Leukocyte Degranulation and Vacuole Formation in Patients with Chronic Granulomatous Disease of Childhood

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ABSTRACT A cellular defect associated with decreased bactericidal activity of the polymorphonuclear leukocyte has been found in a 2½ yr old Negro boy with the typical clinical and pathological findings of chronic granulomatous disease. Unlike previously described patients his polymorphonuclear leukocytes were shown to undergo apparently normal degranulation and vacuole formation after phagocytosis. Metabolic studies of the leukocytes indicated a failure to increase oxygen consumption with phagocytosis or to reduce Nitroblue tetrazolium dye. These metabolic abnormalities are identical with those previously reported in patients with chronic granulomatous disease. Two additional patients with chronic granulomatous disease have also been found to have apparently adequate degranulation of polymorphonuclear leukocytes after phagocytosis. Our studies suggest that failure of degranulation may not be a necessary part of this functional leukocyte abnormality.

INTRODUCTION

Among the chronic granulomatous diseases of children is a group of strikingly similar patients that were first brought together as a clinical entity under the name of "a fatal granulomatous disease of childhood" (1). The polymorphonuclear leukocyte (PMN) of these patients has been shown to have decreased bactericidal activity by in vitro measurement (2). Recent reports have focused attention on morphologic and metabolic abnormalities in the functionally abnormal PMN. In the normal PMN degranulation and vacuole formation occur after phagocytosis (3). Quie, White, Holmes, and Good (4) have reported a failure of these events to occur in the PMN of patients with chronic granulomatous disease (CGD). Holmes, Page, and Good have reported that an acid extract of the PMN had normal bactericidal activity in vitro (5). Baehner and Nathan (6) reported a failure to reduce Nitroblue tetrazolium (NBT) of leukocytes from two patients with CGD. The hexose monophosphate shunt (HMS) also failed to be normally stimulated during phagocytosis. In one patient they measured oxygen consumption of the leukocytes during phagocytosis and found that it was not increased. They attributed these metabolic abnormalities to the lack of a cyanide–insensitive nicotinamide adenine dinucleotide (NADH) oxidase.

Holmes et al. (5) have recently reported on extensive metabolic studies in leukocytes of patients with CGD. They concluded that glucose utilization, lactate production, Kreb's cycle activity, and lipid turnover all were stimulated normally during phagocytosis. However, respiration, hydrogen

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peroxide production, and HMS activity were not normally increased with phagocytosis. They postulated that these impaired metabolic events are related to a failure of degranulation.

However, Zatti, Rossi, and Meneghelli (7) have studied the time of stimulation of the HMS relative to the time of degranulation in phagocytosing leukocytes and reported that increased HMS activity occurred before the act of degranulation, suggesting to these authors that the stimulation to oxidative glycolysis was not due to degranulation but to the act of particle ingestion by the cell.

Holmes and coworkers (5) have also challenged the concept, put forth by Baehner and Nathan, of a lack of NADH oxidase in these abnormal leukocytes. Obviously this degree of controversy indicates that more needs to be learned about the metabolic abnormality in the leukocyte of the patient with CGD.

The purpose of this report is to present evidence that degranulation and vacuole formation may occur normally in a patient who fulfills all the criteria for CGD.

METHODS

Case Report. The patient was a 2½ yr old Negro boy who had his first onset of cervical adenitis at 3½ months of age. Recurrent adenitis required incision and drainage on five subsequent occasions. Seborrheic eczema of the scalp and neck with secondary pyoderma began at 12 months of age. Pulmonary disease with recurrent pneumonia and persistent pulmonary infiltrates has been present since 15 months of age. Other bacterial infections have included an hepatic and abdominal wall abscess and an iliopsoas abscess. Moderate hepatosplenomegaly has been present intermittently at times of infection. There are seven older siblings, three of whom are male. None of these family members had a significant history of repeated infections.

Laboratory findings. Appropriate leukocytosis occurred at the time of bacterial infections with absolute neutrophil counts ranging from 4000 to 25,000/mm^3. Hemoglobin concentration was 9 g/100 ml with hypochromia and microcytosis. Hyperglobulinemia with elevations of IgA, IgM, and IgG was first noted at 15 months of age and has been persistent since. Leukocytes entered into areas of inflammation normally as demonstrated by a normal response to "Rebuck skin window" (8) preparations and the presence of abundant leukocytes in purulent material from abscesses. Normal delayed hypersensitivity was demonstrated by a positive mumps skin test. An excisional biopsy of an inguinal node was done and a caseated granuloma found. Skin tests for tuberculosis, histoplasmosis, and atypical mycobacteria were repeatedly negative as were cultures for these organisms from biopsy material and abscesses. Cultures from abscess material yielded *Staphylococcus aureus* and *Aerobacter aerogenes.*

Phagocytosis studies. Phagocytosis and bactericidal properties of intact leukocytes were determined by a modification of the method described by Maaløe (9) with further modifications as described by Cohn and Morse (10). Leukocyte suspensions were prepared by dextran sedimentation of heparinized venous blood. 20 ml of freshly drawn blood was mixed with 40 ml of 3% sterile dextran (mol wt 228,000) containing 100 U of sterile heparin, and allowed to sediment at 37°C for 20–30 min. The supernatant plasma layer containing the leukocytes was harvested and spun in sterile siliconized graduated centrifuge tubes for 10 min at 500 *g* (1500 rpm, PR-2 International centrifuge, head No. 269). The cell buttons were washed two times with warm Hank's solution. The white cell concentration was determined by hemocytometer counting, smears were stained with Wright's stain, and differential counts were performed. Cell suspensions were then diluted to give a PMN concentration of $1–3 \times 10^7$/ml. Appropriate dilutions were calculated to give approximately the same number of neutrophils per milliliter for patient and control samples.

The bacterial species used in these studies were *Staphylococcus aureus* strain 502A and *Aerobacter aerogenes,* The Staphylococcal strain has previously been described and its biological characteristics defined (11). The *A. aerogenes* species was isolated from drainage from an ileopsoas abscess from the patient. The organism to be used in each study was cultured overnight in trypticase Soy Broth at 37°C for 18 hr. The bacterial suspension was then centrifuged and washed two times with Hank's solution in a manner similar to the cell suspensions. After the second wash, the bacterial button was resuspended in an equal volume of Hank's solution and the concentration of organisms determined using a Petroff-Hausser bacterial counter. The bacterial concentration was then adjusted with Hank's solution to give approximately 1×10^8 bacteria per ml.

As a source of serum opsonins for the phagocytosis mixture fresh blood was obtained at the same blood letting utilized for obtaining the blood for leukocyte isolation and allowed to clot at room temperature. In different experiments, the serum obtained from the clotted blood was collected from either the patient or the control donor.

Phagocytosis studies were done in sterile siliconized 30-ml French bottles. Each bottle contained 1.6 ml of the cell suspension, 0.2 ml of serum, and 0.2 ml of the bacterial suspension. This resulted in an approximately equal ratio of bacteria to leukocytes and a final concentration of 10% serum.

Immediately after the suspensions were made they were thoroughly mixed using a vortex mixer and a 0.1 ml aliquot was obtained from each bottle, mixed with 0.9 ml of saline, and each sample was homogenized for 3–4 min using a teflon homogenizer driven by a high speed motor. Serial dilutions with normal saline were made and 0.1 ml of a 1/10,000 dilution was mixed with melted Trypticase Soy Agar and pour plates made to de-

termine the total number of viable organisms. The leukocyte-bacterial mixtures were then incubated at 37°C in a temperature-controlled water bath shaker, set at 30 agitations/min. At 1 and 2-hr intervals another 0.1 ml aliquot was obtained and processed in the same manner for determining total viable organisms. At the same time, a 0.5 ml aliquot was obtained from each bottle and diluted with 4.5 ml of normal saline. This was centrifuged at 600 rpm for 4 min. The supernatant containing the extracellular bacteria was removed and a pour plate made from a 0.1 ml aliquot of a 1/10,000 dilution. The cell button containing the intracellular bacteria was reconstituted to 0.5 ml in normal saline. A 1/10 dilution was homogenized to release intracellular bacteria and a 1/10,000 dilution was prepared from a 0.1 ml aliquot of the homogenate. Pour plates were prepared as above. The colonies were counted on the following day after overnight incubation at 37°C and total, extracellular, and cell-associated viable bacteria determined for each incubation period.

Wright's-stained cover slip smears were also obtained before incubation and at each incubation period for determination of percentage of PMN participating in phagocytosis, the average number of bacteria per phagocytosing PMN, and the degree of degranulation and vacuole formation. Cover slip smears were also prepared and stained for alkaline phosphatase and peroxidase. Wet preparations of leukocyte-bacterial mixtures were examined under phase microscopy and leukocyte motility and phagocytosis observed.

Preparation for electron microscopy. Leukocyte-bacterial suspensions were fixed in 3% glutaraldehyde in Millonig's phosphate buffer at pH 7.35. They were postfixed in 1% osmium tetroxide in the same buffer and then embedded in araldite. The tissue was sectioned with glass knives and examined using Zeiss EM 9 and Philips 300 electron microscopes. Sections were stained with lead and uranyl acetate. Some were lightly shadowed with carbon.

Procedure for determining oxygen consumption. Leukocytes were separated by dextran sedimentation as described above. No attempt was made to obtain red blood cell–free suspensions. Cells were washed in Krebs-Ringer phosphate buffer and resuspended in 1 ml of this buffer. Equal numbers of neutrophils for the patient and control studies were utilized for each experiment and suspensions were adjusted to contain between 2 and 4×10^7 cells/ml. A glucose solution was prepared in Krebs-Ringer phosphate buffer containing 1 mmole of glucose per ml. Just before the start of each experiment, 1 ml of glucose solution was added to 1 ml of cell suspension. The sample to be tested was placed in an ultramicrosample system (model No. 113-S1 Instrumentation Laboratory, Inc., Boston, Mass.).

We allowed 5 min for equilibration and then took readings every 5 min for 20 min, mixing the sample after every reading. This constituted the resting state. 2/10 ml of polystyrene latex particles (Difco, 0.81 μ) in suspension was then added through a three-way stopcock and after thorough mixing, readings were taken every 3 min for 20 min. The hemoglobin in each sample was then determined and glass cover slip smears obtained and stained with Wright's stain to ascertain phagocytosis. The partial tension of oxygen was measured polarographically. Since all samples were maintained at 37°C no temperature correction factor was necessary. Serial samples were withdrawn for determination of pH.

The pH of the cell suspension decreased during the period of sampling. The measured oxygen tension was corrected for pH change by correction factors obtained from Severinghaus (12) and converted to saturation in per cent by a nomogram (12). The oxygen carrying capacity of the hemoglobin in these samples was calculated from hemoglobin content measured by the cyanmetheglobin method (13) multiplied by the factor 1.34 (14). The oxygen carrying capacity was multiplied by the saturation to obtain the oxygen content in volumes per cent for each sample. The difference in oxygen content from the time of initial measurement to that of the last sample was taken as a measure of oxygen consumption by the leukocytes in the sample.

Cellular uptake of p-Nitroblue tetrazolium chloride (NBT). NBT uptake and reduction intracellularly in leukocytes participating in phagocytosis was studied using the method of Windhorst, Holmes, and Good (15). Leukocyte-rich plasma was obtained from 10 ml of heparinized venous blood by sedimentation with clinical dextran. Leukocyte counts were done on the supernatant plasma and an aliquot calculated to contain 7×10^6 cells was transferred to a siliconized test tube and centrifuged at 500 rpm. The cells were resuspended in 0.35 ml of normal human serum to which 0.16 mg of glucose in 0.05 ml of water was added to ensure adequate oxidative substrate. 3 mg of nicotinamide adenine dinucleotide phosphate (NADPH) and 0.13 mg of NBT in 0.1 mg of phosphate buffer at pH 7.0 were added to the cell suspension which was mixed and allowed to stand at 37°C for several minutes. Latex particles (Difco, 0.81 μ) were then added and the tubes were gently mixed on a tilt table at 37°C for 45 min. The cells were then centrifuged at 500 rpm. Smears were prepared from the cell button and stained with neutral red. Only cells containing 10 or more latex particles were counted. These were counted as positive if a blue precipitate could be seen in the cytoplasm. This method was modified from that of Windhorst and coworkers, in that the cells were incubated with NBT for 45 min and all reagents were kept at 37°C. These modifications produced more consistent results with normal cells which labeled to 70% or more.

Morphology and cytochemistry of leukocytes. Blood smears and smears of leukocyte-bacteria mixtures were made on glass slides and stained with Wright's stain in standard fashion. Slides were prepared for histochemical determination of peroxidase by the method of Graham (16) and for alkaline phosphatase by the method of Ackerman (17).

RESULTS

Phagocytosis. The results of the study to measure intracellular killing of ingested bacteria are

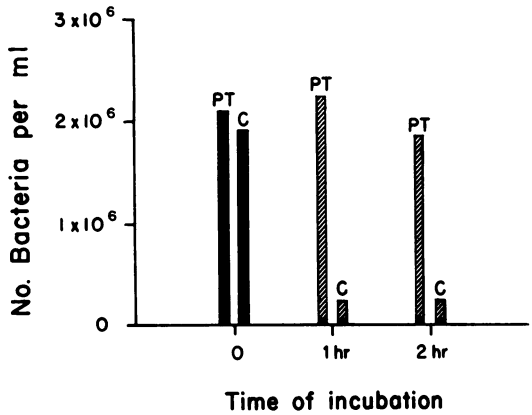

FIGURE 1 Location and number of viable bacteria after 1 and 2 hr incubation of leukocyte-bacteria mixtures. Note that most of the viable bacteria were cell associated with many more viable organisms in the patient's leukocytes than in the control cells. Organism, *Staphylococcus aureus* 502A; serum, control; black bars, extracellular bacteria; striated bars, cell associated bacteria.

shown in Fig. 1. There was a striking persistence of viable cell-associated organisms in the patient's study as compared with the control. The control cells performed normally in other similar experiments when the patient's serum was used as a source of opsonins. The patient's cells were defective against both *Staphylococcus aureus* and *Aerobacter aerogenes*.

Light microscopy. In Wright's-stained blood smears examined at several intervals after incubation of leukocyte-bacterial suspensions there was an equal degree of degranulation and vacuole formation for the patient's and for control leukocytes. There was no significant difference in the percentage of PMN containing ingested bacteria, the average number of bacteria per PMN, or the percentage of PMN in which bacteria were contained within vacuoles (Table I). However, in the control cells a larger percentage of bacteria appeared to be undergoing fragmentation and digestion as compared with a higher percentage of intact bacteria in the cells of the patient. To assess the degree of degranulation in these leukocyte-bacterial suspensions peroxidase and alkaline phosphatase stains were used to highlight the granular morphology. At 5, 30, and 60-min incubation times, slides were prepared with peroxidase and alkaline phosphatase stain, coded, and read by a single observer. 200 leukocytes containing one or more bacteria were counted per slide and the degree of degranulation recorded as minimal, moderate, or marked. No difference in degranulation could be detected between normal cells and those of the patient.

These studies were performed in two additional patients with chronic granulomatous disease. Their diagnoses were based upon the typical clinical features of the syndrome plus the characteristic functional and metabolic abnormalities of the polymorphonuclear leukocytes as described for the first patient. In one of these children the typical granulomatous lesion was demonstrated in a biopsy of an area of chronic suppurative lymphadenitis. As seen in Fig. 2 *a* and *b* the patients' PMN contained vacuoles and a decreased number of cytoplasmic granules after phagocytosis.

Active motility directed towards phagocytosis and followed by degranulation and vacuole formation was seen with phase microscopy.

Electron microscopy. Electron photomicrographs were prepared from suspensions of the

TABLE I

Phagocytosis and Vacuole Formation

Organism, incubation period	*Staphylococcus aureus*				*A. Aerogenes*			
	Patient	Control	Patient	Control	Patient	Control	Patient	Control
	1 hr		*2 hr*		*1 hr*		*2 hr*	
% PMN with bacteria	43	37	54	45	60	32	87	63
Average No. of bacteria per PMN	4	4	4	4	5	3	8	4
% PMN with bacteria having vacuoles	91	92	94	95	94	98	94	92

PMN, polymorphonuclear leukocyte.

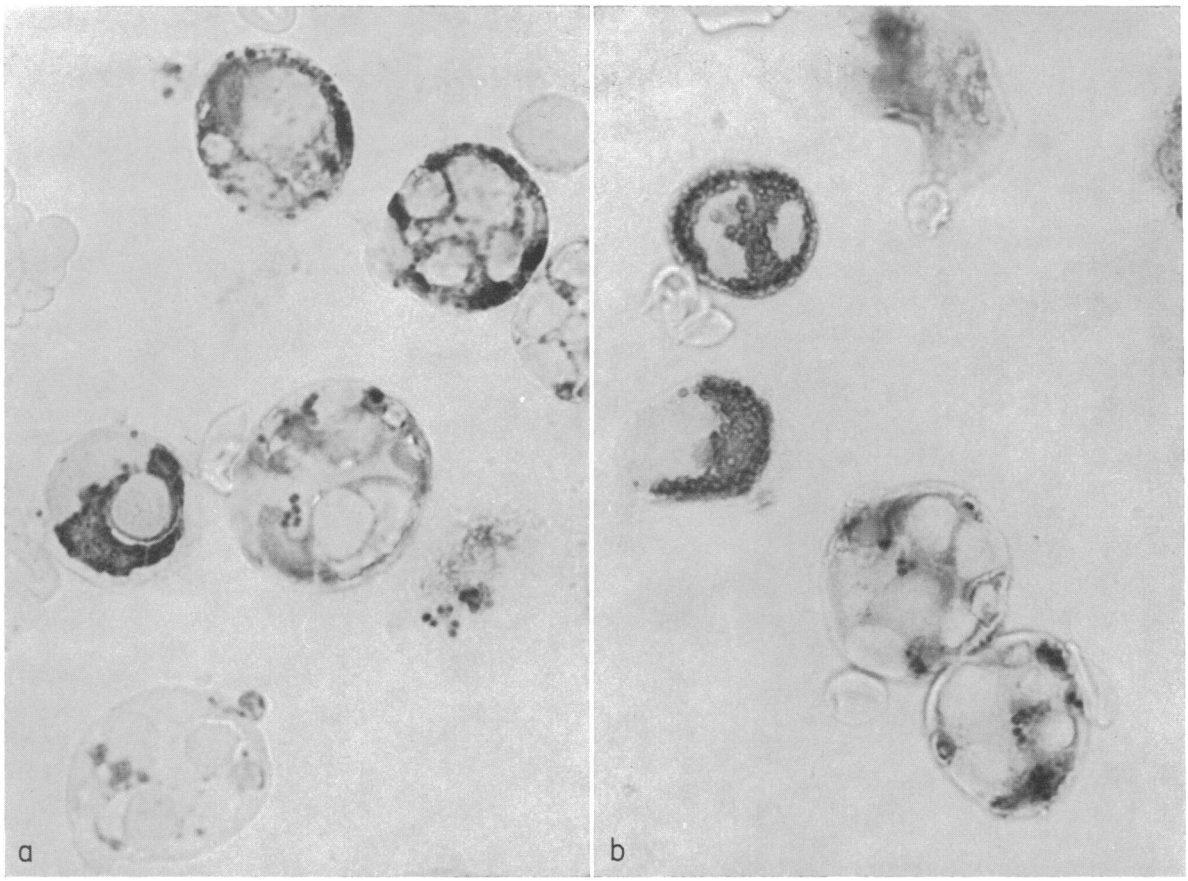

FIGURE 2 *a* and *b* Light microscopy photographs of peroxidase stained 1 hr leukocyte-bacteria mixtures. These photomicrographs from studies of two additional patients with chronic granulomatous disease include cells which have not ingested bacteria (*Staphylococcus aureus* 502A) in which granules are intact and no vacuoles are seen. In contrast most cells have undergone marked degranulation and vacuole formation. × 2000.

patient's leukocytes after 15 and 60-min incubation with *Aerobacter aerogenes*. Approximately 500 leukocytes containing bacteria were examined from each of these time periods. At the 15 min incubation time bacteria could be seen within the cells but cytoplasmic granules were numerous and digestive vacuoles were small (Fig. 3). At the 1 hr incubation time digestive vacuoles were well formed and the number of cytoplasmic granules had decreased (Fig. 4). A higher magnification of the digestive vacuole is shown in Fig. 5 to demonstrate the clear evidence of granular fragments and whole granules emptying into a vacuole from the surrounding cytoplasm. We could detect no difference in the time sequence or the degree to which these events occurred in normal leukocytes studied under the same conditions. A consistent difference between the control and the patient studies was the presence of a higher percentage of intact appearing bacteria in the latter.

Nitroblue tetrazolium study. The number of phagocytic leukocytes containing NBT dye varied from 0–15% in the patient's cells. The patient's mother and two female siblings had values ranging from 26–66%. The range of normal in our laboratory is 70–98% (mean 85%). Three male siblings and two other female siblings had normal NBT studies.

Oxygen consumption study. As shown in Fig. 6, the patient's leukocytes failed to show an increase in oxygen consumption during phagocytosis as compared with normal cells. This demonstration of failure to stimulate respiration with phagocytosis is presented as additional evidence that our patient is similar to those described by Holmes and coworkers (5) and by Baehner and Nathan (6).

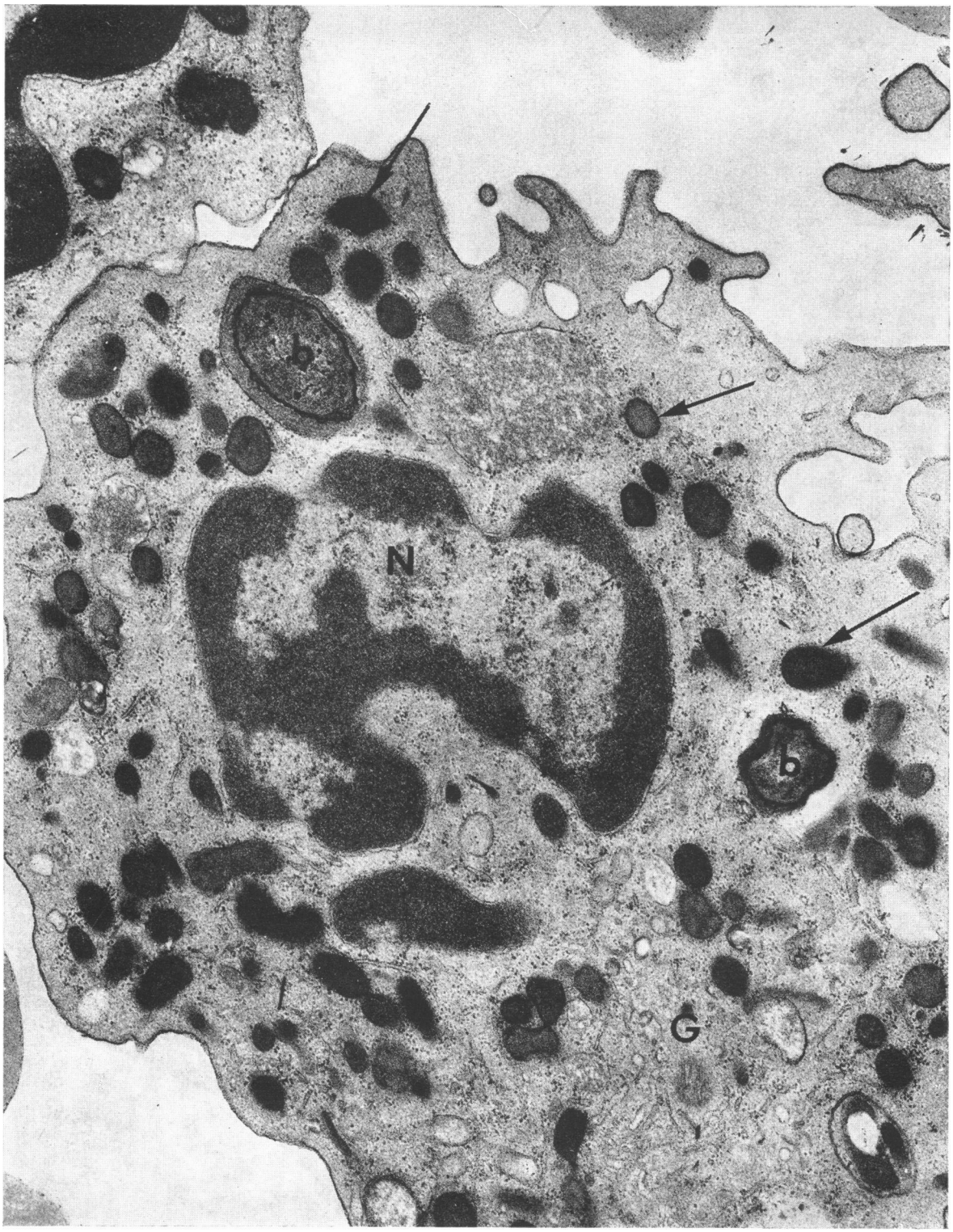

FIGURE 3 Patient's polymorphonuclear leukocyte incubated 15 min with bacteria. Two bacteria (*b*) are within phagocytic vacuoles. Numerous granules (arrows) are present. N, nucleus; G, golgi zone. × 24,000.

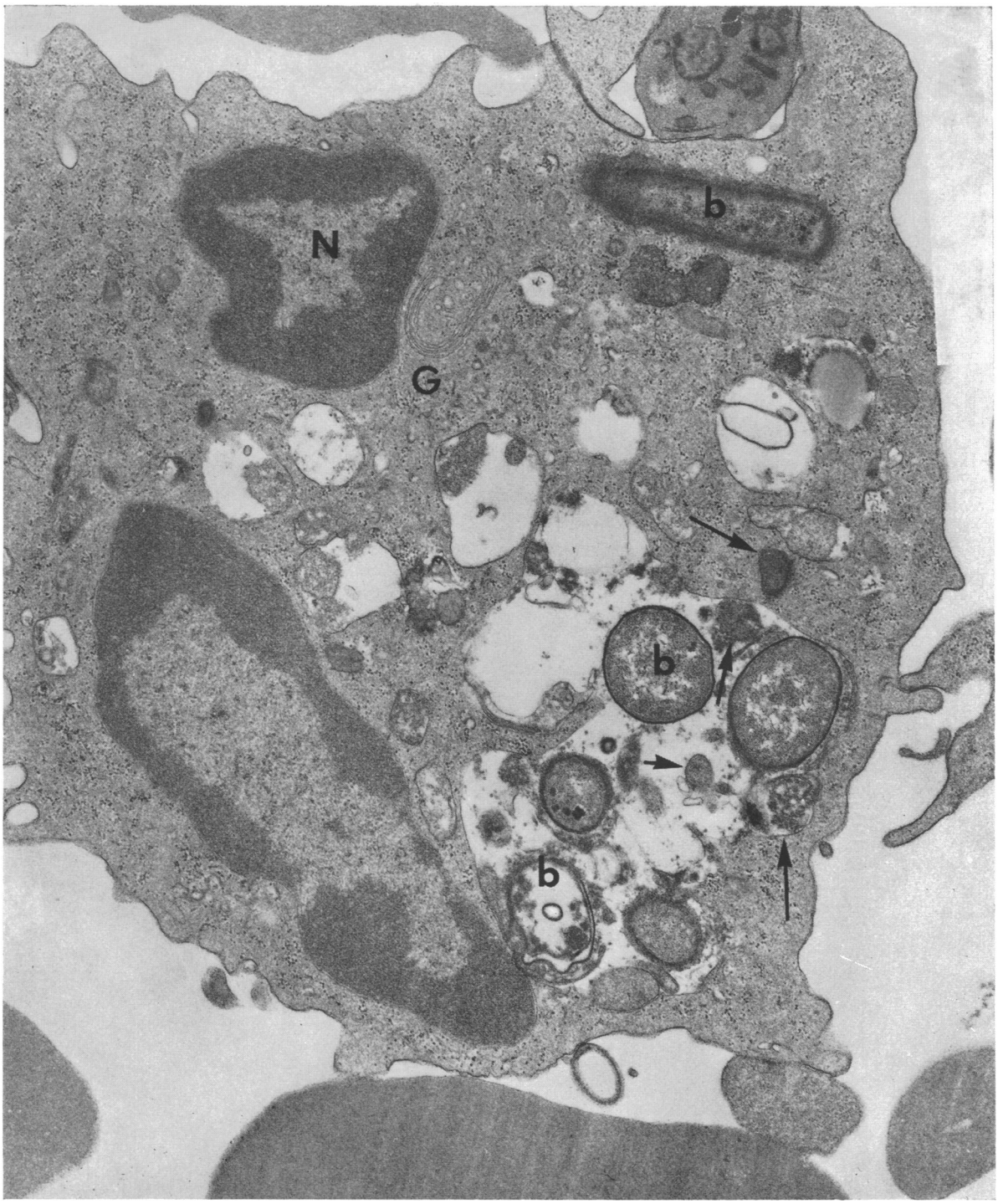

FIGURE 4 Patient's polymorphonuclear leukocyte incubated 1 hr with bacteria. The degree of degranulation is similar to that seen in the normal control leukocytes. Cytoplasmic granules are decreased in number as compared with the 15 min incubation time. The bacterial bodies (*b*) show less evidence of disruption than was seen in controls. Cytoplasmic granules are adjacent to and within the digestive vacuole (arrows). N, nuclear lobes; G, golgi zone. × 24,700.

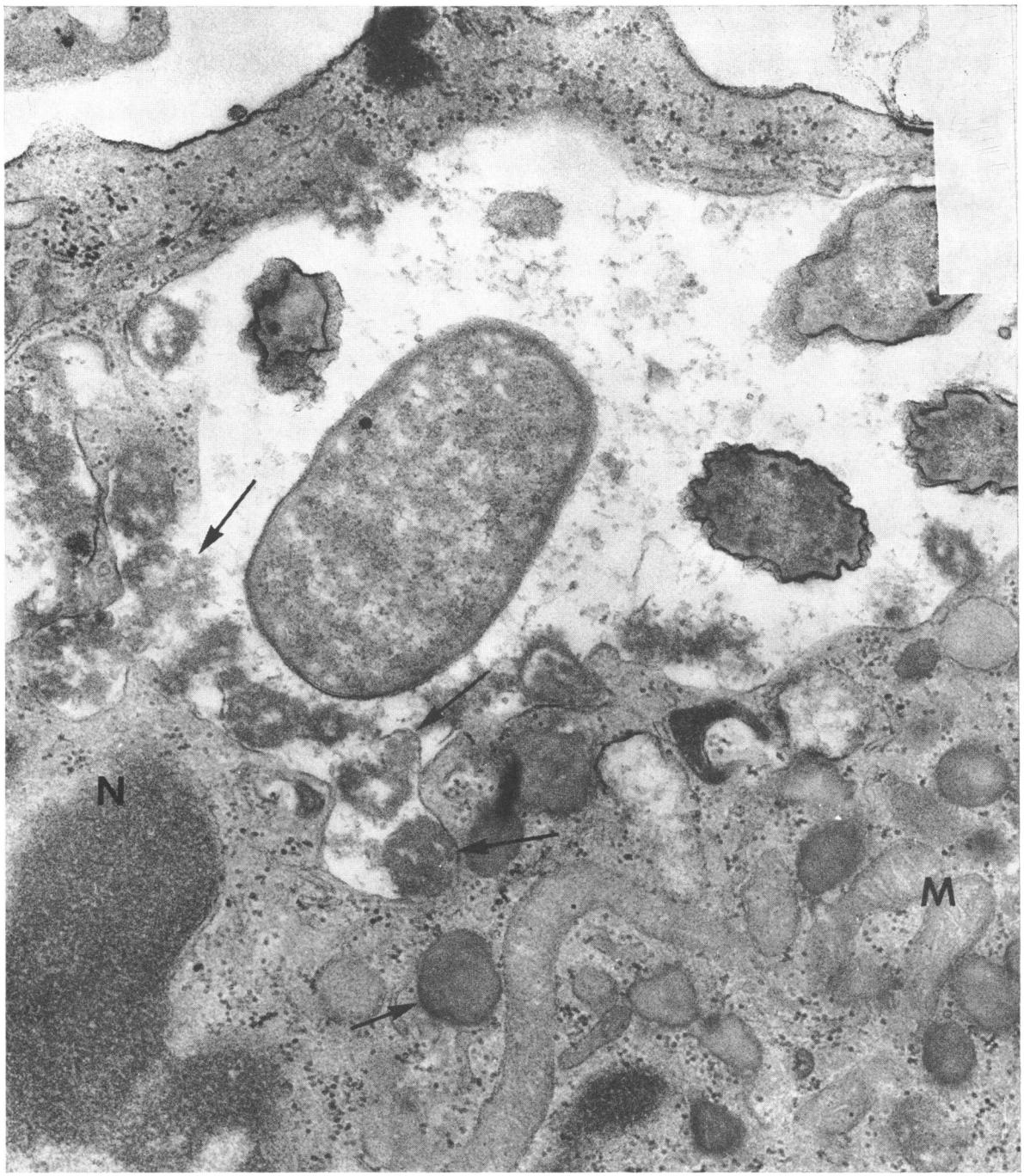

FIGURE 5 Higher magnification (ca. 0000) of digestive vacuole in patient's polymorphonuclear leukocyte after 1 hr incubation with bacteria. This typical digestive vacuole contains five undigested bacterial bodies. Lysomal granules (arrows) are adjacent to, entering, and within the vacuole. N, nucleus; M, Mitochondria. × 37,000.

DISCUSSION

The studies presented in this report document the ability of leukocytes from a patient with CGD to undergo degranulation and vacuole formation in apparently normal fashion. The morphologic evidence for degranulation from light microscopy is supported by the electron microscopic studies.

The most extensive studies of the metabolism of

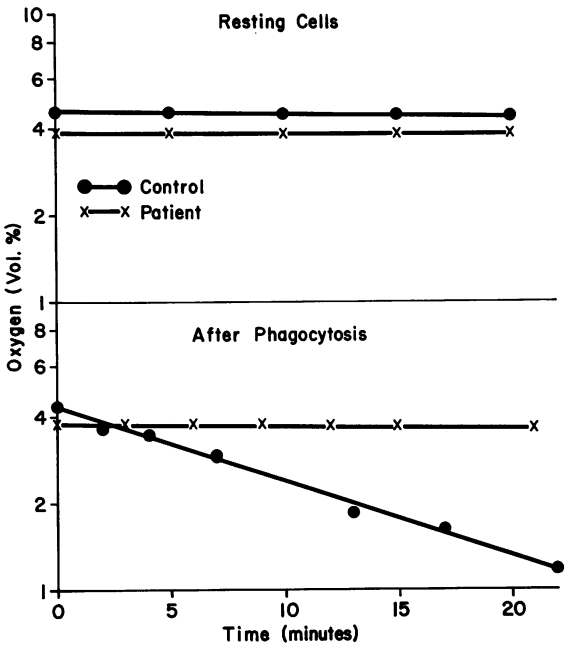

FIGURE 6 Comparison of O_2 consumption by leukocytes of the patient and those of a normal subject. Note that O_2 content decreased after the addition of polystyrene latex particles to normal leukocytes, indicating consumption of O_2. This did not occur with the patient's leukocytes.

PMN from patients with CGD are those of Holmes and associates (5). They have reported that phagocytosis fails to stimulate respiration, hydrogen peroxide production, and HMS activity. Relating these observations with those previously published by Quie and coworkers Holmes et al. have hypothesized that the metabolic abnormalities are tied to the failure of degranulation and of intracellular death of bacteria.

We have demonstrated a lack of stimulation of respiration in the PMN of our patient and a failure of his leukocytes to reduce NBT during phagocytosis. These metabolic abnormalities, in conjunction with the typical clinical course, pathological findings, and suggestion of sex-linked inheritance of his disease, make it most likely that he is suffering the same disease as those patients reported by the investigators cited above. We think it reasonable to suggest that the metabolic abnormality of leukocytes in patients with CGD is not dependent upon a failure of degranulation by these cells. Support for this suggestion comes from the data of Zatti and coworkers (7) who have noted that stimulation of HMS activity as measured by $^{14}CO_2$ production from a glucose-1-^{14}C precursor is temporally unrelated to and clearly precedes the event of degranulation in the test leukocytes.

An alternative hypothesis is that our patient has a different cellular defect than those previously reported and that any metabolic error in the leukocyte leading to defective intracellular killing of bacteria can produce the same clinical syndrome of CGD. Failure to kill intracellular bacteria could result from defective bactericidal substances within the cell or from a failure to deliver the bactericidal material from its lysosomal package in the cytoplasmic granule to the membrane-bound vacuole which is the intracellular environment of the ingested bacteria. In our current state of knowledge we feel, however, that the more tenable hypothesis is one which accepts that the event of degranulation is not causally related to the metabolic dysfunction as described by Holmes and coworkers (5), by Baehner and Nathan (6), and as seen in our patient.

Skarnes and Watson (18) have extracted a protein from the nucleus of the PMN which manifested selective bactericidal activity against Gram-positive organisms. Zeya and Spitznagel (19) have recently extracted a cationic protein from the PMN lysosomes which was equally bactericidal against Gram-positive and Gram-negative organisms. It is therefore possible that specific protein defects might result in susceptibility to only certain strains of bacteria. The definitive answer must await further studies.

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