This event was further confirmed by TEM (Fig. 3 and 4) of infected nonstimulated resident peritoneal macrophages from $C5^+$ and $C5^-$ mice at 120 min postinfection. Numerous morphologically intact gonococci were within the cytoplasm

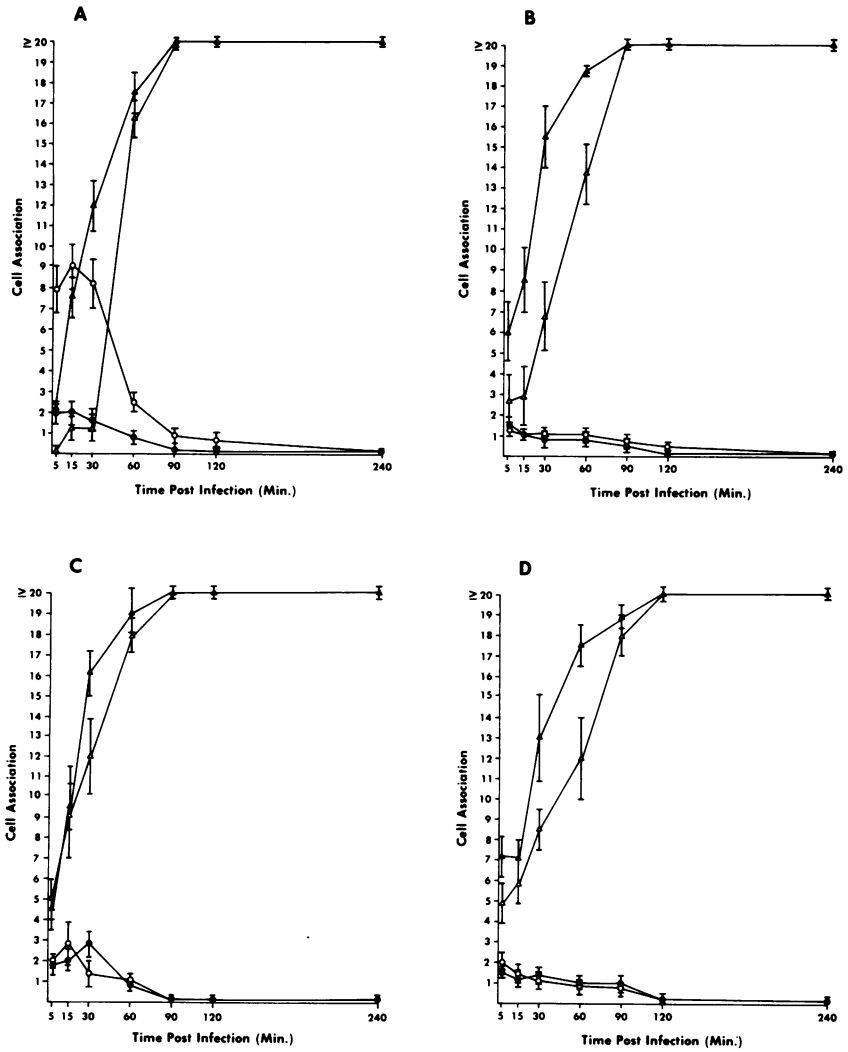

FIG. 1. Attachment and phagocytosis of immune and nonimmune macrophages from $C5^+$ and $C5^-$ B10.D2 mice. (A) Nonimmune mice; (B) immunization with 100 μg of LPS; (C) immunization with 100 μg of outer membranes prepared by the method of Wolf-Watz et al. (20); (D) immunization with 25 μg of ribosomal preparations. △, Phagocytosis by $C5^-$ macrophages; ○, attachment by $C5^-$ macrophages; ▲, phagocytosis by $C5^+$ macrophages; ●, attachment by $C5^+$ macrophages.

of both $C5^+$ and $C5^-$ macrophages. Thus, the early disadvantage of phagocytosis in $C5^-$ macrophages was no longer present by 90 min postinfection.

Macrophages from $C5^+$ and $C5^-$ mice immunized with LPS, outer membranes, or ribosomes (Fig. 1B to D) had low rates of gonococcal attachment and essentially no lag time in their initiation of phagocytosis. The $C5^-$ macrophages, although activated by immunization with LPS or ribosomes, were less efficient in phagocytosis than immune $C5^+$ macrophages during the early kinetics but had achieved parity by 90 min postinfection. However, macrophages from mice ($C5^-$ or $C5^+$) immunized with outer membranes prepared by the method of Wolf-Watz et al. (20) appeared to have similar rates of phagocytosis and attachment. Further, immune $C5^-$ macrophages did not appear to have the lag time in phagocytosis when compared with nonimmune $C5^-$ macrophages.

Intracellular killing of gonococci by normal peritoneal macrophages. The kinetics of intracellular killing of gonococci by resident $C5^+$ and $C5^-$ peritoneal macrophages are shown in Fig. 5. Approximately 8×10^6 transparent gonococci

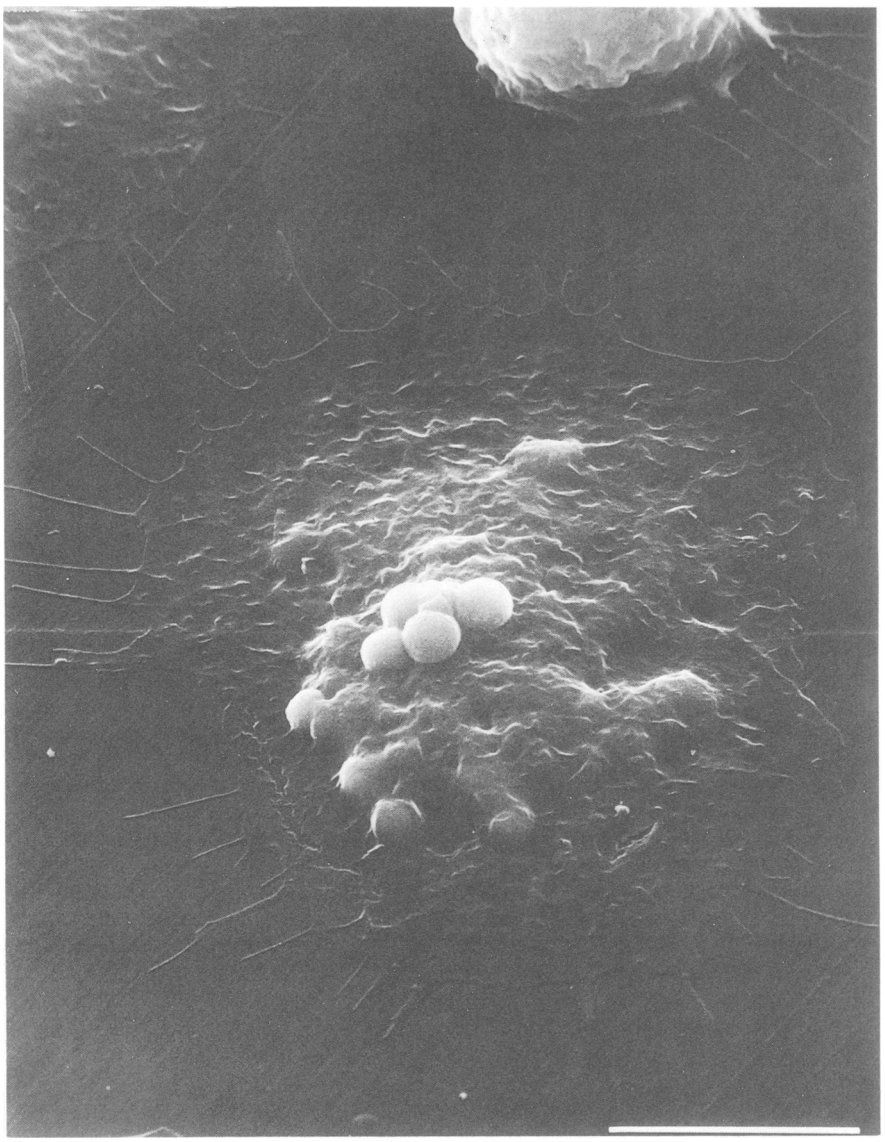

FIG. 2. SEM of an infected nonstimulated resident peritoneal macrophage of a nonimmunized $C5^-$ mouse at 5 min postinfection. Bar, 5 μm.

were used to infect macrophage monolayers. At various time intervals, the culture from the infected monolayers was quantitatively sampled for presence of viable gonococci (Fig. 5A). By 60 min postinfection ≥99.99% of the original inoculum was either attached to or phagocytized by the macrophages. Nonadherent gonococci, as well as externally attached gonococci, were killed by a 30-min pulse of fresh normal rat serum. After the rupture of infected macrophages by osmotic lysis, quantitative plate counts of the lysate were determined (Fig. 5B). Although the macrophages at 120 min postinfection (Fig. 3 and 4) had gonococci which appeared intact, the organisms were clearly incapable of cell division (Fig. 5B). The data indicated that by the earliest sampling period the intracellular gonococci were not viable and that at no later time period were there any viable organisms.

DISCUSSION

The use of mice as models of gonococcal infection has been reported recently by several

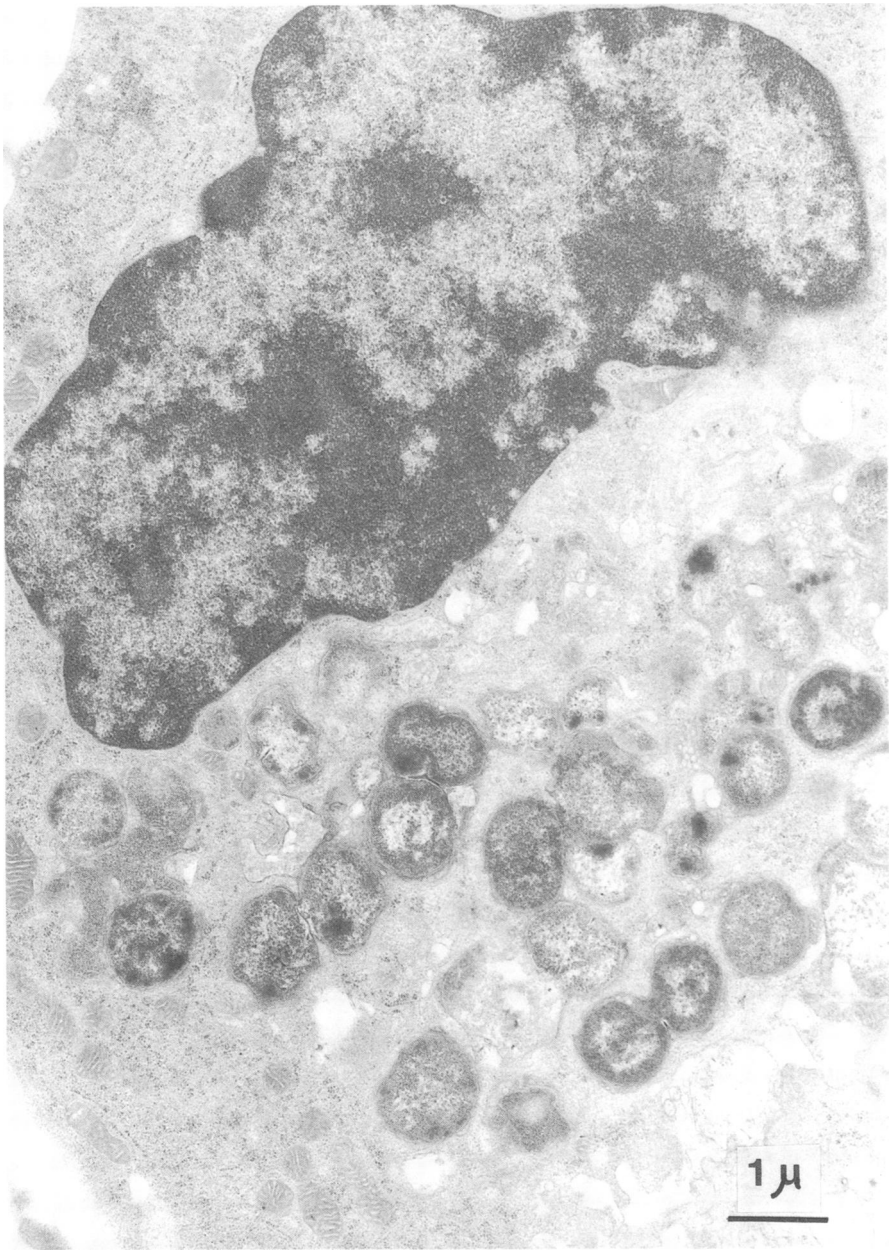

FIG. 3. TEM of an infected nonstimulated resident peritoneal macrophage of a nonimmunized $C5^+$ mouse at 120 min postinfection.

investigators (1, 6, 13). Infections have been induced subcutaneously in implanted chambers (1), peritoneally (6), and vaginally (13). Corbeil et al. (6) produced disseminated gonococcal infection with gonococci suspended in mucin and hemoglobin. We confirmed and extended these results, using $C5^+$ and $C5^-$ mice. These studies demonstrated that $C5^-$ mice are more susceptible to gonococcal infection (50% lethal dose, 10^3 CFU) than $C5^+$ (50% lethal dose, 5×10^4 CFU). Further, in the absence of mucin-hemoglobin, $C5^-$ mice can be infected, but clear the infection in 12 h (M. D. Cooper, M. F. Fedyk, and S. A. Floyd, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B84, p. 28).

It has been reported that gonococci which

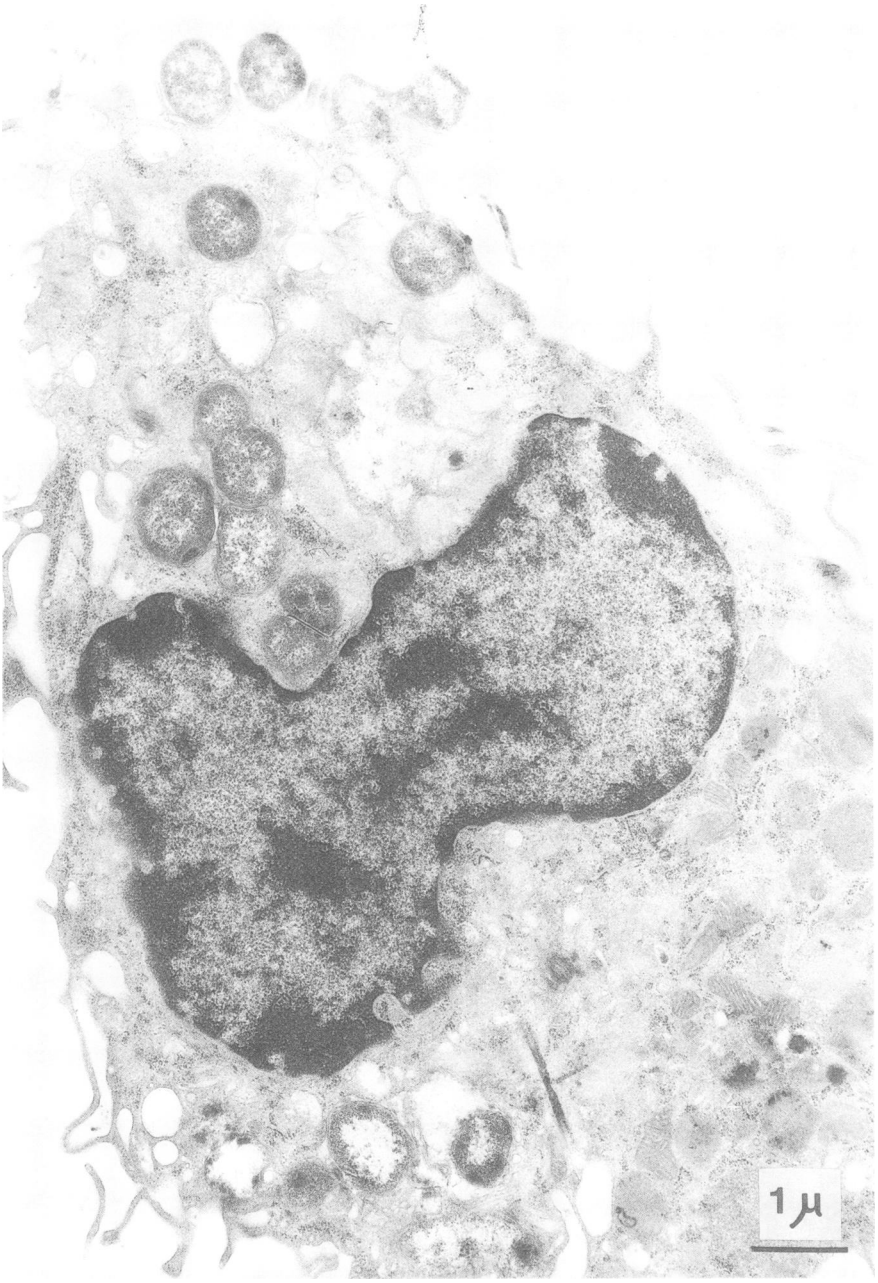

FIG. 4. TEM of an infected nonstimulated resident peritoneal macrophage of a nonimmunized $C5^-$ mouse at 120 min postinfection.

attach to PMN surfaces resist phagocytosis and are not killed by the PMN (7). The apparent failure of PMN to kill attached gonococci appears to be a failure of the PMN to enclose the gonococcus within a phagosome rather than a failure of the intracellular bactericidal mechanism. However, the role of the other phagocytic cell, the macrophage, in the killing of gonococci is less well understood.

Blake and Swanson (2) reported that pili enhance attachment of gonococci to tissue culture cells and reduce their attachment to and ingestion by unstimulated resident mouse peritoneal macrophages. Jones and Buchanan (11), using

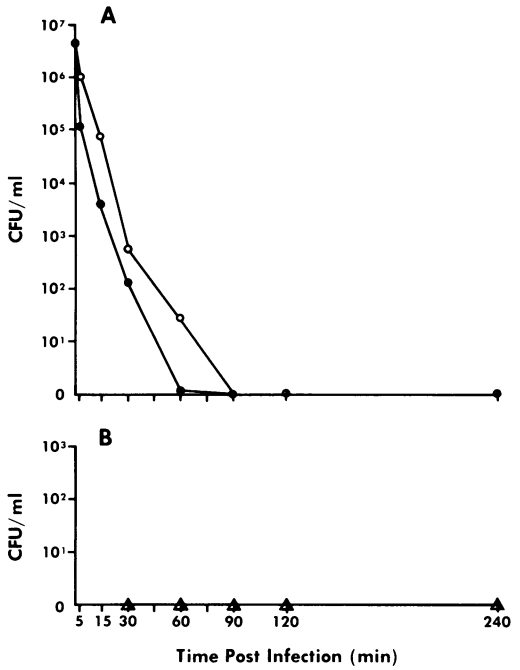

FIG. 5. Kinetics of intracellular killing of *N. gonorrhoeae* 12094 transparent type T_1 by resident peritoneal $C5^+$ and $C5^-$ macrophages. (A) Clearance of gonococci from culture media. ○, Viable gonococci per milliliter of culture medium on $C5^-$ macrophage monolayers; ●, viable gonococci per milliliter of culture medium on $C5^+$ macrophage monolayers. (B) Release of viable gonococci from lysed macrophages. △, Organisms from $C5^-$ macrophages;▲, organisms from $C5^+$ macrophages.

radioactive labels, quantitated the association of gonococci with murine macrophages under various experimental conditions. They found that homologous rabbit antiserum enhanced the association for virulent T_1 gonococci by 70-fold and suggested that this enhancement occurs through the Fc receptor for immunoglobulin G.

Several reports (3, 9, 16, 17) have shown that animals which have deficiencies in their complement components are more susceptible to bacterial infection than those with intact complement systems. Shin et al. (17) reported that $C5^-$ B10.D2 mice were more susceptible to pneumococcal peritonitis than $C5^+$ B10.D2 mice, which showed a 10-fold higher 50% lethal dose than $C5^-$ animals. They also showed an impairment of phagocytosis when $C5^-$ sera was compared with $C5^+$ or $C5^-$ sera with the C5 restored. Our data is analogous to that from their study in that $C5^-$ mice were more susceptible to gonococcal infection than $C5^+$ mice and, further, that macrophages from mice which have a genetic C5 defect in their complement components were at an initial disadvantage in the rate at which they phagocytize gonococci. These $C5^+$ macrophages have seemingly low rates of attachment to the gonococci. However, it is also possible that the lower rate of attachment could be due to the higher rate of phagocytosis. This would result in fewer surface attachments because of a greater rate of engulfment. This does not account for the differences in rates of phagocytosis between $C5^-$ and $C5^+$ macrophages. The electron micrographs showed that unstimulated resident macrophages from both $C5^-$ and $C5^+$ mice were quite capable of engulfing large numbers of gonococci. These gonococci appeared to be morphologically intact within the macrophage. However, the evidence presented clearly showed that although the intracellular gonococci appeared to be relatively intact by TEM, they were not able to produce CFU when released from lysed macrophages, and there was no difference in the intracellular killing of gonococci by $C5^+$ or $C5^-$ macrophages. Activation of the macrophages by immunization of mice with gonococcal subcellular components resulted in an immediate uptake of organisms because of the presence of LPS in the membrane and ribosomal preparations (5). However, although activated, the $C5^-$ macrophages remained less efficient in their rates of phagocytosis than $C5^+$ macrophages. Our data does not support the speculation of Ota et al. (15) that gonococci may be capable of intracellular survival within murine macrophages and thus capable of disseminating the infection.

Petit (16) suggested that $C5^+$ mice are at an advantage because macrophages are thought to produce C5 protein. During uptake of bacteria, some of the C5 molecules are hydrolyzed, producing the chemotactic C5a and thus recruiting more macrophages into the infected area. This may, in part, explain the initial lag in phagocytosis of gonococci in $C5^-$ mice. Another explanation may be that the C5b receptor site may not be on the $C5^-$ macrophage; thus, the C5 which could be generated by potential activation of complement by the alternate pathway from LPS present in the gonococcal inoculum would not be able to attach to these macrophages.

In conclusion, it appeared macrophages of $C5^+$ and $C5^-$ mice were capable of phagocytosis and intracellular killing of gonococci. There appeared to be a different rate of phagocytosis between these macrophages, but it did not compromise their ability to prevent intracellular killing of gonococci.

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