# THE INTERACTION IN VITRO BETWEEN GROUP B MENINGOCOCCI AND RABBIT POLYMORPHONUCLEAR LEUKOCYTES

## Demonstration of Type Specific Opsonins and Bactericidins*

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Plate 60

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Since the earliest descriptions of the bacterium *Neisseria meningitidis*, examination of blood or spinal fluid from patients with meningitis has revealed meningococci within polymorphonuclear leukocytes. Indeed, for many years the generic name of this organism was *Neisseria intracellularis*. Despite these observations, factors required for phagocytosis of meningococci, and the intracellular fate of these organisms have not been delineated.

This report describes studies on the interaction in vitro between group B meningococci, serum factors, and rabbit polymorphonuclear leukocytes. The results indicate meningococci are rapidly killed following ingestion by leukocytes, and that efficient ingestion requires the presence of specific antibody and of heat-labile serum factors. Some antisera exhibit direct bactericidal action on meningococci. The findings furthermore indicate that opsonic and bactericidal antibodies exhibit *type*, rather than *group* specificity.

### *Materials and Methods*

*Medium.*—The medium employed in both phagocytosis and bactericidal experiments consisted of sterile Gey's balanced salt solution (Microbiological Associates, Inc., Bethesda, Md.) and 0.1% gelatin (Difco Laboratories, Detroit, Mich.). Gelatin was dissolved in distilled water (10% concentration), adjusted to pH 7.4 with 0.1 NaOH, and autoclaved. The 10% gelatin was stored in 5 ml aliquots at 4°C; before each experiment it was heated and added 1:100 to Gey's balanced salt solution. This solution is hereafter designated gel-Gey's medium.

*Preparation of Leukocyte Suspensions.*—The technique for obtaining homogenous populations of rabbit polymorphonuclear leukocytes has been described previously (1). New Zealand Red female rabbits were used throughout the study. Employing aseptic techniques, 250 ml of sterile isotonic saline containing 0.1% glycogen (cp, Amend Drug & Chemical Co., Inc., New York) was injected intraperitoneally through a No. 18 short needle. 3- 4 hr later, a No. 15 long perforated needle was inserted into the peritoneal cavity and exudate was drained by gravity into 500 ml Erlenmeyer flasks containing 10 mg of heparin (Abbot Laboratories, Chicago, Ill.). To maintain flow of the exudate, it was often necessary to knead the abdomen.

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The peritoneal drainage was 100–200 ml and contained 3–9 $\times$ $10^3$ leukocytes per $mm^3$ of which 99% were granulocytes. In most cases, cells from one rabbit were utilized in a given experiment.

The exudate was screened through sterile gauze to remove clumped cells and fibrin. Following volume measurements and leukocyte counts, the screened exudate was dispersed into 40 ml sterile conical centrifuge tubes and sedimented (International Centrifuge, model UV, International Equipment Co., Boston, Mass.) at 1000 rpm for 10 min. The cell-free supernatant was drained off, and the cell concentration adjusted to 20,000 per $mm^3$ by resuspending cells in gel-Gey's medium. The preparation of leukocyte suspensions was performed at room temperature.

All rabbits received three initial priming injections before peritoneal exudates were harvested. Animals yielding adequate exudates were used every 10–20 days for 7 months without a decrease in leukocyte production.

TABLE I
*Properties of Group B Meningococci*

| Strain | Source | Place and time of isolation | Carbohydrate fermentations* | | | Oxidase reaction |
|---|---|---|---|---|---|---|
| | | | D | M | S | |
| 78 | Blood | Germany April, 1965 | + | + | − | + |
| 79 | Blood | Germany April, 1965 | + | + | − | + |
| NE-15 | Spinal fluid | United States 1955 | + | + | − | + |
| 55 | Blood | United States April, 1965 | + | + | − | + |
| 161 | Nasopharynx | Ft. Knox, Ky. November, 1966 | + | + | − | + |
| I-3-5 | Nasopharynx | Ft. Dix, N. J. February, 1966 | + | + | − | + |

* D, dextrose; M, maltose; S, sucrose.

*Bacteria.*—All *Neisseria meningitidis* strains were obtained from the Department of Bacteriology, Walter Reed Army Research Institute, Washington, D. C. Characteristics of group B meningococci are shown in Table I. In addition, two group A strains (45 and 98) and two group C strains (47 and 95) were employed for the determination of the specificity of bactericidal activity in immune serum.

The strain of coagulase-negative *Straphylococcus albus* used in this study is a stock laboratory culture and was kindly supplied by Dr. Zanvil A. Cohn.

*Preparation of Bacterial Suspensions.*—Meningococci were maintained by daily subculture on Mueller-Hinton chocolate agar (Difco). Culture plates were incubated at 37°C in the presence of moisture and $CO_2$ (candle jar). In preparation for each test, a 16 hr agar culture was transferred to 125 ml Erlenmeyer flasks containing 25 ml of Eugonbroth (Baltimore Biological Laboratory, Inc., Baltimore, Md.). Broth cultures were placed on an Eberbach reciprocating shaker (6,000 revolutions per hr (rph)) and incubated aerobically at 37°C. 3–4 hr later, 0.1–1.2 ml of the bacterial suspension was diluted in 2.0 ml of gel-Gey's medium.

Staphylococci were maintained on Penassay agar (Difco). Prior to each phagocytosis test,

5 ml of Eugonbroth was inoculated with an agar culture and incubated aerobically at 37°C. After 18 hr, 0.05 ml of the standing broth culture was also diluted in 2.0 ml of gel-Gey's medium. The final concentration of bacteria in gel-Gey's medium was 0.3–3.0 $\times$ $10^8$ organisms per ml.

*Serum.*—Normal rabbit serum was collected from animals used only for this purpose. Immune rabbit serum was obtained at weekly intervals following immunization. The preparation of immunizing antigens and the method of immunization will be described under Results. All serum specimens were obtained by bleeding rabbits from the heart. Blood was placed in 40 ml sterile conical centrifuge tubes which were left standing in a slanted position for 1 hr at room temperature. After rimming the clot, tubes were placed at 4°C for 18 hr to insure maximal clot reaction. Serum was then transferred to 15 $\times$ 120 mm sterile test tubes and centrifuged (International, model UV) at 2000 rpm for 10 min. Aliquots of 1 ml were placed in 45 $\times$ 15 mm screwcap sterile vials (Arthur H. Thomas, Co., Philadelphia) and stored at −20°C. Serum was frozen once and thawed immediately before each experiment.

*Preparation of Phagocytosis and Bactericidal Tests.*—*Phagocytosis tests* were done in 10 $\times$ 75 mm test tubes. To each of these tubes was added 0.8 ml leukocyte suspension, 0.1 ml bacterial suspension, and 0.1 ml serum; thus, the total volume was 1.0 ml and contained 15–20 $\times$ $10^6$ leukocytes per ml, 3–30 $\times$ $10^6$ bacteria per ml, and 10% rabbit serum. The period of time between collection of peritoneal exudates and suspension of cells in the test system never exceeded 45 min.

*Bactericidal tests* were also done in 10 $\times$ 75 mm test tubes. To each tube was added 0.8 ml gel-Gey's medium, 0.1 ml bacterial suspension, and 0.1 ml serum.

All tubes were sealed with rubber stoppers covered with parafilm and tumbled end over end (Spinerette, New Brunswick Scientific Co., New Brunswick, N. J.) for 2 hr at 37°C. This degree of mixing ensured adequate contact between leukocytes and bacteria.

After 2 hr of incubation, the pH of leukocyte-bacteria-serum mixtures remained between 7.2–7.8. More than 95% of leukocytes, as demonstrated by negative nuclear staining with 1% trypan blue, were viable after this period of incubation.

Control tubes were employed in each phagocytosis experiment. In addition to the test sample, a static control (not tumbled) of the same leukocyte-bacteria-serum mixture, and a serum control containing 0.8 ml of gel-Gey's medium without leukocytes, 0.1 ml bacterial suspension, and 0.1 ml serum were used to rule out extracellular killing of bacteria. A *Staph. albus* requiring only the presence of normal rabbit serum for phagocytosis was used as a positive control to insure that each leukocyte preparation was capable of adequate phagocytosis.

*Enumeration of Viable Bacteria.*—

*Phagocytosis test:* The technique employed for the enumeration of viable bacteria is a modification of that used by Hirsch and Strauss (2) and by Cohn and Morse (3). To determine the total number of viable bacteria, a 0.003 ml aliquot was removed with a standardized platinum loop at time intervals of 0, 60 and 120 min and placed in 1.0 ml of distilled water containing 0.01% crystallized bovine plasma albumin (Armour Pharmaceutical Co., Kankakee, Ill.). The sample was serially diluted and 0.1 ml of the dilutions spread on the surface of Mueller-Hinton chocolate agar. Since meningococci remain viable in this diluent whereas granulocytes are lysed, the number of viable bacteria from these dilutions is the total number of extracellular and intracellular viable organisms.

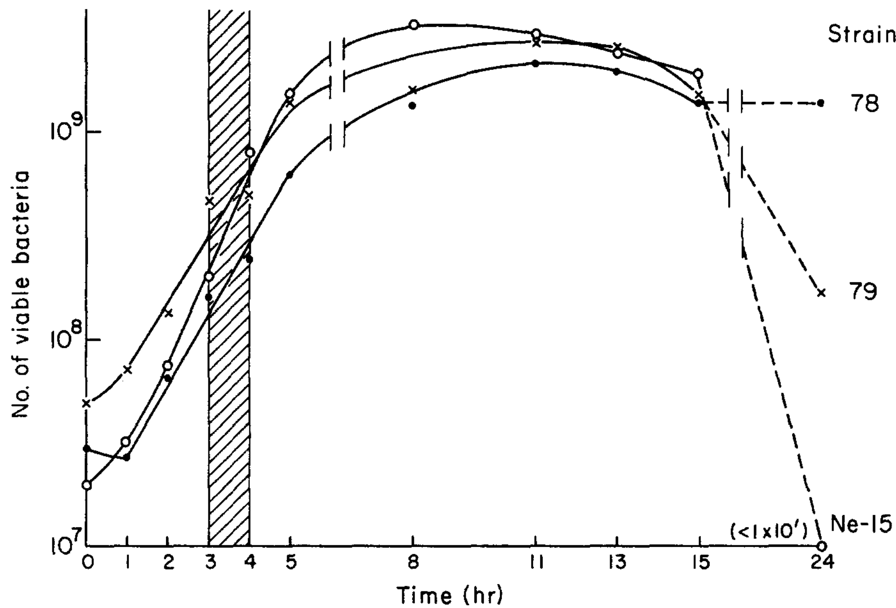
Simultaneously, a 0.003 ml aliquot was removed and placed in 1.0 ml of gel-Gey's medium. After thorough mixing, the suspension was centrifuged at 450 rpm for 4 min, and 0.1 ml of the supernatant fluid removed, serially diluted in gel-Gey's medium and colony plated. Because low speed centrifugation sediments leukocytes but not bacteria (4), the number of viable organisms from these dilutions represented the number of extracellular bacteria in the suspension.

Aliquots removed from the standing control tubes were dispensed into 1.0 ml of 0.01%

albumin water, and those removed from serum and *Staph. albus* control tubes were placed in 1.0 ml of gel-Gey's medium. These suspensions were serially diluted and plated as described above except *Staph. albus* was cultured on Penassay agar.

*Bactericidal test:* At time periods of 0 and 120 min, 0.003 ml samples were removed from these test suspensions, dispensed in 1.0 ml of gel-Gey's medium, serially diluted and colony plated.

All chocolate agar plates were incubated at 37°C in the presence of moisture and $CO_2$ (candle jar) and bacterial colonies counted after 18 hr. Penassay agar plates were incubated aerobically at 37°C for the same period of time.




TEXT-FIG. 1. The growth curves of three group B meningococcal strains in shaking broth cultures.

## RESULTS

*Log Phase Growth of Meningococci.*—To insure that the total population of organisms used in each experiment were in the same growth phase, a growth curve was determined for each group B meningococcal strain.

A 16 hr agar culture was transferred to 125 ml Erlenmeyer flasks containing 25 ml of Eugonbroth. Broth cultures were placed on an Eberbach reciprocating shaker (6000 rph) and incubated aerobically at 37°C. At time intervals between 0 and 24 hr, 0.1 ml was removed, dispensed into 0.9 ml Eugonbroth, serially diluted, and colony plated on chocolate agar. Plates were incubated at 37°C in the presence of moisture and $CO_2$ (candle jar) and bacterial colonies counted after 18 hr.

Text-fig. 1 shows the growth curve for strains 78, 79, and NE-15. A similar log phase of growth was seen for each strain after 2–5 hr of incubation. There-

after, only strain 78 maintained a stationary growth phase whereas strains 79 and NE-15 underwent autolysis. Similar results were observed when Trypticase soy broth (Baltimore Biological Laboratory) was used as the culture medium. Reproducible viable bacterial counts could not be obtained from stationary broth cultures. In all experiments reported in this communication, meningococcal suspensions were prepared from 3–4 hr shaking broth cultures, as demonstrated by the shaded vertical bar.

*The Stability of Meningococci in the Suspending Medium.*—Since experimental results were based on the viability of organisms tested, it was necessary to determine the effect of medium constitutents on the growth and survival of meningococci. The viability of strain 78 in the suspending media is shown in Table II. Meningococci survived, and in fact multiplied slightly in the gel-

TABLE II
*Stability of Meningococcal Strain 78 in Medium Constituents*

| Medium | Bacteria per ml after incubation at 37°C | | | |
|---|---|---|---|---|
| | Zero | 30 min | 60 min | 120 min |
| Gey's BSS* + 0.1% Gelatin | $6.8 \times 10^7$ | — | — | $1.8 \times 10^8$ |
| Distilled $H_2O$ + 0.01% Albumin | $3.4 \times 10^7$ | $3.6 \times 10^7$ | $2.5 \times 10^7$ | $1.2 \times 10^7$ |

* BSS, balanced salt solution.

Gey's solution. Organisms suspended for 30 min in distilled water containing 0.01% albumin ceased to multiply but continued to survive for this period of time. This diluent was used only to determine the total number of viable bacteria in the test suspension; the time for this procedure never exceeded 30 min.

*The Viability of Group B Meningococci in Normal Rabbit Serum.*—The bactericidal effect of normal serum on meningococci has been described previously (5–8). It was therefore imperative prior to all experiments, to determine quantitatively the viability of each group B meningococcal strain in fresh normal rabbit serum.

To each 15 × 120 mm sterile test tube was added either: (*a*) 1.6 ml serum, 0.2 ml bacteria and 0.2 ml gel-Gey's medium or (*b*) 0.2 ml serum, 0.2 ml bacteria and 1.6 ml gel-Gey's medium. The total volume of each tube was 2.0 ml. All tubes were incubated standing at 37°C for 2 hr. At time intervals of 0 and 120 min, 0.1 ml was removed, dispensed in 0.9 ml gel-Gey's medium, serially diluted, and colony plated as described previously.

Results of the bactericidal effect of this serum are shown in Table III. Only strains 78, 79, and 161 were not killed in either serum concentration. Strain NE-15 and I-3-5 were killed to some degree in 80% serum (a 6-fold decrease in the number of viable bacteria after 120 min of incubation at 37°C) but continued to multiply in 10% serum. Strain 55 was completely killed in both

serum concentrations, though this bactericidal activity was lost following treatment of the serum at 56°C for 30 min. Because 10% serum was used in both phagocytosis and bactericidal tests, only those strains that remained viable in this serum concentration were employed in the studies that follow.

*The Effect of Polymorphonuclear Leukocyte Supernatant Fluid on the Viability of Meningococci.*—To insure that bactericidal substances were not released into the medium as leukocyte suspensions were tumbled end over end, the effect of leukocyte supernatant fluid on the viability of strain 78 was determined.

TABLE III
*Viability of Group B Meningococci in Normal Rabbit Serum*

| Strain | Bacteria per ml after incubation at 37°C | | | |
|---|---|---|---|---|
| | 80% Serum | | 10% Serum | |
| | Zero | 120 min | Zero | 120 min |
| 78 | $5.2 \times 10^6$ | $3.8 \times 10^7$ | $5.2 \times 10^6$ | $5.5 \times 10^7$ |
| 79 | $1.0 \times 10^7$ | $7.2 \times 10^7$ | $1.0 \times 10^7$ | $1.0 \times 10^8$ |
| NE-15 | $3.0 \times 10^6$ | $6.0 \times 10^5$ | $3.1 \times 10^6$ | $1.0 \times 10^7$ |
| 55 | $3.1 \times 10^6$ | $<1.0 \times 10^1$ | $4.6 \times 10^6$ | $<1.0 \times 10^1$ |
| 55 | $6.5 \times 10^6$ | $1.4 \times 10^7$ * | | |
| 161 | $1.2 \times 10^7$ | $4.7 \times 10^7$ | $1.1 \times 10^7$ | $8.9 \times 10^7$ |
| I-3-5 | $4.2 \times 10^6$ | $7.0 \times 10^5$ | $5.3 \times 10^6$ | $3.4 \times 10^7$ |

* Serum inactivated at 56°C/30 min.

Leukocytes were suspended in gel-Gey's medium containing 10% normal rabbit serum. Aliquots of 1 ml were dispensed into 10 × 75 mm test tubes which were sealed and tumbled end over end at 37°C for 120 min. At time intervals of 10, 60, and 120 min, two tubes were centrifuged at 450 rpm for 4 min. The cell-free supernatants were removed and 0.9 ml of the pooled supernate transferred to tubes containing 0.1 ml of the bacterial suspension. These tubes were in turn placed on a Spinerette at 37°C, and at 0, 60, and 120 min, 0.003 ml samples were removed, serially diluted, and plated as described previously.

Table IV shows that the number of viable bacteria of each sample increased 2–8-fold after 120 min of incubation with leukocyte supernatant fluids. These results thus demonstrate that extracellular leukocyte products did not affect the viability of meningococci.

### *Opsonic Activity*

*The Interaction of Group B Meningococci with Rabbit Polymorphonuclear Leukocytes in the Presence of Normal Rabbit Serum.*—The phagocytosis of meningococci in previous studies has been based on stained smears (9–12). Conflicting results have been reported and phagocytosis in the presence of normal animal serum has been described (10, 11).

Text-fig. 2 shows the interaction of three representative meningococcal strains with homogenous populations of rabbit granulocytes in the presence of fresh normal rabbit serum. The total number of viable bacteria increased 8–12-fold during the 2 hr period of incubation. Similar viable counts were observed in supernatant samples; thus, these meningococci were neither ingested nor killed by granulocytes in the presence of normal serum.

*The Interaction of Group B Meningococci with Rabbit Polymorphonuclear Leukocytes in the Presence of Immune Serum.*—Phagocytosis of meningococci in the presence of immune serum has been described previously (9, 13). In fact, during World War I, studies of phagoctosis by the stained smear method were used for the standardization of horse antiserum in the treatment of epidemic meningitis (14). In these early studies, quantitative techniques were not

TABLE IV
*Effect of Polymorphonuclear Leukocyte Supernatant Fluid on the Viability of Meningococcal Strain 78*

| Period of incubation of leukocyte suspension | Bacteria per ml after incubation in leukocyte supernatant fluid at 37°C | | |
|---|---|---|---|
| | Zero | 60 min | 120 min |
| *min* | | | |
| 10 | $9.6 \times 10^6$ | $1.7 \times 10^7$ | $2.9 \times 10^7$ |
| 60 | $7.2 \times 10^6$ | $1.3 \times 10^7$ | $3.3 \times 10^7$ |
| 120 | $4.5 \times 10^6$ | $8.4 \times 10^6$ | $3.3 \times 10^7$ |

employed; thus, the specificity of opsonic activity in immune serum has not been delineated.

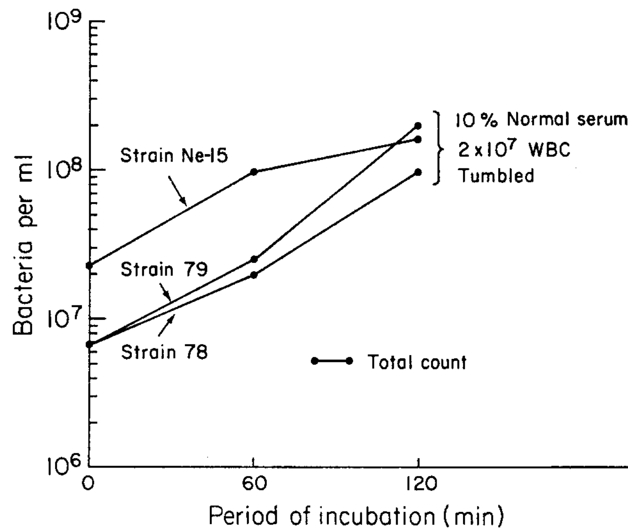
The immunizing antigens were prepared as follows. Flasks containing 25 ml Eugonbroth were inoculated with a 16 hr agar culture of meningococci and incubated on a reciprocating shaker at 37°C. 3 hr later, 10 ml were removed, centrifuged at 2000 rpm for 10 min, washed once with pyrogen-free normal saline, and the bacterial pellet resuspended in 5 ml of normal saline. The bacterial concentration in these suspensions was 4–8 $\times 10^8$ organisms per ml.

New Zealand Red female rabbits weighing 7–9 lb. were immunized by injecting subcutaneously 1 ml of freshly prepared bacterial suspensions. Only one injection was given so that agglutinating antibodies would not be present in immune sera. All rabbits tolerated immunizations well; neither subcutaneous nor dermal reactions were observed.

Immune sera collected 7 days following immunization did not demonstrate either opsonic or bactericidal activity. The interaction between strain 78 and rabbit polymorphonuclear leukocytes in the presence of 12–21 day homologous antiserum is shown in Text-fig. 3. The closed circle lines represent the total number of viable bacteria and the open circle lines the number of extracellular viable bacteria. In the presence of specific immune serum, the total number of viable bacteria rapidly decreased in 60 min. The extracellular bacterial count

closely paralleled the total count, indicating that following ingestion of meningococci by leukocytes, prompt intracellular killing occurred.

In the static tubes containing leukocyte-bacterial suspensions and immune serum, little or no phagocytosis occurred since there was limited opportunity for contact between leukocyte and bacterial populations. The constant bacterial counts in this static specimen and in an immune serum control containing no leukocytes demonstrated that no extracellular bactericidal or clumping activity were present in the antiserum.




TEXT-FIG. 2. The interaction between three representative group B meningococcal strains and rabbit granulocytes in the presence of normal rabbit serum.

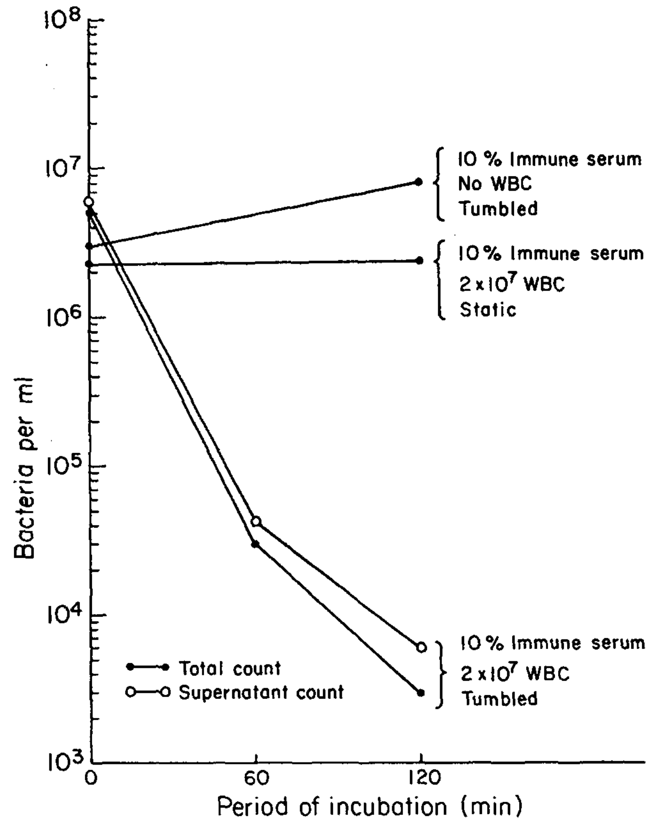
Similar results were obtained when strain 79 was added to leukocyte suspensions in the presence of strain 78 antiserum. It should be noted that both strains 78 and 79 were isolated from patients at the same army installation in April 1965.

Phagocytosis of strain NE-15 did not occur in the presence of strain 78 antiserum. However, as demonstrated in Text-fig. 4, ingestion and intracellular killing of strain NE-15 was observed when leukocytes and bacteria were mixed in the presence of NE-15 antiserum.

Antiserum to strain NE-15 did not promote phagocytosis of strain 78 or 79, as shown in Text-fig. 5.

A summary of the studies on phagocytosis and killing of several group B meningococci by rabbit polymorphonuclear leukocytes is shown in Table V. Phagocytosis of these strains did not occur in systems containing either no

serum or normal rabbit serum. Ingestion and intracellular killing did occur when strain 78 and NE-15 were mixed with leukocytes in the presence of homologous antisera. Phagocytosis of strain 79 and 161 also occurred in the presence of strain 78 but not strain NE-15 antiserum, whereas strain I-3-5 was

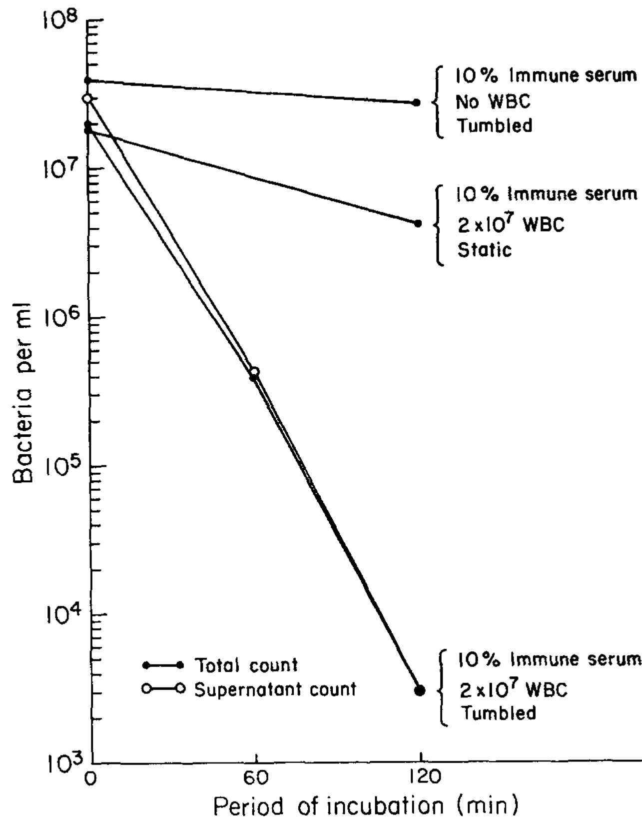



TEXT-FIG. 3. The interaction between meningococcal strain 78 and rabbit granulocytes in the presence of 12-day homologous immune serum.

engulfed and killed in the presence of strain NE-15 but not strain 78 antiserum. Thus, there are at least two serologic types of group B meningococci based on specific opsonic activity of immune rabbit serum.

*The Morphologic Study of the Interaction of Meningococci and Rabbit Granulocytes in the Presence of Normal and Immune Rabbit Serum.*—In the past, morphologic study of the phagocytosis of meningococci has been based on techniques initially described by Wright and Douglas and by Neufeld as cited in references 9 and 15, respectively. Suspensions of leukocytes, bacteria, and serum were mixed in capillary or small test tubes for 15–60 min, after which

samples were removed, smeared on slides, and stained. Owing to severe clumping and retraction of cells, reliable evaluation of the degree of phagocytosis is difficult or impossible by these methods.

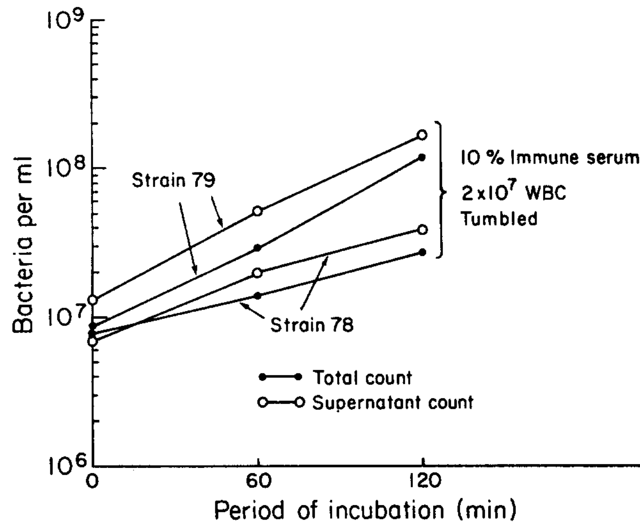



TEXT-FIG. 4. The interaction between meningococcal strain NE-15 and rabbit granulocytes in the presence of 21-day homologous immune serum.

The morphologic preparations described in this report were patterned after the method of Hirsch and Cohn (16). Screened rabbit peritoneal exudates were placed on small cover slips and incubated for 30 min at 37°C in Felsen quadrant Petri dishes which served as moist chambers, allowing leukocytes to become firmly attached as a monolayer on the glass surface. After gently washing in gel-Gey's medium, meningococci suspended in rabbit serum were immediately placed on the cover slips, and incubation was continued in moist chambers for 1 hr at 37°C. The cover slips were then drained, dried with an air jet to prevent cell retraction, and stained with Wright's. When examined microscopically, cell aggregation was minimal and cell morphology distinct.

Fig. 1 *a* illustrates the interaction of strain 78 and granulocytes incubated for 1 hr in the presence of fresh normal rabbit serum. Though occasional

organisms were seen within cells, most meningococci remained extracellular. Fig. 1 *b* demonstrates the interaction of strain 78 and granulocytes in the presence of 12 day homologous antiserum. In this preparation, granulocytes




TEXT-FIG. 5. The interaction between meningococcal strains 78 and 79 and rabbit granulocytes in the presence of heterologous (strain NE-15) immune serum.

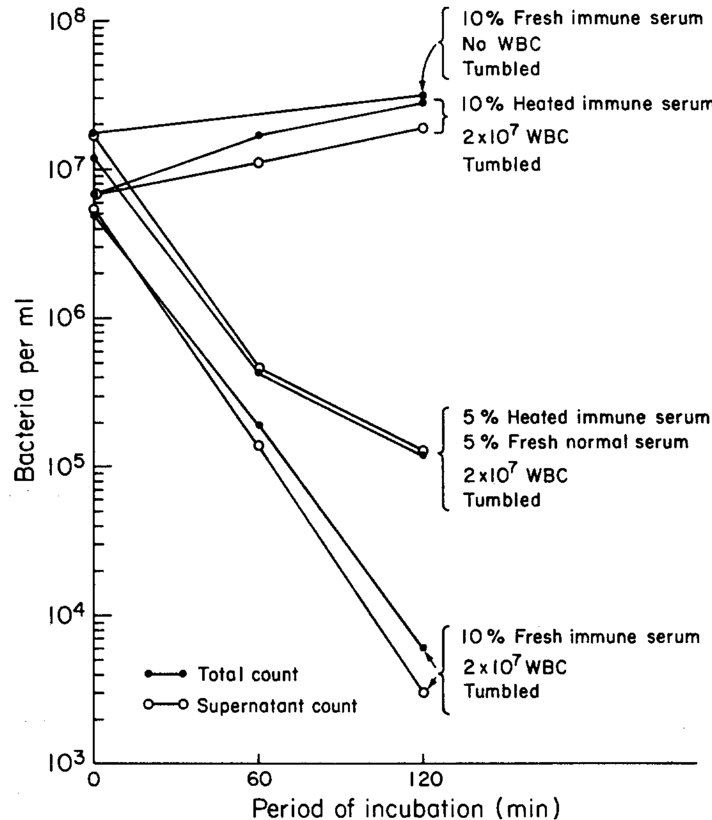
TABLE V
*Phagocytosis of Group B Meningococci by Rabbit Polymorphonuclear Leukocytes*

| Strain | Phagocytosis* by rabbit granulocytes in presence of: | | | |
|---|---|---|---|---|
| | No serum | Normal rabbit serum | Immune (78) rabbit serum | Immune (NE-15) rabbit serum |
| 78 | 0 | 0 | + | 0 |
| 79 | 0 | 0 | + | 0 |
| NE-15 | 0 | 0 | 0 | + |
| 161 | 0 | 0 | + | 0 |
| I-3-5 | 0 | 0 | 0 | + |

* 0, no phagocytosis; +, >90% of organisms engulfed and killed after 1 hr of incubation at 37°C.

were packed with meningococci. Furthermore, within granulocytes that contain many organisms, cytoplasmic granules were not seen, indicating extensive degranulation after ingestion of bacteria. Similar morphologic findings have been described following the ingestion of other bacteria or zymosan by polymorphonuclear leukocytes (16).

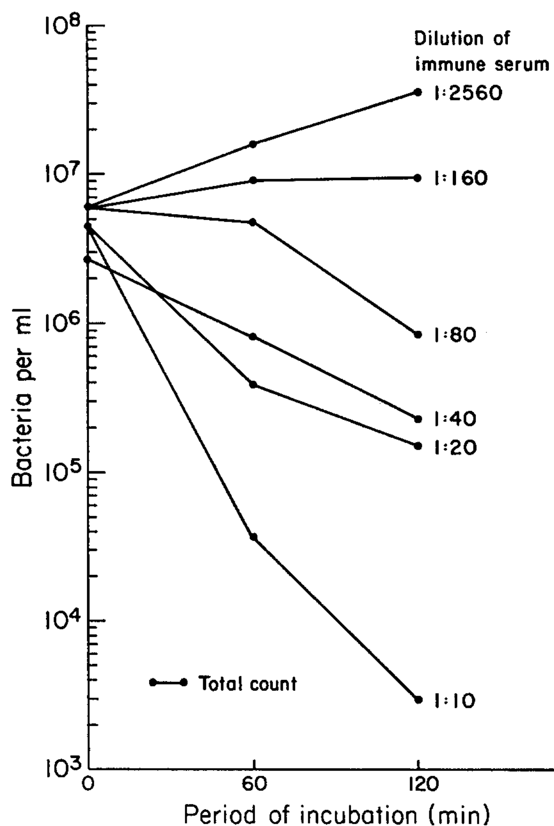
*The Effect of Heat Inactivation on the Opsonic Activity in Immune Rabbit Serum.*—The role of heat-labile factors in phagocytosis of microorganisms is well-known. Whereas in certain systems this factor appears to be complement or complement-like (17), a heat-labile opsonin in normal rabbit serum that is neither complement nor antibody has also been reported (2).




TEXT-FIG. 6. The effect of heat-inactivated, 12-day homologous antiserum on the interaction between meningococcal strain 78 and rabbit granulocytes.

The effect of heat inactivation of fresh immune rabbit serum on the phagocytosis of meningococcal strain 78 is shown in Text-fig. 6. Homologous antiserum heated at 56°C for 30 min completely lost its phagocytosis-promoting property. This property could be restored by the addition of unheated fresh normal rabbit serum. The slightly less effective phagocytosis and killing in the inactivated immune serum plus fresh normal serum specimen, as compared to the fresh immune serum sample, probably is a reflection of the lower concen-

tration of immune serum in the former specimen (see Text-fig. 7 below). Therefore, opsonic activity of meningococcal antiserum depends on both heat-labile and heat-stable components. Characterization of this heat-labile factor awaits further study.

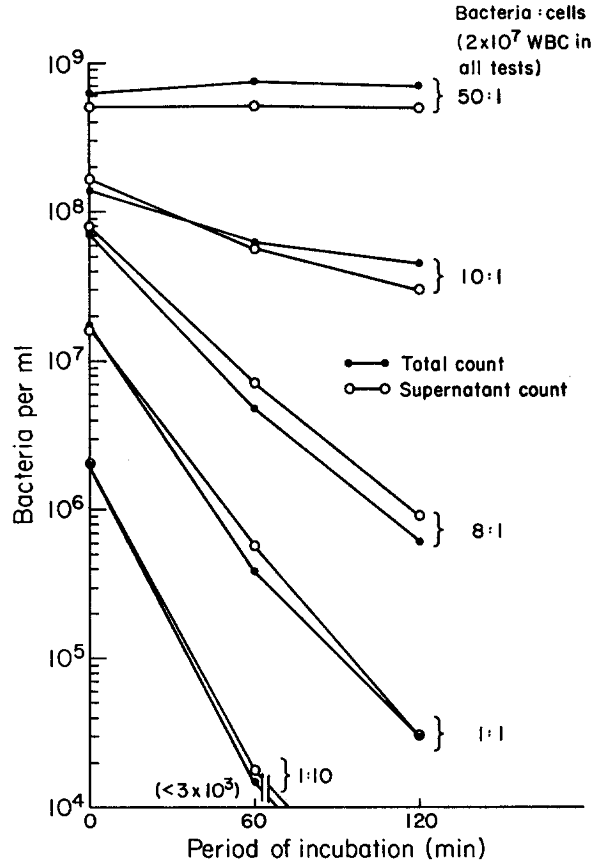



TEXT-FIG. 7. The effect of serial dilutions of 12-day homologous antiserum on the interaction between meningococcal strain 78 and rabbit granulocytes.

*The Titer of Specific Opsonic Activity in Immune Rabbit Serum.*—The titer of the opsonic activity in immune serum was determined in the following manner.

Serial dilutions of strain 78 antiserum were prepared in fresh normal rabbit serum. The final dilutions of immune serum ranged from 1:10 to 1:2560 while the total serum concentration in each tube remained at 10%. Aliquots were removed at 0, 60, and 120 min and serially diluted in distilled water with 0.01% albumin to determine the total number of viable bacteria. A phagocytosis test was considered positive when greater than 90% of the organisms were killed after 1 hr of incubation at 37°C.

As shown in Text-fig. 7, phagocytosis occurred only in very low dilutions of immune serum (1:10 and 1:20). Similar low titers of opsonic activity were also observed in the sera of rabbits receiving either successive inoculations of



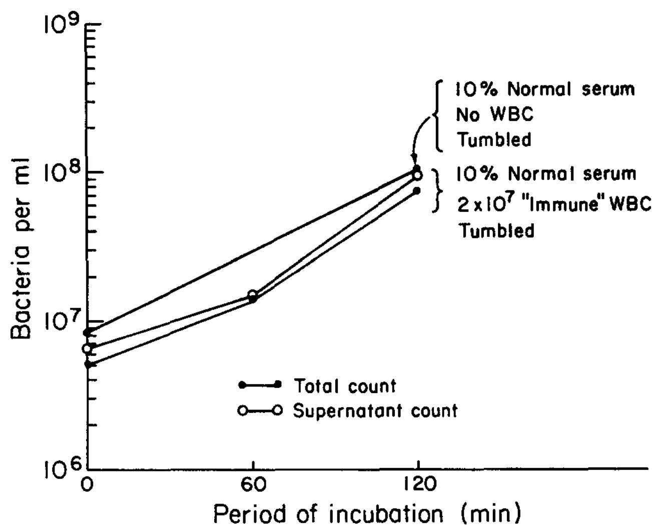

TEXT-FIG. 8. The effect of the ratio of bacteria to leukocytes on the ingestion and killing of strain 78 by rabbit granulocytes in the presence of 12-day homologous antiserum.

bacteria or one inoculation with complete Freund's adjuvant added to the bacterial suspension.

*The Effect of the Ratio of Bacteria to Leukocytes on the Interaction between Meningococci and Polymorphonuclear Leukocytes.*—Based on the degree of staining of intracellular bacteria, previous reports have suggested that polymorphonuclear leukocytes are unable to kill meningococci when large numbers of organisms are ingested (18). The following experiment was designed to investigate this possibility.

Exudate granulocytes were prepared in the usual manner and concentrated to 20,000 per $mm^3$. Various concentrations of log phase meningococci were added to cell suspensions so that the ratio of bacteria to leukocytes ranged from 1 bacteria per 10 leukocytes to 50 bacteria per leukocyte. The range of concentrations of bacteria were $10^6$ to $10^9$ organisms per ml. The final serum concentration in each bacteria-leukocyte suspension was 10%.

The results of such an experiment with strain 78 in the presence of its homologous antiserum is shown in Text-fig. 8. Regardless of the ratio of the number of bacteria to leukocytes, all meningococci ingested by polymorphonuclear leukocytes were promptly killed. Similar rates of ingestion and intracellular




TEXT-FIG. 9. The interaction, in the presence of normal rabbit serum, between meningococcal strain 78 and granulocytes obtained from rabbits injected with meningococcal strain 78.

killing were seen when the concentration of bacteria was approximately $1 \times 10^8$ organisms per ml (8 bacteria per leukocyte) or less. Higher concentrations of bacteria per leukocyte resulted in progressively less phagocytosis. This apparent loss of opsonic activity may have been due to the limited concentrations of either heat-labile or heat-stable factors available in immune rabbit serum.

*The Role of Polymorphonuclear Leukocytes from Immune Rabbits*.—To ascertain whether or not phagocytosis by polymorphonuclear leukocytes from immune animals was similar to that observed with cells from normal animals the following experiment was performed.

Rabbits were immunized with strain 78 as previously described. Peritoneal exudates were harvested 12 days after immunization and prepared in the usual manner. To 0.8 ml of the leukocyte suspension was added 0.1 ml bacteria (strain 78) and 0.1 ml fresh normal rabbit serum.

As demonstrated in Text-fig. 9, phagocytosis of meningococcal strain 78 by leukocytes obtained from immune rabbits was not observed in the presence of normal rabbit serum. These findings are similar to those seen in systems containing cells from normal animals. Thus there appears to be no significant difference in the functional phagocytic properties between normal and "immune" polymorphonuclear leukocytes. Similar observations on other bacteria have been reported by Cohn and Morse (3).

### *Bactericidal Activity*

Attempts to study the interaction between strain 78 and granulocytes in the presence of late homologous antisera (collected from rabbits 4 or more wk after immunization) were thwarted by the fact that these antisera regularly exhibited bactericidal activity, i.e., there was killing of meningococci in the control specimen containing no leukocytes.

*Titer of Bactericidal Activity in Homologous Antiserum.*—In order to quantitate bactericidal activity in immune sera, the viability of meningococci in dilutions of homologous antiserum was determined.

Serial dilutions of homologous antiserum obtained 16 wk after immunization were performed in fresh normal rabbit serum. To each 15 × 75 mm test tube was added 0.8 ml gel-Gey's medium, 0.1 ml bacteria (strain 78) and 0.1 ml of the immune serum dilution. The total volume of each tube was 1.0 ml and contained 10% serum. Aliquots of 0.003 ml were removed at time intervals of 0 and 120 min, dispensed in 1.0 ml gel-Gey's medium, serially diluted, and plated as described previously. Bactericidal activity of immune serum was not observed when initial concentrations of bacteria in the test system exceeded approximately $1 \times 10^8$ organisms per ml.

Results of such an experiment are shown in Table VI. Meningococci were killed in low immune serum dilutions (1:10 to 1:40). The titers of bactericidal activity in relation to time of serum collection are shown in Table VII. Titers are expressed as the reciprocal of the highest 2-fold dilution in which greater than 90% of meningococci are killed after 2 hr of incubation at 37°C. Titers of such activity appeared at 4 wk and remained at relatively low values through the 16th wk following immunization.

*The Specificity of Bactericidal Activity in Immune Serum.*—Though bactericidal activity of meningococcal antiserum has been described previously (5, 7, 8) the specificity of this activity has not been determined. Table VIII summarizes the viability of meningococci in 16 wk strain 78 antiserum. In addition to the five group B strains previously used in phagocytosis experiments, two group A and two group C strains were also tested. No bactericidal activity was observed either against group B strains NE-15 and I-3-5, or against the group A or C strains tested.

Further confirmation of the specificity of the bactericidal activity in immune sera was examined by absorption studies.

All absorbing strains were grown in 25 ml Eugonbroth and incubated on a reciprocating shaker at 37°C. After 4 hr of incubation, each broth culture (25 ml) was sedimented at 2000 rpm for 10 min (International, model UV), washed, resuspended in 2 ml of pyrogen-free normal saline, and dispensed in 10 × 80 mm lusteroid tubes (Lourdes Instrument Corp., New York). Suspensions were then sedimented at 10,000 rpm (Lourdes, model AB) for 10 min at 4°C, the supernate removed with a sterile capillary pipette, and 0.4 ml of immune serum added to the pellet. The bacteria were resuspended gently in the serum to avoid bubble formation and the

TABLE VI
*The Viability of Strain 78 in Dilutions of Homologous Antiserum**

| Dilutions of strain 78 antiserum | Bacteria per ml after incubation at 37°C | |
|---|---|---|
| | Zero | 120 min |
| 1:10 | $4.5 \times 10^6$ | $<3.0 \times 10^3$ |
| 1:20 | $5.1 \times 10^6$ | $3.0 \times 10^3$ |
| 1:40 | $4.5 \times 10^6$ | $1.2 \times 10^5$ |
| 1:80 | $7.2 \times 10^6$ | $3.3 \times 10^6$ |
| 1:160 | $4.8 \times 10^6$ | $2.1 \times 10^7$ |

* Serum obtained 16 wk following immunization with strain 78.

TABLE VII
*Titer of Bactericidal Activity in Strain 78 Antisera*

| Strain 78 antisera: time after immunization | Titer* of bactericidal activity on strain 78 |
|---|---|
| *wk* | |
| 3 | <5 |
| 4 | 5 |
| 6 | 10 |
| 8 | 10 |
| 12 | 40 |
| 16 | 40 |

* Reciprocal of highest 2-fold dilution in which >90% of organisms are killed after 2 hr of incubation at 37°C.

tubes incubated at 4°C. After 1 hr with occasional agitation the bacteria-serum mixture was centrifuged at 10,000 rpm for 10 min and the serum removed with a capillary pipette, placed in 15 × 45 mm sterile vials and stored at 4°C. All bactericidal tests were performed 18–24 hr after serum absorption. To each 15 × 75 mm test tube was added 0.7 ml gel-Gey's medium, 0.1 ml bacteria suspension, 0.1 ml absorbed serum, and 0.1 ml fresh normal rabbit serum. The latter serum was added to insure an adequate amount of heat labile factor in the test system.

The results of these experiments are shown in Table IX; the viability of strain 78 was determined in 10% homologous antiserum which had prevously been absorbed with the strains listed. All strains except 78, 79, and 161 failed to remove the bactericidal activity thus confirming the type specificity observed

in direct bactericidal testing (Table VIII). The type specificity of bactericidal activity in immune sera appears to be identical with that of opsonic activity.

*The Effect of Heat Inactivation on the Bactericidal Activity of Immune Serum.*— Studies on the role of heat-labile factors in the bactericidal activity of meningococcal antiserum have given confusing results (6, 8). As shown in Table X, the bactericidal activity of homologous antiserum against strain 78 was completely lost following heat inactivation at 56°C for 30 min, and this activity was restored by the addition of fresh normal rabbit serum. Thus, the bactericidal activity of meningococcal antiserum depends on heat labile as well as heat stable factors.

TABLE VIII
*Viability of Meningococci in Strain 78 Antiserum**

| Meningococcal strain | Bacteria per ml after incubation at 37°C | |
|---|---|---|
| | Zero | 120 min |
| Group B | | |
| 78 | $9.9 \times 10^6$ | $3.0 \times 10^3$ |
| 79 | $3.0 \times 10^6$ | $3.0 \times 10^3$ |
| NE-15 | $3.3 \times 10^7$ | $1.5 \times 10^8$ |
| 161 | $3.6 \times 10^7$ | $6.0 \times 10^3$ |
| I-3-5 | $1.2 \times 10^7$ | $5.1 \times 10^7$ |
| Group A | | |
| 45 | $3.3 \times 10^7$ | $8.7 \times 10^7$ |
| 98 | $3.0 \times 10^7$ | $1.1 \times 10^8$ |
| Group C | | |
| 47 | $6.6 \times 10^6$ | $6.6 \times 10^7$ |
| 95 | $3.6 \times 10^7$ | $1.2 \times 10^8$ |

* Serum obtained 16 wk following immunization with strain 78.

## DISCUSSION

Previous studies on phagocytosis of meningococci by polymorphonuclear leukocytes have yielded conflicting results (9–11). Phagocytosis is a complicated biologic process, and reliable quantitation of this process is often difficult (reviewed in reference 2). One technical factor probably determining the conflicting results obtained by prior workers is the fact that they employed suspensions of meningococci prepared from 12–24 hr old colonies on agar media. Such suspensions contain many degenerated or dead organisms. Recent observations made in this laboratory indicate that several strains of meningococci are susceptible to killing by normal rabbit serum when old cultures

are tested, whereas log phase cultures of these same strains survive and grow in this serum. It is obviously desirable to use a population of bacteria as homogenous as possible in order to obtain reproducible results in a quantitative system, and liquid medium cultures in the logarithmic phase of growth appear

TABLE IX
*Viability of Meningococcal Strain 78 in Absorbed Homologous Antiserum**

| Immune serum absorbed with: | Test organism | Bacteria per ml after incubation at 37°C | |
|---|---|---|---|
| | | Zero | 120 min |
| Group B | | | |
| 78 | Strain 78 | $3.0 \times 10^6$ | $5.1 \times 10^6$ |
| 79 | " | $3.3 \times 10^6$ | $4.8 \times 10^6$ |
| NE-15 | " | $3.3 \times 10^6$ | $1.2 \times 10^5$ |
| 161 | " | $4.2 \times 10^6$ | $1.2 \times 10^7$ |
| I-3-5 | " | $3.9 \times 10^6$ | $2.7 \times 10^4$ |
| Group A | | | |
| 45 | " | $3.9 \times 10^6$ | $4.8 \times 10^4$ |
| 98 | " | $4.5 \times 10^6$ | $1.8 \times 10^4$ |
| Group C | | | |
| 47 | " | $7.8 \times 10^6$ | $3.6 \times 10^4$ |
| 95 | " | $4.2 \times 10^6$ | $2.4 \times 10^4$ |

* Serum obtained 16 wk following immunization with strain 78.

TABLE X
*Viability of Meningococcal Strain 78 in Heat-Inactivated Immune Serum**

| Serum | Bacteria per ml after incubation at 37°C | |
|---|---|---|
| | Zero | 120 min |
| Immune serum | $5.7 \times 10^6$ | $2.1 \times 10^4$ |
| Heated immune serum (56°C/30') | $5.1 \times 10^6$ | $5.4 \times 10^7$ |
| Heated immune serum + fresh normal serum | $5.4 \times 10^6$ | $1.5 \times 10^4$ |

* Serum obtained 6 wk following immunization with strain 78.

to be best suited for this purpose. Another technical factor of importance in study of phagocytosis of meningococci is the concentration of the various reactants in the test system. For example several meningococcal strains are killed in 80% normal rabbit serum, but survive well in 10% serum. The specific opsonic and bactericidal activities which appear in immune sera are of low titer, and can not be reliably detected if the initial dilution is greater than 1:20, or

if too large (> $10^8$) a concentration of meningococci are employed in the test.

Prior studies on phagocytosis of meningococci have all employed mixed populations of phagocytic cells, whereas the present observations were made with pure populations of polymorphonuclear leukocytes. The interaction between meningococci and mononuclear phagocytes remains to be defined.

The methods employed in the present studies allowed quantitative assessment of the interaction between meningococci, leukocytes, and serum factors. The results indicate that the ingestion and intracellular killing of many strains of meningococci by rabbit polymorphonuclear leukocytes require, among other factors, the presence of immune serum. The observations thus suggest that meningococci possess antiphagocytic surface factors which almost certainly are important in determining the virulence of this organism. Attempts to isolate and identify these surface factors are planned.

The presence of bactericidal activity in the "late" immune sera made it impossible to observe opsonic effects of these sera by the method which uses intracellular killing of bacteria as an index of phagocytosis. However, two observations confirm that opsonic activity is still present in 16 wk homologous antiserum. First, morphologic studies as previously described have shown that phagocytosis of meningococcal strain 78 does occur in the presence of 16 wk homologous antiserum. Secondly, incubation of strain 78 in serial dilutions of 16 wk antiserum with and without leukocytes revealed that at a serum dilution in which bactericidal activity was no longer detected, 90% of organisms were killed when incubated with leukocytes, i.e., the titer of opsonic activity was somewhat higher that that of bactericidal activity. Isolation and characterzation of opsonic and bactericidal antibodies in immune sera are indicated. The difference in their time appearance suggests, of course, that the opsonic and the bactericidal antibodies may belong to different classes of immunoglobulins.

Muschel has stated that the bactericidal reaction constitutes a precise and sensitive method for the measurement of antibody (19). The studies reported in this communication demonstrate that the bactericidal activity of meningococcal antiserum can be detected in rabbits receiving only one subcutaneous injection of living log phase meningococci. Though present in only relatively low titers, this activity persists for at least 24 wk after immunization. Repeated attempts to demonstrate agglutinating antibodies in these sera have been unsuccessful. Properties of the bactericidal activity of immune serum are similar to those of opsonic activity; that is, both activities have the same type specificity and depend on heat-labile and heat-stable factors. Furthermore, similar technical factors are critical in the detection of both serum activities, i.e., the growth phase of the organism and the concentrations of bacteria and serum in the test system.

Previous studies have reported that meningococci resist intracellular digestion (18), and that this property may determine in part the pathogenicity of this organism (20). In these earlier studies, intracellular viability of menin-

gococci was demonstrated by stained films, though as mentioned previously, reliable evaluation by these methods is impossible. Employing (*a*) low speed centrifugation to separate leukocyte from bacterial populations and (*b*) direct colony plating, results reported herein demonstrate that all meningococci are promptly killed after being ingested by rabbit polymorphonuclear leukocytes. Though phagocytes from different species may not exhibit identical properties, these findings do suggest that meningococci neither survive nor multiply within granulocytes. Studies of the fate of meningococci within human polymorphonuclear leukocytes are planned.

Meningococcal infections have continued to be an important public health problem during the past century. Despite numerous epidemiologic surveys, factors related to disease transmission and host susceptibility remain obscure. In recent years, sulfadiazine resistence of meningococci and an increased incidence of group B meningococcal disease have been well documented. From January 1964 to May 1966, 86% of meningococcal isolates from the U. S. Army were group B strains (21). In 1966, 70% of meningococcal strains submitted to the Communicable Disease Center were also group B organisms (22). From published data such as these, it is apparent that a more specific serological classification must be defined before meaningful epidemiological studies can be undertaken. Branham has suggested that serologic types based on agglutinating antibodies may exist within all groups of meningococci (23). Only the serotype Boshard has been described to date (21). Evans described a serologic classification on the basis of immune "tropins" (24), but later studies demonstrated that their specificity was identical with that of agglutinins (25). The quantitative results of this investigation describe type-specific opsonins and bactericidins for group B meningococci. Examination of other group B meningococcal strains may well uncover additional serologic types. Studies of the type specificity of opsonic and bactericidal activity in immune sera to group A and C meningococci are now in progress. It is hoped that these type-specific antibodies may prove useful in future epidemiologic studies of meningococcal infections.

## SUMMARY

The interaction in vitro between group B meningococci and rabbit polymorphonuclear leukocytes has been described. Phagocytosis did not occur in the presence of normal rabbit serum. Antiserum collected 12–21 days following one subcutaneous inoculation of living log phase meningococci exhibited opsonic activity with type specificity; this opsonic action depended on both heat-labile and heat-stable factors. Following ingestion by granulocytes, meningococci were rapidly killed. These studies suggest that group B meningococcal strains contain specific antiphagocytic surface factors of an as yet unknown chemical nature.

Antisera obtained 4 or more wk after immunization showed bactericidal

activity with the same type specificity as opsonic activity. This bactericidal activity was also lost after heating and restored by the addition of normal serum.

Further studies on opsonins and bactericidins for meningococci may shed light on virulence factors in these microorganisms, and may prove useful for a more precise classification of meningococci according to type rather than group specificity.

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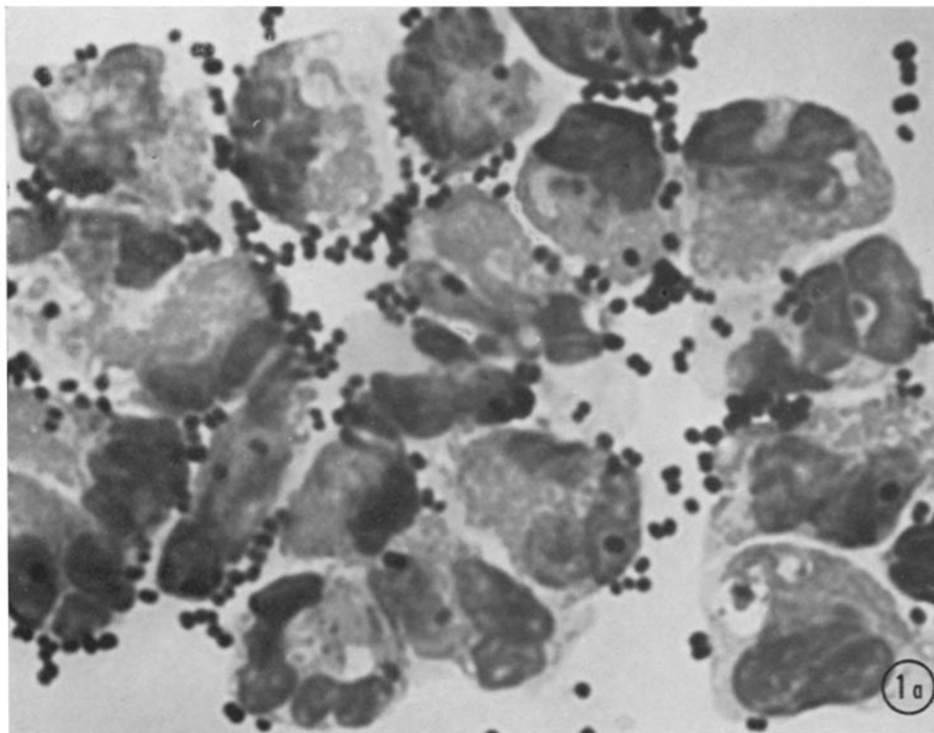
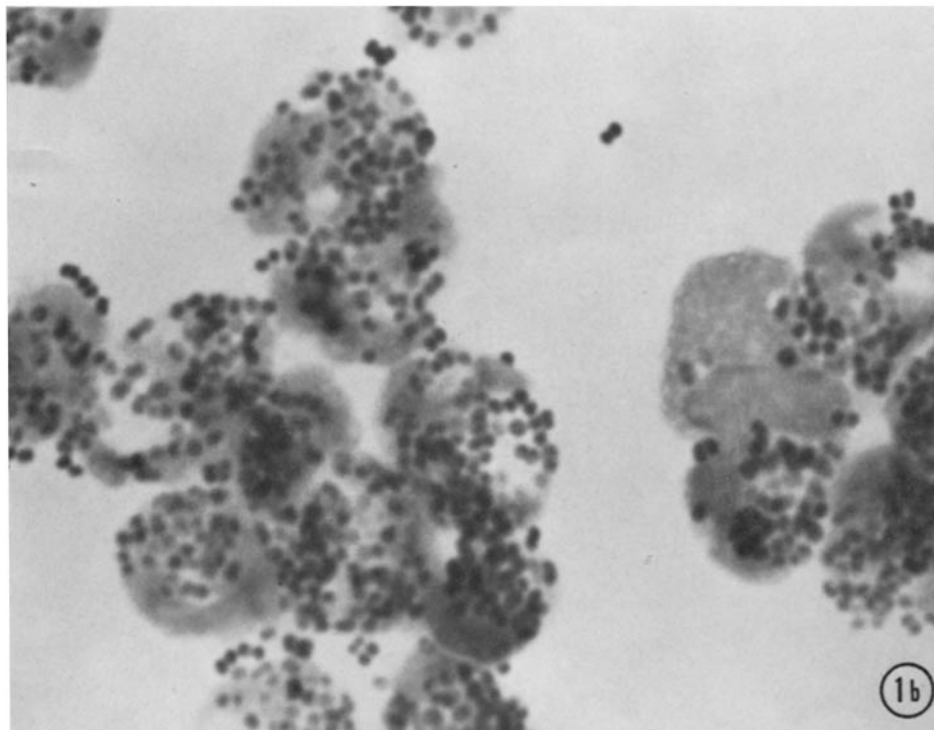
## EXPLANATION OF PLATE 60

FIG. 1. Photographs were taken on Kodak Panatomic-X film.

*a*. Illustrates the interaction between meningococci and rabbit polymorphonuclear leukocytes in the presence of normal rabbit serum. After 1 hr of incubation at 37°C, most meningococci remain extracellular. The cytoplasmic granules of leukocytes are intact. × 2000.

*b*. Illustrates the interaction between meningococci and rabbit polymorphonuclear leukocytes in the presence of 12-day homologous antiserum. After the same period of incubation granulocytes are packed with meningococci, and extensive degranulation has occurred in the cells containing many bacteria. × 2000.




(Roberts: Opsonins and bactericidins for meningococci)