DELAYED PHAGOCYTOSIS AND BACTERIAL KILLING IN CHEDIAK-HIGASHI SYNDROME NEUTROPHILS DETECTED BY A FLUOROCHROME ASSAY. ULTRASTRUCTURAL ASPECTS

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The few studies already published about phagocyte functions in Chediak-Higashi syndrome (CHS) has stated that neutrophils present slow rate of bacterial killing but normally ingest microorganisms.

In the present study, both phagocytosis and killing of Staphylococcus aureus *were verified to be delayed in neutrophils from two patients with CHS when these functions were simultaneously evaluated by a fluorochrome phagocytosis assay. Electron microscopic examination showed morphologic differences among neutrophils from CHS patients and normal neutrophils regarding the cytoplasmic structures and the aspects of the phagolysosomes. It was noteworthy the presence of giant phagolysosomes enclosing bacteria in active proliferation commonly observed in CHS neutrophils after 45 min of phagocytosis, which corresponded with the impaired bactericidal activity of these leukocytes. The present results suggest that phagocytosis may also be defective in CHS, and point out to the sensitivity of the fluorochrome phagocytosis assay and its application in clinical laboratories.*

Key words: neutrophils – Chediak – Higashi syndrome – phagocytosis – bacterial killing

Chediak-Higashi syndrome (CHS) is an autosomal recessive defect recognized as a generalized cellular dysfunction characterized by increased fusion of cytoplasmic granules (White & Clawson, 1980). The diagnosis and probably all the functional abnormalities spring from the formation of abnormal giant granules, seen in circulating leukocytes and platelets but widespread in other tissues (Barak & Nir, 1987). Patients with CHS present partial albinism (Blume & Wolff, 1972), central nervous system abnormalities (Pettit & Berdal, 1984), and have a peculiar propensity for limphohistiocytic proliferation (Blume & Wolff, 1972; Conley & Henle, 1987). They also have manifestations characteristic of defective neutrophil function-recurrent bacterial infections of skin, respiratory tract and mucous membrane, caused by both gram-nega-

tive and gram-positive bacteria (Barak & Nir, 1987; Boxer & Smolen, 1988).

A number of functional alterations have been observed in CHS neutrophils *in vitro:* they spontaneously aggregate surface molecules into caps, exhibit higher resting rates of oxygen consumption, demonstrate impaired adherence to surfaces, reduced chemotactic responses and kill microorganisms slowly (Clark & Kimball, 1971; Root et al., 1972; Stossel et al., 1972; Clawson et al., 1979; Boxer & Smolen, 1988). Phagocytosis has been referred to be normal in CHS neutrophils (Root et al., 1972; Stossel et al., 1972; Gale et al., 1986).

In the present work, the phagocytosis and killing of *Staphylococcus aureus* were simultaneously evaluated in neutrophils from two patients with Chediak-Higashi syndrome through a modified fluorochrome phagocytosis assay (Bellinati-Pires et al., 1989). Some morphologic aspects of bacterial phagocytosis were examined by electron microscopy.

MATERIALS AND METHODS

Patients – A 30-month-old girl (Patient 1) and a 24-month-old boy (Patient 2) admitted to the Department of Pediatrics of the School of Medicine, University of São Paulo, were directed to Adolfo Lutz Institute for laboratorial investigations. Both patients presented partial oculo-cutaneous albinism, photophobia and recurrent pyodermitis. The boy also presented lymphadenopathy, hepatosplenomegaly and frequent episodes of supurated otitis, cutaneous abscesses and sinopulmonary infections. Details about the clinical features of the patients were previously published by Carneiro-Sampaio et al. (1988).

Normal subjects – Each patient was compared with a normal adult volunteer simultaneously evaluated (day control). Unfortunatelly, age-matched healthy controls could not be used because of ethical problems. In addition, the patients and their respective day controls were compared with a group of 50 healthy adult blood donors (Blood Bank – Hospital of Clinics, School of Medicine, University of São Paulo) of both sexes, aged 18-45 years, previously evaluated in the same phagocytosis assay. This group served to establish the normal range values for the phagocytic and bactericidal parameters used in the present study.

Leukocyte preparation – Leukocytes were separated from heparinized venous blood by sedimentation with 4.5% Dextran T-500, washed twice with Hanks' balanced salt solution (HBSS) at 160 xg for 10 min, and resuspended in TC 199 medium buffered with 0.02M Hepes (TC 199/Hepes) to 2×10^6 neutrophils/ml.

Phagocytosis assay – Phagocytosis and killing of *S. aureus* Cowan I (ATCC 12598) by neutrophils were simultaneously assessed through the method reported by Bellinati-Pires et al. (1989). Briefly, bacteria opsonized with 10% pool of human fresh sera were mixed with the leukocyte suspension in siliconized glass tubes at a bacteria: neutrophils ratio of 10:1. The tubes were incubated for 5, 15, 30 and 45 min at 37 °C with mild agitation in a rotatory water bath shaker. After centrifugation at 160 xg, 4 °C for 7 min, part of the cell pellet was prepared for electron microscopic examination. The remaining pellet was stained with 14.4 mg/l acridine orange in phosphate-buffered saline, pH 7.2 for 1 min, washed twice in ice-cold HBSS, and wet-mounted on microscope slides. Intracellular viable and non-viable bacteria were scored by their green (living) or red/yellow (non-living) fluorescence through examination in a ultraviolet microscope. Three parameters were calculated:

(1) Percent phagocytizing neutrophils = percentage of neutrophils which ingested at least three bacteria (a).

(2) Phagocytosis index = $\dfrac{(a) \times (b)}{100}$

where:

(b) = mean number of ingested bacteria/neutrophil.

(3) Percent killed bacteria =

$= \dfrac{\text{number of intracellular dead bacteria}}{\text{total number of ingested bacteria}} \times 100$

Electron microscopy – After phagocytosis, the leukocyte-bacteria pellet was washed twice in ice-cold HBSS by centrifugation at 160 x g for 7 min and fixed by the addition of 1 ml of 2% glutaraldehyde in 0.15 M phosphate buffer, pH 7.2. Glutaraldehyde fixation was followed by 1% osmium tetroxide fixation and block staining in 1% uranyl acetate and lead citrate and studied with a Phillips 400 electron microscope.

Analysis of the data – The values of phagocytosis index and percent killed bacteria obtained for the patients and their respective day controls were analysed regarding their probability of belonging to the normal range of these parameters (mean ± 2 SD) for the healthy group of 50 adults. The data of this healthy population followed normal distribution as verified by the Kolmogorov-Smirnov Test and used to statistically compare the CHS patients (for an explanation see Zar, 1984).

RESULTS

Phagocytosis and bacterial killing – Phagocytosis of *S. aureus* by neutrophils from the CHS patients appeared to be reduced in comparison with normal neutrophils at the first 15 min of the leukocyte incubation with the bacteria, as observed in Fig. 1. At 5 min of phagocytosis, the CHS neutrophils showed phagocy-

tosis indices and percentages of phagocytizing neutrophils below the minimum values of these parameters for the healthy group previously evaluated – the phagocytosis indices have a maximum 2% probability of belonging to the normal range for this healthy population. The data obtained 30 min after mixing the leukocytes with staphylococci showed that only the Patient 1 neutrophils had phagocytosis index comparable to the normal control (data not shown). Readings taken at 45 min period of phagocytosis were not considered for quantitative analysis because the increased bacterial multiplication.

Fig. 2: killing of *Staphylococcus aureus* by neutrophils from the Patient 1 (P1), Patient 2 (P2) (filled bars) and their respective day controls (C1 and C2) (hatched bars) after 30 min of phagocytosis. The values of % killed bacteria for the patients and their respective day controls are compared with the normal range values (mean ± 2SD) (═══) of this parameter obtained for 50 healthy adults (55.1 ± 25.1).
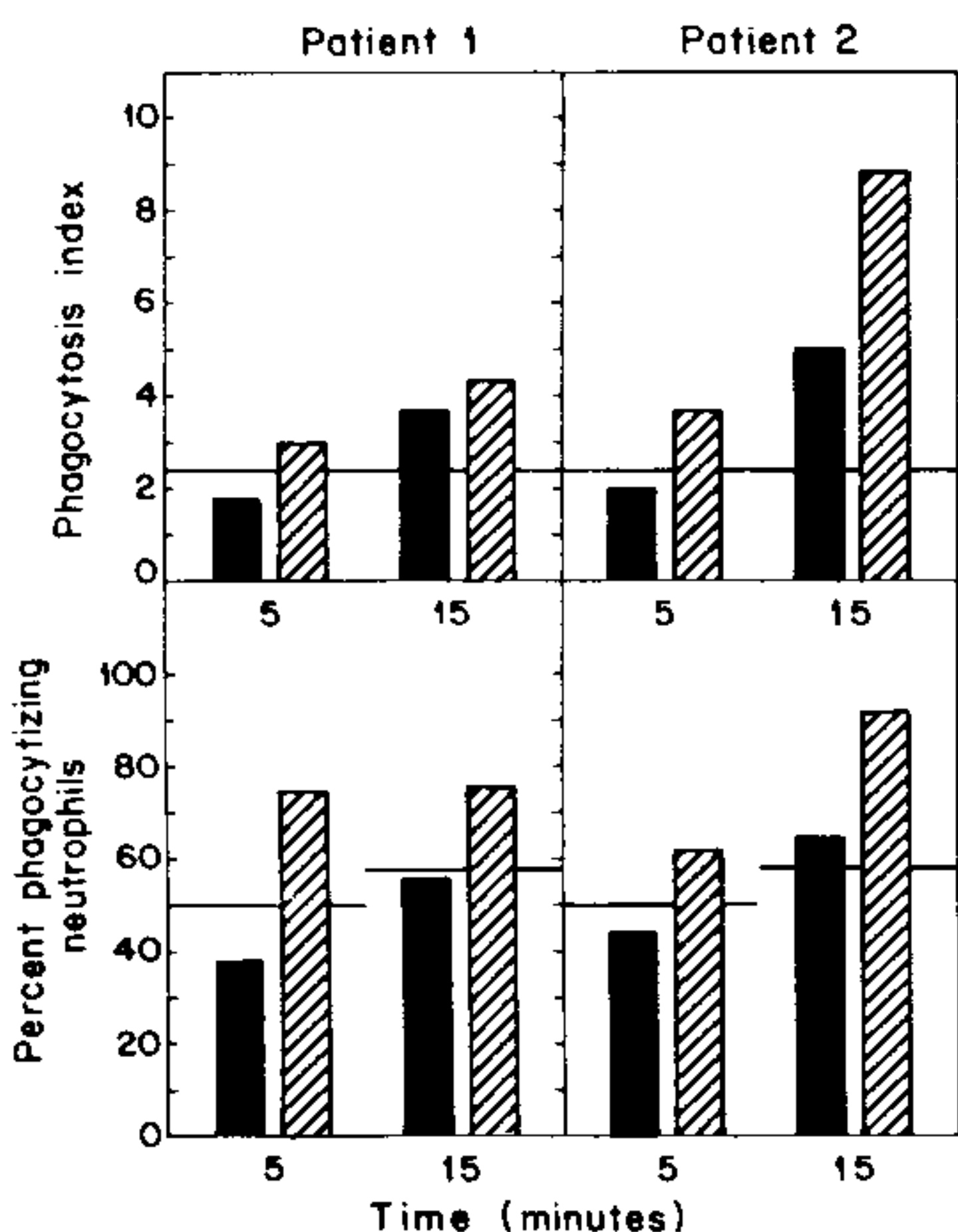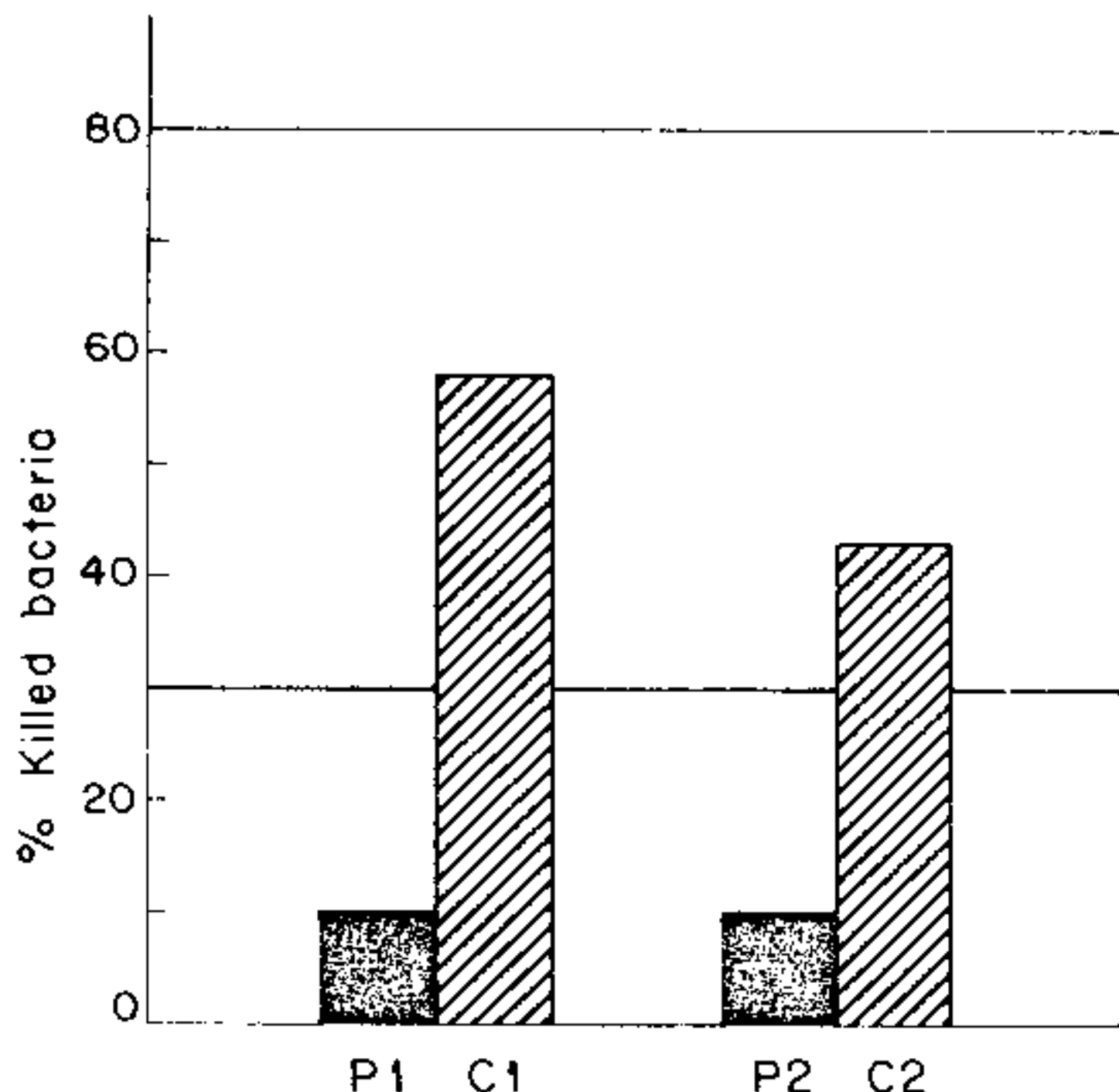

Fig. 1: phagocytosis indices and percentages of phagocytizing neutrophils obtained for the patients (filled bars) and their respective day controls (hatched bars) at 5 and 15 min of phagocytosis. The values of these two parameters are compared with the minimum values (──) of those obtained for 50 healthy adults. The normal range values (mean ± 2SD) for phagocytosis index were 5.7 ± 3.5 and 6.8 ± 4.6 for 5 and 15 min, respectively.

As expected for CHS, both patients showed very low bactericidal activity in comparison with their respective day controls (Fig. 2). The values of percent killed bacteria obtained for the patients had a maximum 0.2% probability of belonging to the normal range for the healthy group.

Electron microscopy – The ultrastructural aspects of normal and CHS neutrophils after different time intervals of *S. aureus* phagocytosis are illustrated in the Figs 3 and 4. Abnormal cytoplasmic inclusion bodies could be seen in approximately 90% of the CHS neutrophils. However, the neutrophils showed to be heterogeneous with respect to the number, size and morphology of their aberrant cytoplasmic structures. We point out to the frequent appearance of giant phagolysosomes enclosing bacteria in active proliferation in CHS neutrophils after 45 min of phagocytosis (Figs 3 and 4). High numbers of intraphagosomal dividing bacteria were commonly observed in neutrophils from the Patient 2 as presented in Fig. 4.

DISCUSSION

Although other previous reports which have stressed that phagocytosis is normal in CHS neutrophils (Root et al., 1972; Stossel et al., 1972; Gale et al., 1986; Boxer & Smolen, 1988), the results of the present study suggest that in addition to impaired bactericidal activity, delayed phagocytosis of bacteria may be another possible defect of CHS neutrophils. This finding would not be surprising taking into consideration the abnormal blood cell membrane fluidity seen in CHS (Haak et al.,
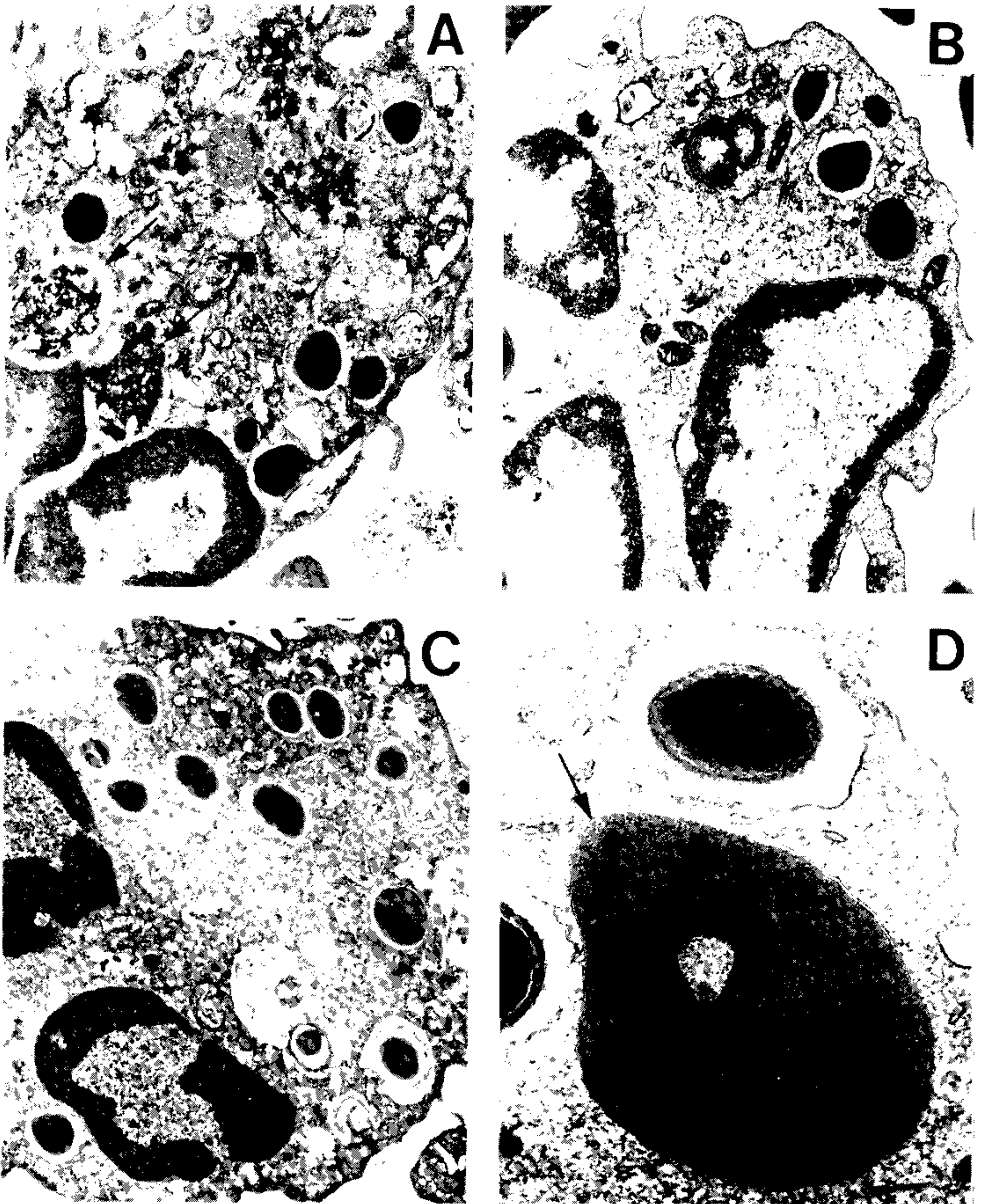
Fig. 3: electron micrographs of normal and CHS neutrophils 5 min after incubation with *Staphylococcus aureus* – A and B: are representative sections of neutrophils from the Patient 1. Few ingested bacteria can be seen within small vacuoles. Giant inclusion bodies with heterogeneous morphology are pointed in A (arrows). C: depicts section of normal neutrophil (day control of the Patient 1). Note the high number of ingested bacteria. D: neutrophil section of the Patient 2 showing an abnormal giant cytoplasmic inclusion consisting of an electron dense ring enclosing cytoplasm and surrounded by lamellar structures resembling myelin figures. Uranyl acetate-lead citrate stain (x 4,200 A and B; x 3,500 C; x 9,050 D).
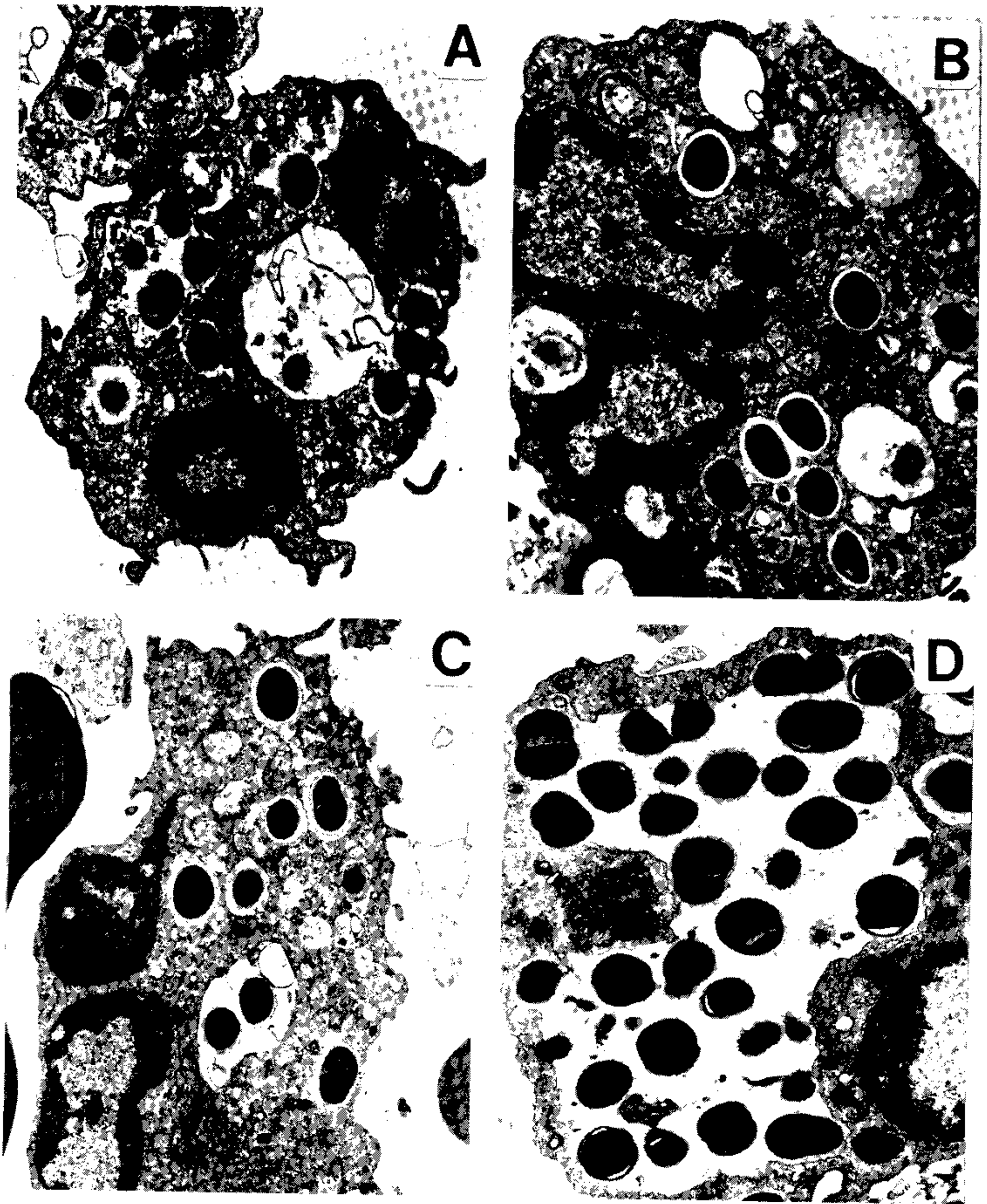
Fig. 4: electron micrographs of neutrophils 45 min after incubation with *Staphylococcus aureus* – A: depicts section of a neutrophil from the Patient 1. A giant phagolysosome enclosing bacteria (↑ ↑) as well as giant lysosomes which appear to be fusing with phagosome (↑) are shown. B: representative section of a normal neutrophil (day control of the Patient 1). Some phagolysosomes present signals of bacterial digestion (↑). Phagolysosomal bodies (↑ ↑) are shown that appear to consist of two concentric structures, which possibly represent septated bacterial cell that has been split in half along a division septum during the lysis process. C: normal neutrophil from the day control of the Patient 2. D: neutrophil from the Patient 2 which presents a giant phagolysosome containing numerous bacteria in active proliferation as seen by the septated coccal forms. Uranyl acetate-lead citrate stain (x 2,500 A; x 3,500 B; x 2,700 C and D).

1979). In this regard, a unifying hypothesis for the aberration of CHS is that the elevated cell membrane fluidity may influence surface receptor expression, which could possibly lead to elevated levels of cAMP, disordered assembly of microtubules and defective interaction of microtubules with lysosomal membranes, all of which occur in this disorder (Oliver, 1976, 1978; White & Clawson, 1980; Ostlund et al., 1980; Pryzwansky et al., 1985). Furthermore, it has been demonstrated that colchicine, which promotes microtubules disassembly can inhibit phagocytosis, chemotaxis, surface adhesion and degranulation (Ukena & Berlin, 1972) suggesting that these structures are important in all these processes.

The reduced phagocytosis indices obtained from the CHS patients studied in here were attributed not only to the decreased number of ingested bacteria but mainly to the reduced percentage of phagocytizing neutrophils. We point out that the present fluorochrome phagocytosis assay allows the simultaneous evaluation of the phagocytic and bactericidal activities of neutrophils in a single test tube. One of the advantages of this method in comparison with conventional assays (Leijh et al., 1986) is that it permits the determination of the number of ingested and killed bacteria by each cell, as well as the proportion of cells that effectively phagocytize and kill microorganisms in a phagocytic cell population. We think that the lack of phagocytosis impairment in CHS neutrophils seen in other studies could be more the result of inadequacy of phagocytosis assays that may have the capacity only to detect relatively gross alterations of this function.

Some authors have been stated that neonates already present neutrophil phagocytosis and killing at levels similar of those observed in adult neutrophils (Forman & Stiehm, 1969; Mc Craken & Eichenwald, 1971; Pross et al., 1977; Miller, 1979). Moreover, in previous experiments we observed that neutrophils from children aged 1-3 years phagocytized and killed S. aureus at rates within the range observed for the healthy adult population (Bellinati-Pires, 1990). With basis on these observations, we think that it would not be conflicting the comparison of our present patients with adult controls.

Having used living bacteria for the ultrastructural examination, it became possible to observe the ability of neutrophils of controlling intracellular bacterial growth. As noted in the electron microscopic observations, giant phagolysosomes enclosing bacteria in active proliferation was a commom feature in CHS neutrophils taken 45 min after mixing with S. aureus. This observation was consistent with the impaired bactericidal activity of these leukocytes. Moreover, the remarkable increased number of intraphagosomal dividing bacteria seen in Patient 2 neutrophils paralleled the severity of his infections when compared with the Patient 1.

Since extracellular bacteria were not removed during the time interval of phagocytosis, the neutrophil uptake of bacteria already in proliferation must be considered in the present study. However, the ultrastructural aspect of the normal neutrophils differed from those of CHS at least in regard to the number of intracellular dividing bacteria and to the size of phagolysosomes. Bacterial cell in division was usually seen alone within small phagolysosome in normal neutrophils, suggesting recent ingestion of the organism.

In view of the present observations, it seems that the slow rate of bacterial killing in CHS neutrophils may be attributed not only to the granular abnormalities but also to the delayed ingestion of the microorganism. However, another studies must be developed to clarify these preliminary findings.

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