

## *In Vitro* Bactericidal Capacity of Human Polymorphonuclear Leukocytes: Diminished Activity in Chronic Granulomatous Disease of Childhood \*

P. G. QUIE,† J. G. WHITE, B. HOLMES, AND R. A. GOOD ‡

(From the Departments of Pediatrics and Microbiology, University of Minnesota Medical School, Minneapolis, Minn.)

**Summary.** Diminished bactericidal capacity was found to be characteristic of polymorphonuclear leukocytes (PMN) from five children with the clinical syndrome of granulomatous disease of childhood. The PMN from these children demonstrated nearly normal phagocytic capacity, and the majority of viable bacteria, after 2 hours of incubation in the phagocytosis system, were found associated with leukocytes.

The morphology of the unstimulated polymorphonuclear leukocytes from patients with chronic granulomatous disease was similar to those from normal persons of similar ages by light and electron microscopy. In addition, the total lysozyme and phagocytin activity of leukocyte extracts from these patients was similar to those from equal numbers of leukocytes from controls.

A striking difference in the cytoplasmic response after phagocytosis characterized the PMN of the patients with granulomatous disease. Whereas degranulation, vacuole formation, and rapid bacterial digestion were the rule in the PMN from controls, little degranulation and persistence of intact bacteria in the cytoplasm characterized disease.

The deficiency of bactericidal capacity and the minimal degranulation after active phagocytosis by the PMN of these children with an inherited syndrome suggest that separate metabolic processes are involved in phagocytosis and in intracellular digestion. Continuing study of the metabolic function of leukocytes from these children should provide an opportunity for increased understanding of the metabolic basis for degranulation and intracellular digestion in phagocytic cells.

### Introduction

The necessity of sufficient quantities of phagocytic cells for bacterial resistance has been estab-

lished by clinical experience with patients leukopenic from a variety of causes. A small number of patients, however, suffer recurrent bacterial disease in spite of a vigorous leukocyte response to infection. Adequate bactericidal function of phagocytic cells, as well as the quantity of available leukocytes, appears to be a critical factor in bacterial resistance.

\* Submitted for publication October 31, 1966; accepted December 30, 1966.

Supported in part by National Foundation, American Heart Association, and U. S. Public Health Service (AI-06931, HE-02085, AI-00798, and NB-02042) grants and the Commission on Streptococcal and Staphylococcal Diseases, Armed Forces Epidemiological Board, Office of the Surgeon General, Dept. of the Army, Washington, D. C.

Presented in part at the 58th Annual Meeting of the American Society for Clinical Investigation, Atlantic City, N. J., May 2, 1966.

† John and Mary R. Markle Scholar in Academic

Medicine. Recipient of U. S. Public Health Service Career Development Award (5-K3-AI-10, 109-05).

Address requests for reprints to Dr. P. G. Quie, Dept. of Pediatrics, University of Minnesota Medical School, Minneapolis, Minn. 55455.

‡ American Legion Memorial Heart Research Professor of Pediatrics and Microbiology.

The present investigation was designed to study the *in vitro* functional capacity of the polymorphonuclear leukocytes during the early stages of cellular-bacterial interaction under conditions that were optimal for phagocytosis and bacterial killing. Patients included in the study were those with disease syndromes characterized by persistent or recurrent bacterial infections.

The capacity of polymorphonuclear leukocytes to phagocytize and kill bacteria *in vitro* was comparable to normal controls in most of these patients; however, one group of patients, all apparently suffering from the same clinical disease, showed a defect in leukocyte function. These patients presented a clinical syndrome originally defined as "a fatal granulomatous disease of childhood" when separated from other granulomatous processes of known etiology (1, 2). Other studies have established the clinical identity of these patients and the familial nature of this disorder (3, 4). Unlike the polymorphonuclear leukocytes of normal persons and persons with many different diseases, PMN from the patients of this group demonstrated phagocytic capacity but impaired ability to kill phagocytized bacteria.

The activity of bactericidal factors lysozyme and phagocytin was quantitatively comparable in extracts of leukocytes from patients with this disease and from normal controls. It was observed, however, that polymorphonuclear leukocytes from patients with granulomatous disease of childhood demonstrated little degranulation after phagocytosis of bacteria. Although the intracytoplasmic bacteria were surrounded by membranes, typical phagocytic vacuoles were not observed in the polymorphonuclear leukocytes from patients with this disease.

### Methods

Twenty-nine patients with recurrent bacterial disease or chronic systemic disease associated with increased susceptibility to bacterial infection were studied. An additional 22 children and adults without bacterial infections were included as normal controls.

*Polymorphonuclear leukocyte function.* Phagocytic and bactericidal capacities of human PMN were determined by the Maaløe method (5) with the modifications described by Cohn and Morse (6) and Hirsch and Strauss (7).

Human peripheral leukocytes were prepared by dextran sedimentation of heparinized venous blood. Ten ml

of blood containing 2 mg heparin was mixed with 5 ml 6% dextran in saline<sup>1</sup> and incubated at room temperature for 1 hour. The plasma containing leukocytes, platelets, and few erythrocytes was withdrawn. Leukocytes were deposited by centrifugation at 1,000 rpm (International centrifuge model U.V. head 219) and were washed with heparinized saline. After the first wash the cell suspension was counted in a hemocytometer, and the proportion of polymorphonuclear leukocytes was determined by chamber differential. The leukocytes were centrifuged and suspended in Hanks balanced salt solution<sup>2</sup> with 0.1% gelatin to give a concentration of  $5 \times 10^6$  PMN per ml.

The bacterial species used in these studies were *Staphylococcus aureus* strain 502A and *Paracolon hafnia*. Biologic characteristics of the staphylococcal strain have been reported previously (8). The *P. hafnia* strain was recently recovered from the osteomyelitic lesion of one of the patients (D.B.). The bacterial strains were cultured overnight in Penassay broth (Difco) and then suspended to give an optical density of 0.6 at 620 m $\mu$  in a Colman Jr. photocolormeter. This optically adjusted suspension was diluted 1-50 in balanced salt solution with 0.1% gelatin. This dilution of staphylococci or paracolon bacteria contained 3 to  $6 \times 10^7$  bacteria per ml. Both the staphylococci and paracolon strains required opsonin for engulfment, and this was provided by fresh human adult sera. Sera from four or more healthy adult volunteers were pooled, divided into 1-ml aliquots, frozen, and stored for no longer than 30 days. An aliquot of pooled sera was thawed immediately before use and diluted to 20% in balanced salt solution.

Phagocytic tests were done in 15  $\times$  75 siliconized tubes. Each tube contained 0.5 ml leukocyte suspension, 0.1 ml bacteria suspension, and 0.4 ml opsonin. This provided approximately one bacterium per PMN and a final concentration of 10% serum.

Each assay included control tubes of leukocytes and bacteria without opsonin and tubes held stationary to determine extracellular bacterial killing. The tubes for assay of bactericidal activity were incubated at 37° C with an end over end rotation. The total viable bacteria at the beginning of and during the incubation period was determined by transferring samples with a calibrated 0.002-ml platinum loop to 1 ml distilled water for cell lysis and dilution. At the end of the incubation period (usually 120 minutes), the tubes containing the phagocytic mixtures were centrifuged at 800  $\times$  g for 5 minutes. Samples were removed from the supernatant fraction for determination of extracellular bacteria, and portions of the washed resuspended leukocyte pellet were placed in distilled water for lysis and dilution as a measure of leukocyte-associated bacteria.

Sera from patients with increased susceptibility to infection were tested for opsonic activity by using dilutions of the opsonin fraction in phagocytic mixtures containing staphylococci and leukocytes from normal donors. Each

<sup>1</sup> Cutter Labs., Chattanooga, Tenn.

<sup>2</sup> Baltimore Biological Labs., Baltimore, Md.

TABLE I  
*Phagocytic and bactericidal capacity of polymorphonuclear leukocytes (PMN) for Staphylococcus aureus*

| Source of PMN | Viable bacteria ( $\times 10^6$ ) per ml |        |        |         | Supernatant fluid after centrifugation $800 \times g$ for 10 minutes | Resuspended leukocyte pellet |
|---------------|--|--------|--------|---------|--|------------------------------|
|               | 0  | 30 min | 60 min | 120 min |  |                              |
| D.B.          | 4.0                                      | 3.50   | 3.6    | 2.20    | 0.26   | 2.00                         |
| Control       | 2.2                                      | 0.12   | 0.1    | 0.09    | 0.12   | 0.01                         |
| J.B.          | 5.0                                      | 2.5    | 2.70   | 2.00    | 0.80   | 1.20                         |
| Control       | 3.9                                      | 0.9    | 0.15   | 0.07    | 0.04   | 0.03                         |
| L.J.          | 4.0                                      | 1.4    | 1.50   | 0.50    | 0.24   | 1.70                         |
| Control       | 5.8                                      | 0.26   | 0.18   | 0.09    | 0.04   | 0.08                         |
| K.S.          | 2.5                                      | 2.5    | 1.80   | 1.10    | 0.40   | 0.70                         |
| Control       | 2.0                                      | 1.6    | 0.18   | 0.06    | 0.04   | 0.02                         |
| T.W.          | 3.5                                      | 2.0    | 2.00   | 2.4     | 0.60   | 1.10                         |
| Control       | 5.8                                      | 0.8    | 0.29   | 0.2     | 0.15   | 0.20                         |

serum was compared with a sample of pooled normal sera and tested at final concentrations of 10%, 5%, and 1% in the phagocytosis system.

*Morphology and cytochemistry of leukocytes.* Smears of the leukocyte-bacteria mixtures were made on glass slides and stained with Wright's stain in standard fashion.

Viability of leukocytes during the incubation period was determined by incubation of a loopful of bacteria-leukocyte suspension and 1% trypan blue on a glass slide under a mineral oil-sealed cover slip for 1 minute. Failure of nuclear staining was regarded as evidence for viability.

Leukocytes from patients and from normal controls were fixed and stained with Sudan black and periodic acid-Schiff. Slides were also prepared for histochemical determination of peroxidase, alkaline phosphatase, and acid phosphatase with standard methods described by Hayhoe (9).

*Preparation for electron microscopy.* Parallel tubes of bacteria-leukocyte mixtures from patients and controls, incubated as described above, were removed at 30 and 60 minutes. The tubes were transferred immediately to a refrigerated centrifuge ( $4^\circ\text{C}$ ) and sedimented at 800 rpm for 5 minutes. Supernatant media were decanted, and 3 ml of cold ( $4^\circ\text{C}$ ) 3% glutaraldehyde in 0.05 ml cacodylate buffer with 1% sucrose, pH 7.3, was added to each cell pellet. After 15 minutes the pellets were loosened from the base of the tubes, the glutaraldehyde was decanted, and the cells were washed with cacodylate buffer. Phosphate-buffered 1% osmic acid, pH 1.3, was then added, and the pellets of cells were incubated in this fixative for  $1\frac{1}{2}$  hours. The preparations were dehydrated and embedded by techniques previously recorded from this laboratory (10), and after sectioning they were examined with a Phillips 200 electron microscope.

*Assay for bactericidal activity of leukocyte extracts.* The method for preparation of leukocyte extracts for assay of lysozyme and phagocytin was that described by

Hirsch and Church (11). Leukocytes in the plasma layer after dextran sedimentation were partially separated from platelets by differential centrifugation and from erythrocytes by lysis with hypotonic saline and neutralization with hypertonic saline. After differential counting of the leukocytes, they were centrifuged, and the leukocyte pellets were frozen and thawed six times. A saline extract of the disrupted leukocytes was made by incubation in saline for 30 minutes at  $37^\circ\text{C}$  at a concentration of approximately  $10^7$  leukocytes per ml. The saline-insoluble residue was separated by centrifugation at  $10,000 \times g$  for 15 minutes, and the supernatant (saline extract) was used for assay of lysozyme by the method of Shugar (12).

The sediment was washed with saline, suspended in 1 ml of 0.01 M citric acid, and incubated for 30 minutes at  $37^\circ\text{C}$  with constant shaking. After centrifugation at  $10,000 \times g$ , the supernatant (citric acid extract) was assayed for phagocytin activity by the method of Hirsch (13).

## Results

*Comparison of bactericidal capacity of polymorphonuclear leukocytes from patients with chronic granulomatous disease and from normal controls.* A comparison of the number of viable staphylococci after incubation for 30, 60, and 120 minutes with PMN from five children with chronic granulomatous disease and with PMN from normal controls is shown in Table I. In preparations with PMN from the patients with this syndrome, at least 50% of the organisms inoculated remained as viable colony-forming units, even after 120 minutes' incubation. In contrast, less than 10% and frequently less than 1% of the staphylococci

TABLE II  
*Phagocytic and bactericidal capacity of polymorphonuclear leukocytes for Paracolon hafnia*

| Source of PMN | Viable bacteria ( $\times 10^6$ ) per ml |        |        |         | Supernatant fluid after centrifugation $800 \times g$ for 10 minutes | Resuspended leukocyte pellet |
|---------------|--|--------|--------|---------|--|------------------------------|
|               | Total phagocytic mixture                 |        |        |         |  |                              |
|               | 0  | 30 min | 60 min | 120 min |  |                              |
| D.B.          | 5.5                                      | 3.0    | 3.00   | 2.70    | 1.60   | 2.60                         |
| Normal        | 6.0                                      | 0.7    | 0.19   | 0.04    | 0.03   | 0.01                         |
| J.B.          | 4.0                                      | 3.00   | 3.00   | 2.50    | 1.00   | 1.50                         |
| Normal        | 4.4                                      | 1.00   | 0.30   | 0.05    | 0.03   | 0.02                         |
| L.J.          | 6.0                                      | 4.9    | 4.80   | 4.20    | 2.20   | 2.20                         |
| Normal        | 5.9                                      | 1.3    | 0.13   | 0.07    | 0.01   | 0.09                         |
| K.S.          | 3.7                                      | 3.5    | 2.50   | 2.60    | 0.40   | 2.00                         |
| Normal        | 3.4                                      | 0.5    | 0.06   | 0.05    | 0.03   | 0.01                         |
| T.W.          | 8.5                                      | 7.0    | 3.6    | 2.00    | 0.60   | 3.00                         |
| Normal        | 8.8                                      | 0.4    | 0.1    | 0.40    | 0.17   | 0.33                         |

incubated with PMN from normal controls were viable after 60 minutes.

The results of similar studies with *P. hafnia* are shown in Table II. There was rapid killing of 90 to 99% of these gram-negative organisms by PMN from normal controls, and less than 50% were killed by PMN from patients with chronic granulomatous disease.

A comparison of bactericidal capacity for *S. aureus* and *P. hafnia* of polymorphonuclear leukocytes from children with chronic granulomatous disease and from normal controls is demonstrated in Figure 1. The PMN from these patients demonstrated an impaired bactericidal capacity for either the gram-positive staphylococci or the gram-negative paracolon bacteria.

PMN from patients whose diagnoses are listed in Table III demonstrated a normal bactericidal capacity for staphylococci and paracolon. These patients were in various stages of their underlying diseases when the *in vitro* leukocyte studies were done; several of them were on long-term therapy with corticosteroids or in terminal stages of their diseases. Indeed, it has not been possible thus far to identify a defect in bactericidal capacity with normal phagocytic capacity in any patients except those with chronic granulomatous disease of childhood.

The decreased bactericidal capacity appeared to be due to intraleukocyte survival of the bacteria during the 120 minutes of the study, not inability to phagocytize bacteria. As shown in Figure 2,

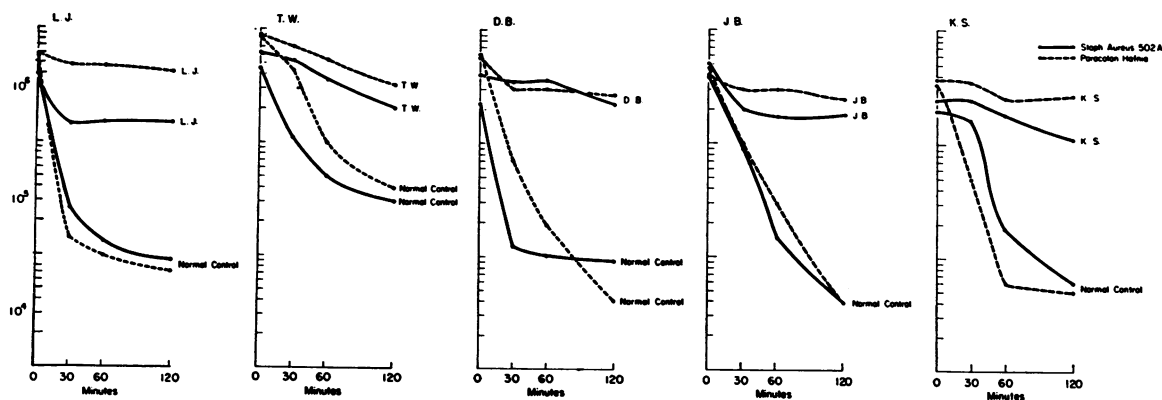


FIG. 1. VIABLE BACTERIAL COUNTS AFTER INCUBATION FOR 30, 60, AND 120 MINUTES WITH POLYMORPHONUCLEAR LEUKOCYTES (PMN) FROM FIVE PATIENTS WITH CHRONIC GRANULOMATOUS DISEASE. Comparison with PMN from normal control patients of similar ages. There was approximately one bacterium per PMN in the phagocytosis system.

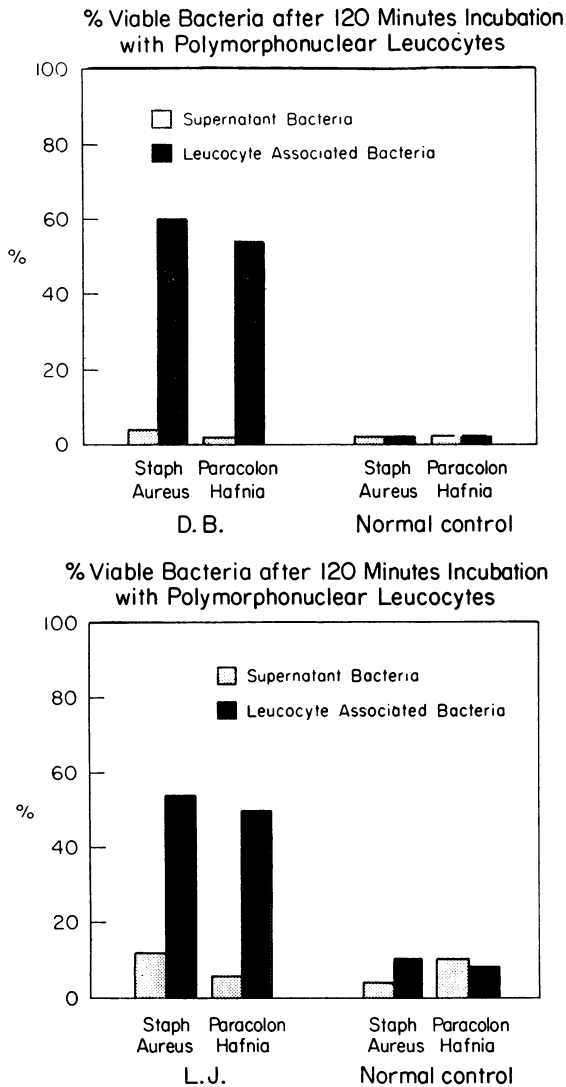


FIG. 2. LOCATION OF THE VIABLE BACTERIA AFTER INCUBATION FOR 120 MINUTES WITH POLYMORPHONUCLEAR LEUKOCYTES FROM TWO OF THE PATIENTS WITH GRANULOMATOUS DISEASE. Comparison with PMN from normal controls. Note that most of the bacteria still viable after 120 minutes' incubation in the phagocytic system were located in the leucocyte pellet of the patients with granulomatous disease.

the greatest number of viable bacteria after 2 hours' incubation was found associated with the leukocytes. Figure 3 shows the rate of phagocytosis during the first 60 minutes of incubation with PMN from two patients with granulomatous disease and a normal control. There was a rapid decrease in the number of supernatant bacteria

TABLE III  
*Patients studied with normal bactericidal activity of PMN*

| Diagnosis                   | Patients studied | Age range    |
|-----------------------------|------------------|--------------|
|                             |                  | <i>years</i> |
| Normal children             | 10               | 2-14         |
| Normal adults               | 12               | 20-40        |
| Plasma cell hepatitis       | 1                | 22           |
| Dysgammaglobulinemia        | 1                | 14           |
| Lupus erythematosus         | 1                | 12           |
| Rheumatoid arthritis        | 1                | 13           |
| Ataxia telangiectasia       | 1                | 2            |
| Diabetes with nephrosis     | 1                | 9            |
| Chédiak-Higashi syndrome    | 1                | 9            |
| Aldrich's syndrome          | 2                | 3-4          |
| Hodgkin's disease           | 1                | 11           |
| Hemolytic anemia            | 2                | 2            |
| Sarcoidosis                 | 2                | 22-40        |
| Cystic fibrosis             | 2                | 8-10         |
| Down's syndrome             | 2                | 1-4          |
| Recurrent pulmonary disease | 4                | 2-15         |
| Brain damage (moribund)     | 2                | 2-15         |
| Total                       | 42               |              |

with PMN from the patients as well as the controls. The viable bacteria in preparations with PMN from patients with chronic granulomatous disease were found associated with the leukocytes during the first few minutes of incubation, and there was no evidence for delayed phagocytosis of *S. aureus* or *P. hafnia* in the presence of normal serum in this *in vitro* system.

Attempts to delineate the nature of the abnormality in the polymorphonuclear leukocytes included the assay of bactericidal factors lysozyme and phagocytin. Comparison of the activities of these granule-associated factors in the extracts of leukocytes from three children with granulomatous disease and in extracts from two normal

TABLE IV  
*Enzyme activity of leukocyte extracts*

| Patient                    | Total leukocytes  | % PMN | Lysozyme* | Phagocytin† |
|----------------------------|-------------------|-------|-----------|-------------|
| L.J. Granulomatous disease | $4.2 \times 10^7$ | 60    | 12        | 20          |
| D.B. Granulomatous disease | $5.7 \times 10^7$ | 40    | 48        | 40          |
| T.W. Granulomatous disease | $5.6 \times 10^7$ | 35    | 24        | 40          |
| L.S. Normal adult          | $4.6 \times 10^7$ | 40    | 12        | 40          |
| E.A. Normal adult          | $4.0 \times 10^7$ | 55    | 24        | 10          |

\* Reciprocal of dilution of leukocyte extract in distilled water that gave activity equivalent to  $2 \mu$  egg white lysozyme.

† Reciprocal of dilution of leukocyte extract in citric acid that killed greater than 50% of  $1 \times 10^8$  salmonella per ml.

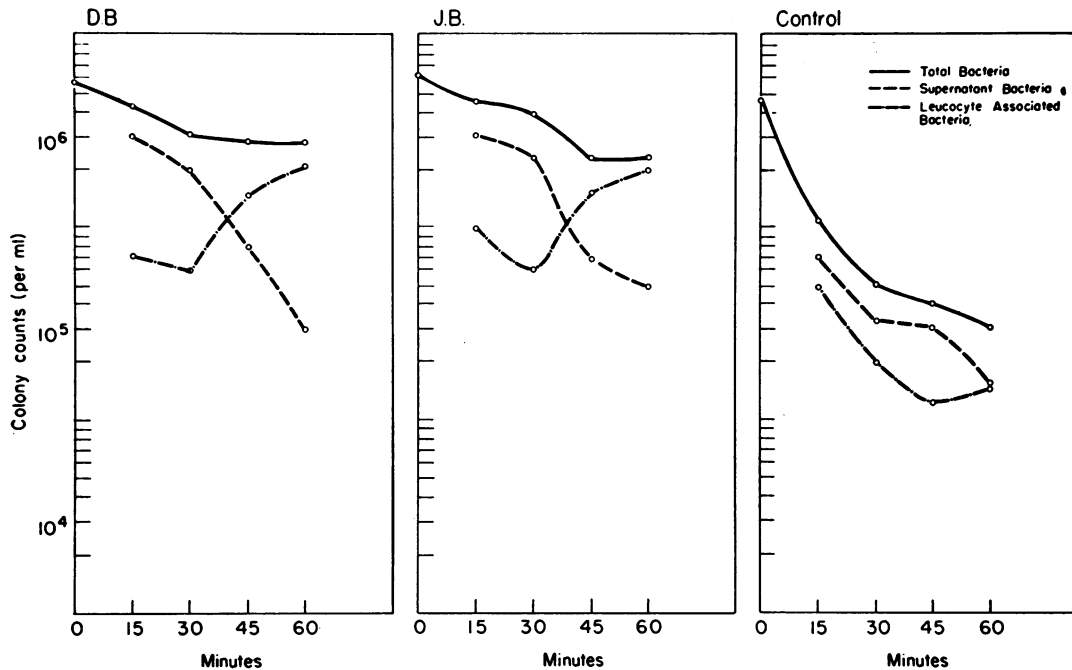


FIG. 3. TOTAL VIABLE BACTERIA, BACTERIA ASSOCIATED WITH LEUKOCYTES, AND EXTRACELLULAR BACTERIA AT 15, 30, 45, AND 60 MINUTES' INCUBATION WITH POLYMORPHONUCLEAR LEUKOCYTES. Two patients with chronic granulomatous disease, D.B. and J.B., and a normal control are illustrated. Note the rapid decrease in extracellular bacteria incubated with leukocytes from the two patients and the control and the increase in viable leukocyte-associated bacteria in the patients as contrasted with the control.

controls are shown in Table IV. Levels of activity of these factors were similar in both groups. Therefore, the leukocyte abnormality in these patients did not appear to be a result of deficiency of these factors.

The role that humoral factors may have played in the unusual susceptibility of these patients, in their leukocyte defect, or in both, was also investigated. There was no deficiency of serum opsonins for staphylococci or paracolon bacteria. As shown in Figure 4, there was a similar rate of phagocytosis and bacterial killing by normal cells incubated with sera from two of the children with granulomatous disease and with a sample of pooled sera from normal controls.

*Morphology and cytochemistry of polymorphonuclear leukocytes.* The morphology of the PMN from the patients with chronic granulomatous disease and those from normal controls of similar ages was identical by several staining methods. Smears of peripheral leukocytes stained with Wright's stain showed normal neutrophilia of

the cytoplasmic granules, and there was no detectable difference in the number of granules.

Sudan black staining showed typical sudanophilia of the granules, and the quantity and location of granules were similar in patients and controls. There were typical peroxidase and alkaline phosphatase reaction products associated with the granules in cells from patients with chronic granulomatous disease, and no reaction product of acid phosphatase activity could be demonstrated in either patients or controls when intact cells were studied. Cells prepared with periodic acid-Schiff stain demonstrated typical distribution of intracellular glycogen.

There was a striking difference in the appearance of the PMN from patients with chronic granulomatous disease and those from normal controls when Wright's stains were made of cells removed at various times of incubation during the bactericidal assay. There were many degranulated leukocytes and obvious vacuole formation in the control patients' PMN that had been incu-

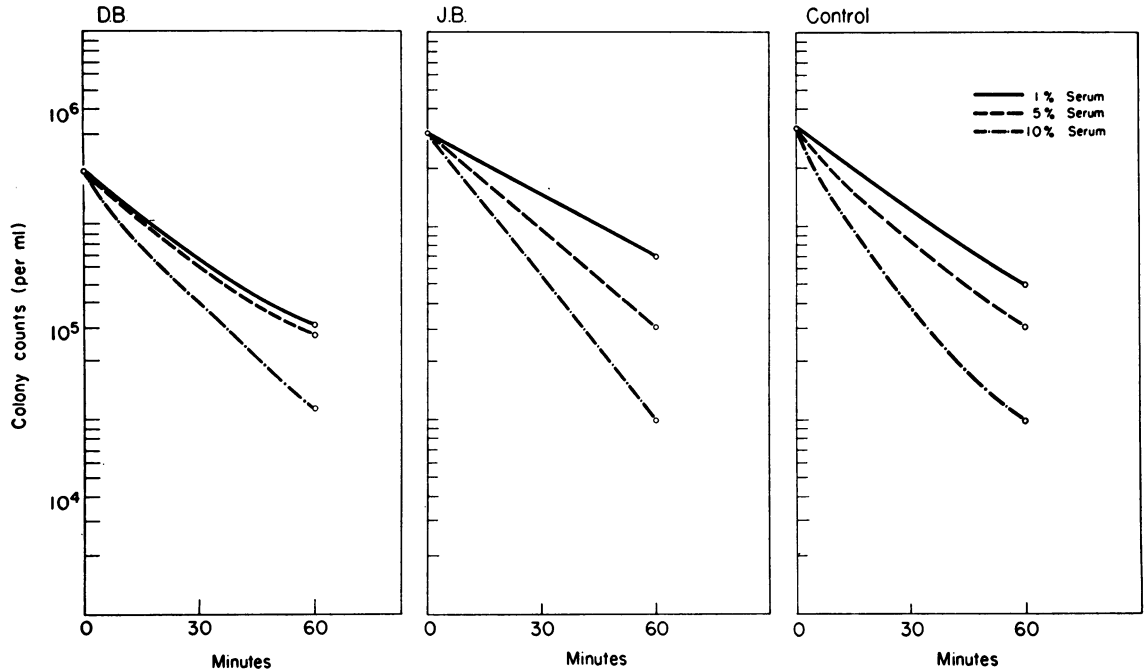


FIG. 4. COMPARISON OF SERUM AS OPSONIN FOR *STAPHYLOCOCCUS AUREUS* FROM TWO OF THE PATIENTS WITH CHRONIC GRANULOMATOUS DISEASE AND SERUM POOLED FROM NORMAL CONTROLS. Note the similar efficient bactericidal capacity of the normal PMN with 10% serum from either patient and controls. There was also a similar decreased rate of bactericidal activity with lower concentrations of sera from the patients and from the controls.

bated with staphylococci or paracolon bacteria for 60 minutes. Paracolon organisms were rapidly digested, and even at 30 minutes' incubation there were few intact intracellular bacteria. In contrast, when PMN from the children with chronic granulomatous disease were incubated with paracolon organisms, the intracellular bacteria appeared relatively intact and there had been little degranulation of the cytoplasm of the phagocytes.

This difference in morphology was most strikingly demonstrated in studies with the electron microscope. Figure 5 shows a PMN from a normal control after 30 minutes' incubation with staphylococci. Extensive degranulation and vacuole formation are obvious. The early stages of digestion of staphylococci in the large vacuoles can be seen. Paracolon organisms were almost completely destroyed within the neutrophil vacuole at the same time interval.

Figure 6 shows a PMN from one of the patients with granulomatous disease. The intracellular location of the bacteria is apparent within phagocytic vacuoles. However, unlike Figure 5, there

is little degranulation of the neutrophil and virtually no evidence of digestion of the intracellular bacteria. Even the paracolon bacteria, which were rapidly digested by the PMN from normal controls, were found to be morphologically intact after 60 minutes' incubation with PMN from patients with granulomatous disease (Figure 7).

### Discussion

This investigation of the bactericidal capacity of polymorphonuclear leukocytes from patients with increased susceptibility to bacterial infection allowed determination of engulfment of bacteria and the fate of ingested organisms. A consistent defect in bactericidal capacity after ingestion of bacteria was demonstrated in five patients with a disease entity termed chronic granulomatous disease of childhood. This syndrome was thoroughly studied and separated as a specific disease entity by Berendes, Bridges, and Good (1, 2) and was described by Landing and Shirkey (3) and Carson and associates (4). All of the patients in-

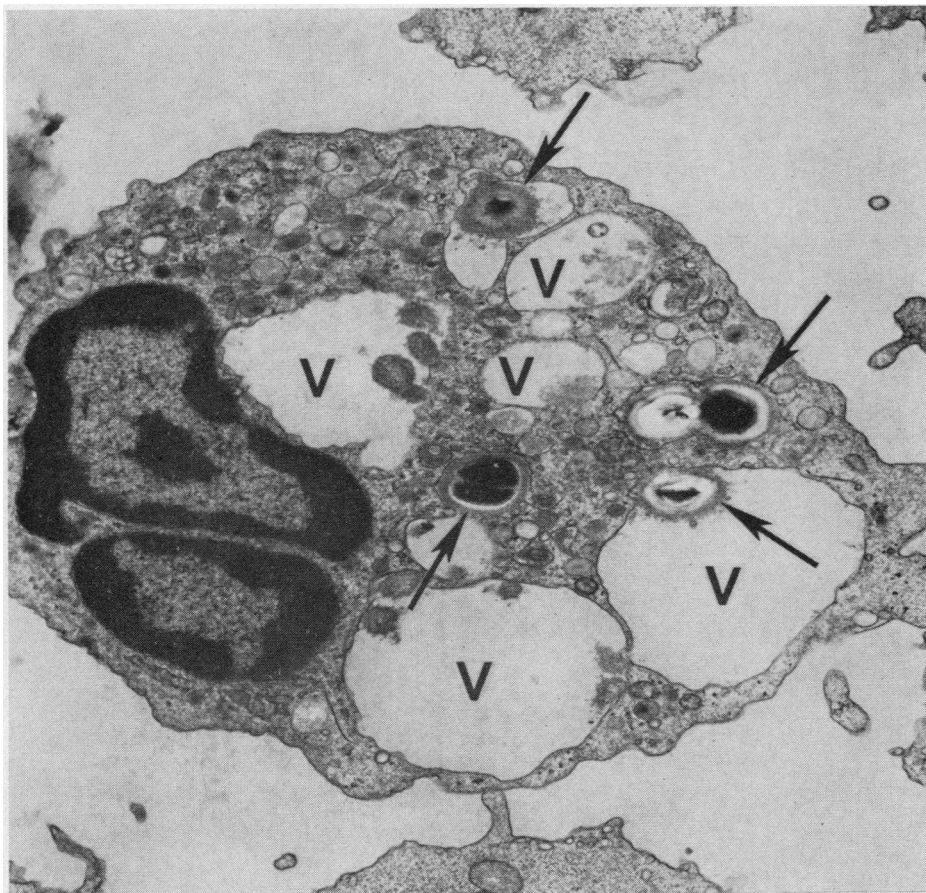


FIG. 5. POLYMPHONUCLEAR LEUKOCYTE FROM A NORMAL CONTROL PATIENT INCUBATED WITH STAPHYLOCOCCI FOR 30 MINUTES. Many bacteria (arrows) in various stages of destruction are evident within the cell. Note cytoplasmic vacuoles (V) around or adjacent to the degenerating bacteria.  $\times 18,000$ .

cluded in this study demonstrated the following characteristics, which separated this disease from other granulomas: *a*) they were males with onset of severe bacterial disease early in life, *b*) they exhibited normal to elevated immunoglobulins with normal response to antigenic stimulation, and *c*) granulomas were demonstrated in histologic sections of lesions.

Case summaries of three patients included in this study, D.B., K.S., and L.J., were published in a preliminary report (14). The two additional patients, J.B. (18 months) and T.W. (17 months), were hospitalized for the first time, and the diagnosis of chronic granulomatous disease was made during the investigation of leukocyte function. Both of these patients are maternal

cousins of other patients with chronic granulomatous disease of childhood. History before hospitalization indicated recurrent respiratory illness and facial dermatitis. PMN from J.B. demonstrated diminished bactericidal capacity when he was well. Several weeks after his initial study he was hospitalized for the first time with suppurative cervical lymphadenopathy. T.W. was hospitalized for several weeks with chronically draining inguinal lymph nodes. Cultures of the drainage yielded an *Aerobacter aerogenes*.

*P. hafnia*, an endogenous enterobacterial species, was recovered from the suppurating cervical lymph nodes of two brothers with the syndrome (D.B. and J.B.). Paracolon organisms were also recovered from the osteomyelitis lesion of the



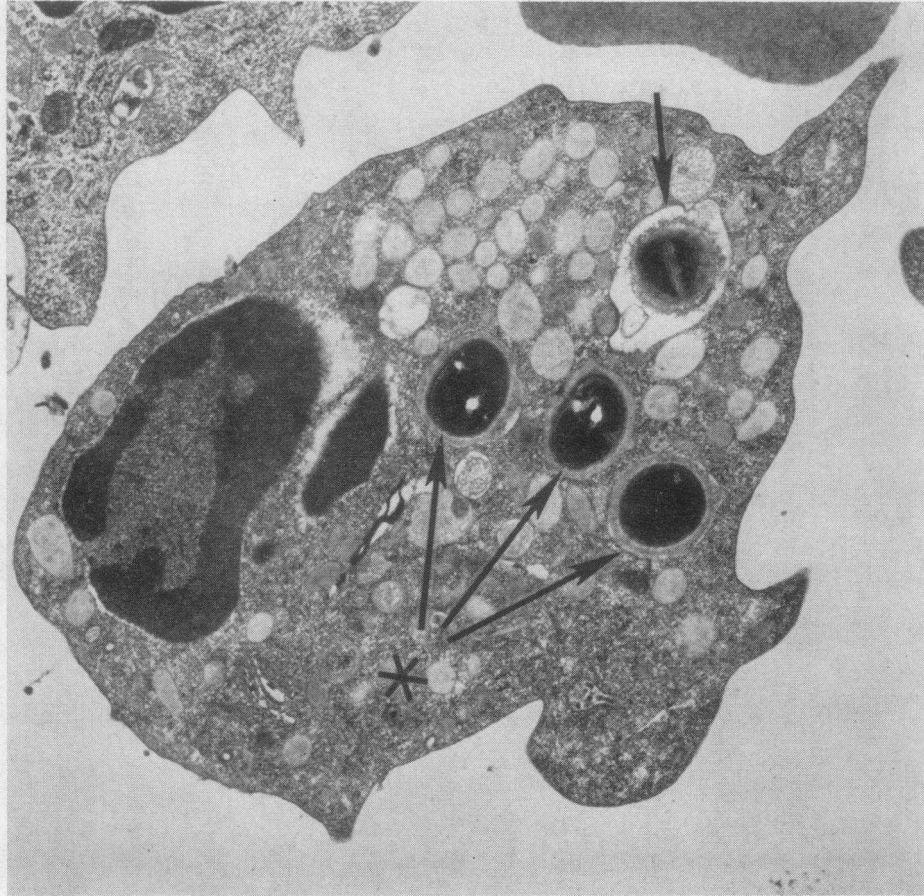


FIG. 6. POLYMORPHONUCLEAR LEUKOCYTE FROM A CHILD WITH CHRONIC GRANULOMATOUS DISEASE INCUBATED WITH STAPHYLOCOCCI FOR 1 HOUR. Several bacteria (arrows) are visible in the cytoplasm. Little degranulation is evident.  $\times 22,500$ .

metacarpal and calcaneus of D.B. A maternally related cousin of these two boys (K.S.) suffered severe recurrent infiltrative pulmonary disease over a 6-year period. He was hospitalized during part of the time of this study for drainage of a rectal abscess, from which an aerobacter species was cultured.

The bacteria associated with the suppurative lesions in these children were species indigenous to the upper respiratory and gastrointestinal tracts of man and species that are usually rapidly phagocytized and killed by polymorphonuclear leukocytes *in vitro* and presumably *in vivo*. Therefore, the existence of an abnormality of function of leukocytes was postulated on the basis of clinical evidence when this disease entity was described (2). This prediction is borne out in the present

study by the demonstration of prolonged viability of *S. aureus* and *P. hafnia* in the polymorphonuclear leukocytes of these patients.

All of these children had male family members who also demonstrated the syndrome of chronic granulomatous disease. Brothers of two of the children (L.J. and K.S.) died from sepsis and with granulomatous disease in infancy. Two of the patients (D.B. and J.B.) were brothers and maternal cousins of K.S. Patient T.W. is the maternal cousin of two brothers, one living and one dead, who presented the clinical features of chronic granulomatous disease.

As was the case with patients previously described, these five patients with chronic granulomatous disease were normal in capacity to respond to antigenic stimulation with heterologous

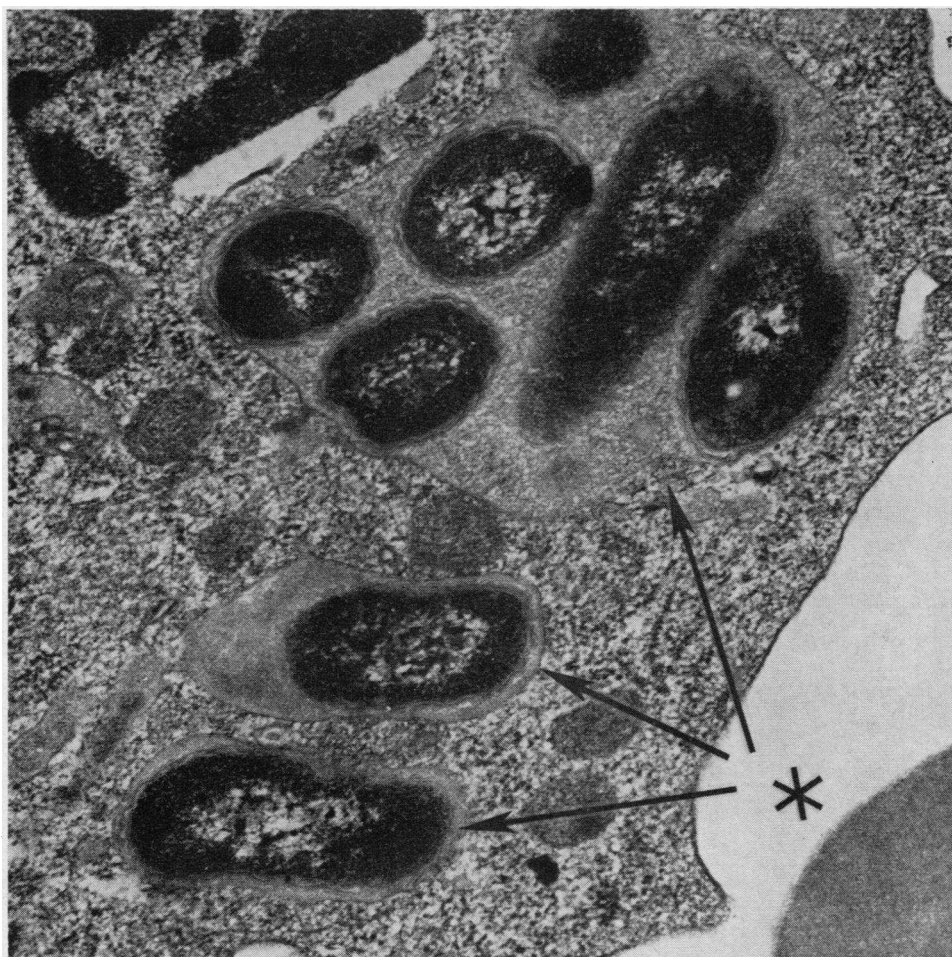


FIG. 7. POLYMORPHONUCLEAR LEUKOCYTE FROM A CHILD WITH CHRONIC GRANULOMATOUS DISEASE INCUBATED FOR 1 HOUR WITH *PARACOLON HAFNIA*. Intact intracellular bacteria are evident (arrows).  $\times 46,000$ .

antigens and had normal or elevated levels of circulating antibody and complement and the normal spectrum of immunoglobulins. Ability to develop delayed hypersensitivity was also found to be normal. The patients also demonstrated typical polymorphonuclear leukocytosis during episodes of systemic illness. Their total peripheral leukocyte counts ranged from 6 to 30,000 per  $\text{mm}^3$ . There was obvious suppuration of lesions, and PMN migrated normally to the skin windows when the inflammatory cycle in these patients was studied by the technique of Rebeck and Crowley (15). Furthermore, the phagocytic capacity of polymorphonuclear leukocytes appeared to be normal as determined by observation of intracellular

bacteria on stained smears of PMN-bacteria mixtures.

The significance of prolonged viability of bacteria in polymorphonuclear leukocytes in our patients in the pathogenesis and the presence of granulomas in the tissues is uncertain. It is known that long continued presence of intracellular viable microorganisms commonly results in chronic inflammatory response. For example, there are many species of microorganisms that have the capacity to survive in human PMN, e.g., tubercle bacillus, brucella, and listeria. The presence of these organisms commonly results in a chronic granulomatous process (16). Therefore, the prolonged presence of bacteria or bacterial

products that are ordinarily quickly detoxified and digested by normal PMN may be basic to the development of a granulomatous inflammatory response in these patients with diminished capacity for intracellular digestion of ubiquitous bacteria. These bacteria then would behave in children with chronic granulomatous disease in a fashion similar to tubercle bacilli or brucella in infected individuals having normal function of their polymorphonuclear leukocytes.

The metabolic basis for the defect in bactericidal capacity is not known; however, there was a striking and consistent difference in the extent of cytoplasmic reaction to phagocytosis in the PMN from patients with granulomatous disease and controls. In the controls, there was extensive degranulation and vacuole formation with visible disintegration of the ingested bacteria, even after short periods of incubation. The polymorphonuclear leukocytes from patients with chronic granulomatous disease of childhood did not respond to phagocytosis with similar extensive degranulation. In the electron microscopic examination, there were visible membranes surrounding ingested particles, but with intact granules in the surrounding cytoplasm. In addition, bacteria in PMN from the granulomatous patients were generally intact, indicating failure or delay of digestive processes. The presence of intact intracellular organisms in the electron micrographs support data obtained by colony counts of viable bacteria associated with leukocytes after differential centrifugation of incubated phagocyte-bacteria mixture.

The morphologic evidence of intact cytoplasmic granules after phagocytosis is of particular interest, since the total quantity of the bactericidal factors lysozyme and phagocytin in extracts of the leukocytes from the patients and from normal controls was comparable. These bacterial factors, as well as many of the hydrolytic digestive enzymes, are known to be associated with the leukocyte granules (17). It has been demonstrated that after phagocytosis there is a release of enzymes from the granule fraction into the supernatant fraction as active soluble enzyme (18). Hence, the demonstration of minimal degranulation after phagocytosis within leukocytes with defective antibacterial activity suggested a lack of availability of bactericidal factors and digestive enzymes rather than absence of these factors.

Evidence that phagocytosis and degranulation in cells after particle ingestion is associated with several metabolic changes has been presented (16, 18). The studies presented here provide evidence for a distinct separation of the metabolic events involved in phagocytosis and those necessary for degranulation and particle digestion. Thus, these patients provide a unique opportunity, permitting investigation of relationships between metabolic events that are related to phagocytosis per se and those that are associated with degranulation and digestion of ingested material.

### Acknowledgments

The technical help of Miss O. Linda Schneider was essential for these studies. We are indebted to Dr. Dorothy Sundberg for histochemical studies on blood smears.

### References

1. Berendes, H., R. A. Bridges, and R. A. Good. A fatal granulomatosis of childhood: the clinical study of a new syndrome. *Minn. Med.* 1957, **40**, 309.
2. Bridges, R. A., H. Berendes, and R. A. Good. A fatal granulomatous disease of childhood: the clinical, pathological, and laboratory features of a new syndrome. *Amer. J. Dis. Child.* 1959, **97**, 387.
3. Landing, B. H., and H. S. Shirkey. A syndrome of recurrent infection and infiltration of viscera by pigmented lipid histiocytes. *Pediatrics* 1957, **20**, 431.
4. Carson, M. J., D. L. Chadwick, C. A. Brubaker, R. S. Cleland, and B. H. Landing. Thirteen boys with progressive septic granulomatosis. *Pediatrics* 1965, **35**, 405.
5. Maaløe, O. On the Relation between Alexin and Opsonin. Copenhagen, Ejnar Munksgaard, 1946.
6. Cohn, Z. A., and S. I. Morse. Interactions between rabbit polymorphonuclear leukocytes and staphylococci. *J. exp. Med.* 1959, **110**, 419.
7. Hirsch, J. C., and B. Strauss. Studies on heat-labile opsonin in rabbit serum. *J. Immunol.* 1964, **92**, 145.
8. Shinefield, H. R., J. C. Ribble, M. Boris, and H. F. Eichenwald. Bacterial interference: its effect on nursery-acquired infection with *Staphylococcus aureus*. I. Preliminary observations on artificial colonization of newborns. *Amer. J. Dis. Child.* 1963, **105**, 646.
9. Hayhoe, F. G. J., D. Quaglino, and R. Doll. Cytology and cytochemistry of acute leukaemias. *Spec. Rep. Ser. med. Res. Coun. (Lond.)* 1964, no. 304, 11.

10. White, J. G., and W. Krivit. The ultrastructural localization and release of platelet lipids. *Blood* 1966, **27**, 167.
11. Hirsch, J. G., and A. B. Church. Adrenal steroids and infection: the effect of cortisone administration on polymorphonuclear leukocytic functions and on serum opsonins and bactericidins. *J. clin. Invest.* 1961, **40**, 794.
12. Sugar, D. The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme. *Biochim. biophys. Acta (Amst.)* 1952, **8**, 302.
13. Hirsch, J. G. Further studies on preparation and properties of phagocytin. *J. exp. Med.* 1960, **111**, 323.
14. Holmes, B., P. G. Quie, D. B. Windhorst, and R. A. Good. Fatal granulomatous disease of childhood. An inborn abnormality of phagocytic function. *Lancet* 1966, **1**, 1225.
15. Rebeck, J. W., and J. H. Crowley. A method of studying leukocytic functions *in vivo*. *Ann. N. Y. Acad. Sci.* 1955, **59**, 757.
16. Cohn, Z. A., and K. F. Austen. Contribution of serum and cellular factors in host defense reactions. II. Cellular factors in host resistance. *New Engl. J. Med.* 1963, **268**, 1056.
17. Cohn, Z. A., and J. G. Hirsch. The isolation and properties of the specific cytoplasmic granules of rabbit polymorphonuclear leukocytes. *J. exp. Med.* 1960, **112**, 983.
18. Cohn, Z. A., and J. G. Hirsch. The influence of phagocytosis on the intracellular distribution of granule-associated components of polymorphonuclear leukocytes. *J. exp. Med.* 1960, **112**, 1015.