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AN ULTRASTRUCTURAL ABNORMALITY OF NEUTROPHILS PRESENTING WITH IMPAIRED FUNCTION*

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Recent advances in the analysis of polymorphonuclear leukocyte (PMN) function generally concern clinical defects of its components. Though this seems to be an indirect way of finding out the physiology of PMNs, the reported cases of PMN dysfunction have enabled us to classify the principal activities of PMNs as adhesion to vascular endothelium, chemotaxis, ingestion and killing. We report a primary intrinsic chemotactic and bactericidal defect of PMNs which have ultrastructural atypical bodies, increased surface ruffling and rudimentary pseudopodia formation.

Material and Methods

The clinical features and detailed chemotaxis studies of this patient have been previously reported (Yeğin et al, 1981)[1].

An 11-year-old boy was referred to us because of a productive cough, chronic otitis media of seven years' duration and recurrent episodes of lymphadenitis. Physical examination revealed his height and weight to be below the third percentile. He had signs of periodontitis and chronic tonsillitis. The left tympanic

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membrane was perforated and the cervical lymph nodes were enlarged. Multiple scars of drained lymph nodes or abscesses were observed on the submandibular and cervical regions. There were crepitant rales on both lung fields and hepatosplenomegaly (both five cm below the costal margins). Roentgenogram studies revealed bilateral maxillary sinusitis, chronic mastoiditis and bronchiectasis.

Laboratory studies showed hemoglobin 11.25 g/dl and white blood cell counts 18.000-25.000/µl with 63-82 percent neutrophils and 18-28 percent lymphocytes. Eosinophil, monocyte and basophil counts were normal. No obvious morphological abnormality of PMNs e.g., toxic granulations, nuclear abnormality, vacuolization and Döhle bodies could be observed under light microscopy. Bone marrow examination was normal. Liver needle biopsy showed nonspecific hepatitis. Sweat chloride concentration, serum alpha-l-antitrypsin level, erythrocyte glucose-6-phosphate dehydrogenase level and complement hemolytic activity (CH 50) were normal. Studies on humoral and cellular immunity including serum IgG, IgA, IgM and IgE concentrations, isohemagglutinin titers, antibody responses to diphteria and tetanus toxoids, skin test responses to phytohemagglutinin (PHA), streptokinase-streptodornase, E, EA, EAC rosette values, and in vitro lymphocyte blastogenic transformation response to PHA gave normal results.

The patient was one of five children of a nonconsanguineous marriage. No other member of the family showed an increased susceptibility to infections. PMN chemotaxis studies of the father and four siblings were normal.

The studies of PMN function described below were performed both during hospitalization and during periods of well-being. The results were similar in both clinical situations. Informed consent was obtained from the families of the patient and healthy controls after approval by the Human Experimentation Committee of Hacettepe University Institute of Child Health.

Studies of PMN Function: PMNs were obtained by the two-step technique of Boyum[2]. Glass adherence of PMNs[3], quantitative NBT dye reduction[4], leukocyte alkaline phosphatase activity[5], myeloperoxidase staining of PMNs[6], and lysosomal enzymes of primary granules including, beta-D-glucuronidase[7], acid phosphatase[8], alpha mannosidase[9], and beta-N-acetylglucosaminodase[10] were determined by the previously described methods.

PMN chemotaxis was tested by the under agarose method[11] as modified by Yeğin[12]. Both distance and cell counts were determined while evaluating the chemotaxis and random migration. Monocyte chemotaxis was assessed by the membrane filter method[12], and evaluated by the leading front method[13]. The patient's PMN chemotaxis studies were repeated before and after levamisole (2 mg/kg per oral, three days every fortnight for six weeks) and ascorbic acid (250 mg x 2/day, per oral, two weeks) treatments. PMN chemotaxis studies were also

repeated in the presence of patient, control serum or plasma in the cell suspension. Zymosan (Sigma, St. Louis, Mo. U.S.A) activated pooled human serum was used as the standard chemotactic factor. After activation with zymosan, chemotactic activity of the patient's serum was tested against control PMNs.

Candida phagocytosis and killing were determined by the method of Lehrer and Cline[14]. Phagocytosis was assessed at the 15th minute of the test. In vitro bactericidal capacity of the PMNs against *Staphylococcus aureus,* in the presence of the patient's or controls' serum was evaluated by the method of Quie et al[15], following incubation periods of 30, 60 and 120 minutes.

Electron Microscopy: E.M. was performed according to Watanabe[16], with some modifications. Briefly, the cells were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7) for six hours, then post-fixed in 1% osmium tetroxide in the same buffer. The cells were subsequently dehydrated through ethanol and propylene oxide and embedded in araldite. Thin sections were stained with uranyl acetate and lead citrate and examined under a Carl Zeiss EM 9 S2 electron microscope. Samples of the patient and two healthy controls were also studied at the 15th minute of the candida phagocytosis test as described above.

Results

As shown in Table I the results of NBT dye reduction and candida phagocytosis tests were found to be within normal limits while chemotaxis, candida killing and bactericidal activity against *Staphylococcus aureus* were significantly depressed. Although random migration was also lower than normal, it was within the two standard deviations. PMN chemotactic activity was also studied by the membrane filter method and evaluated by both the lower surface count (patient 28 ± 4 cells per high power field (h.p.f) versus 126 ± 16 h.p.f in five controls) and the leading front methods (patient 35 ± 2 μm versus 83 ± 15 μm in 13 controls). These values were also lower than the two standard deviations of the controls. Oral levamisole and ascorbic acid treatments were given separately, but failed to affect the in vitro PMN chemotaxis and no obvious clinical beneficial effect could be observed. Monocyte chemotaxis of the patient was normal (63 ± 5 μm) when compared with healthy age-matched controls (n = 12, 75 ± 15 μm).

Chemotaxigeneticity of the patient's serum after activation with zymosan was normal. No inhibitory effect on the chemotaxis or bactericidal capacity could be detected when the patient's serum or plasma was added to control PMNs. Neither did the serum of the controls have any reconstitutive effect on the patient's PMNs.

Glass adhesiveness of the patient's PMNs was not different from the controls (6.7 ± 1 percent of the patient's and 4.9 ± 2 percent of the controls' PMNs applied to the glass bead column were recovered).

TABLE I: Results of Polymorphonuclear Neutrophil Function Studies

		Chemotaxis		Random migration				Bactericidal activity (percent living *S. aureus* after the following intervals)		
	NBT ΔOD+	μm	cell count	μm	cell count	Candida phagocytosis (%)	Candida killing (%)	30 min	60 min	120 min
Patient	0.094	350±64*	211±57*	264±68*	138±51*	79±3**	7±2**	79±9**	53±12**	45±10**
Control values (mean ± 1 SD	0.081±0.026	950±269	874±220	493±123	457±175	87±6	28±7	20±7	12±7	5±2
(Number of healthy controls studied)	14	20	20	20	20	20	20	20	20	20
P value	>0.05	<0.01	<0.01	>0.05	>0.05	>0.05	<0.01	<0.01	<0.01	<0.01

*: Mean ± 1 S.D. of seven separate determinations during the six-month observation period

**: Mean ± 1 S.D. of three separate determinations during the six-month observation period.

+: Δ optic density (OD) = O D stimulated-OD unstimulated.

Leukocyte alkaline phosphatase activity was determined as a marker of the secondary granules on two separate occasions and found to be normal on the first ($98/10^8$leukocyte/per hour) and increased on the second occasion ($210/10^8$ leukocyte/per hour; normal value = $91 \pm 45/10^8$ leukocyte/per hour, n = 20).

PMN myeloperoxidase activity was normal histochemically. Lysosomal enzymes of primary granules (including glucuronidase 204 fluorescence units/mg protein in the patient versus 231 (180-271) in ten age-matched controls, acid phosphatase 2176 fluorescence units/mg protein in the patient versus 2189 (1420-3835) in the controls, alpha mannosidase 0.57 unit/mg protein in the patient versus 0.53 (0.35-0.71) in the conrols; beta-N-acetylglucosaminidase 9.7 units/mg protein in the patient versus 8.3 (6.6-11.0) in the controls were found to be within normal limits.

The most striking abnormality observed under the EM was the presence of round or elongated, membrane bound granule-like atypical bodies composed of electron dense material, having tubulovesicular structures of various sizes (Fig. 1). These seem to have a close relation to the Golgi complex (Figs. 2a, 2b) and fuse with phagocytic vacuoles together with secondary granules (Figs. 3a, 3b). The atypical

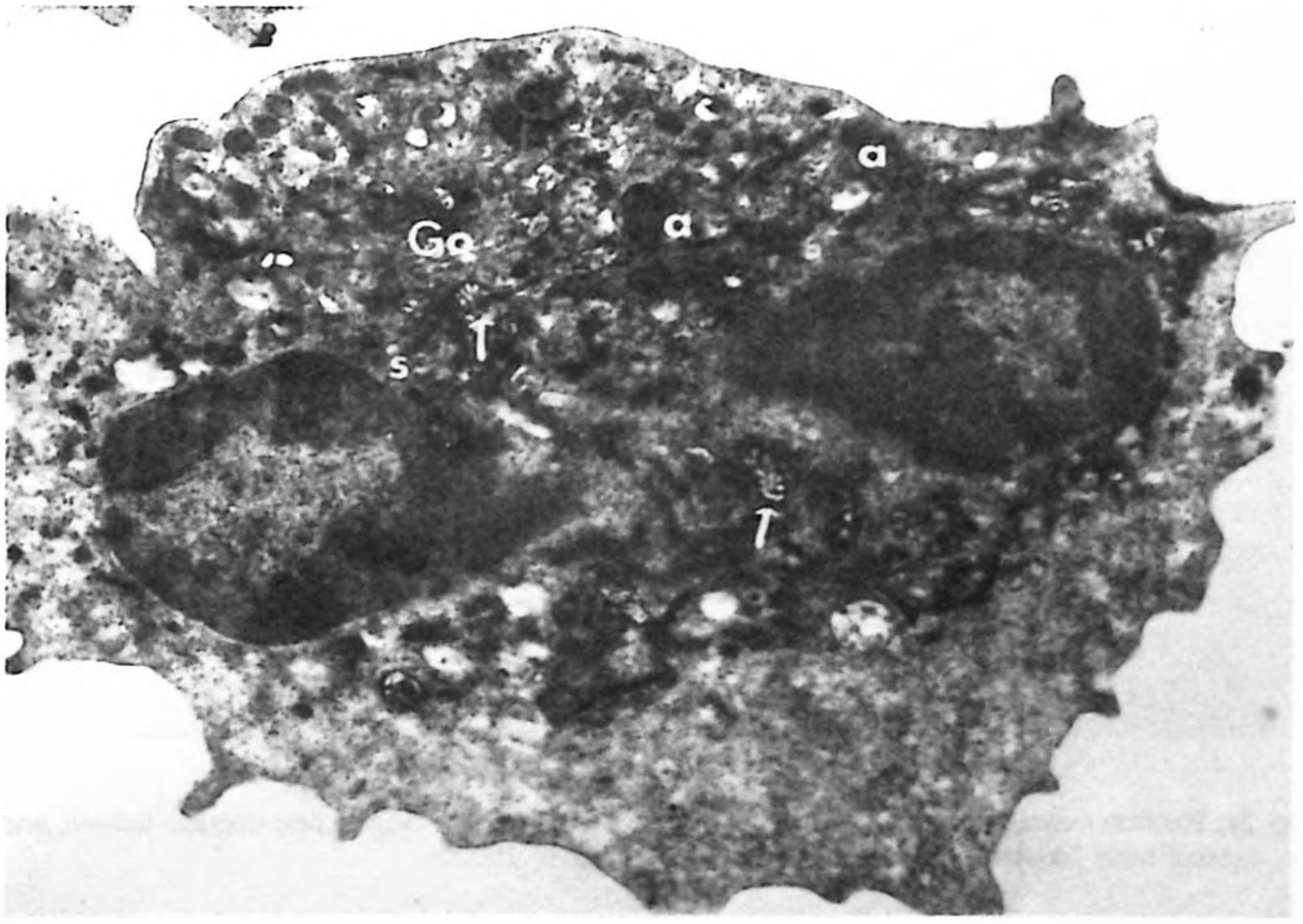

Fig. 1: Cytoplasmic details of PMN from the patient. Arrows indicate the atypical bodies, one of which is seen in the vicinity of the Golgi complex (Go). The cytoplasm contains both secondary (s) and primary (a) granules. Short narrow pseudopodia are also seen (x 25000)

bodies disappear after phagocytosis (Fig. 4). The patient's PMNs had both types of granules (primary and secondary, Figs. 1-4). In addition to these atypical bodies the patient's PMNs had many short, narrow rudimentary pseudopodia and increased surface ruffling as if they were chemotactically stimulated (Figs. 1, 2). No ultrastructural abnormality was observed in the monocytes and lymphocytes of the patient.

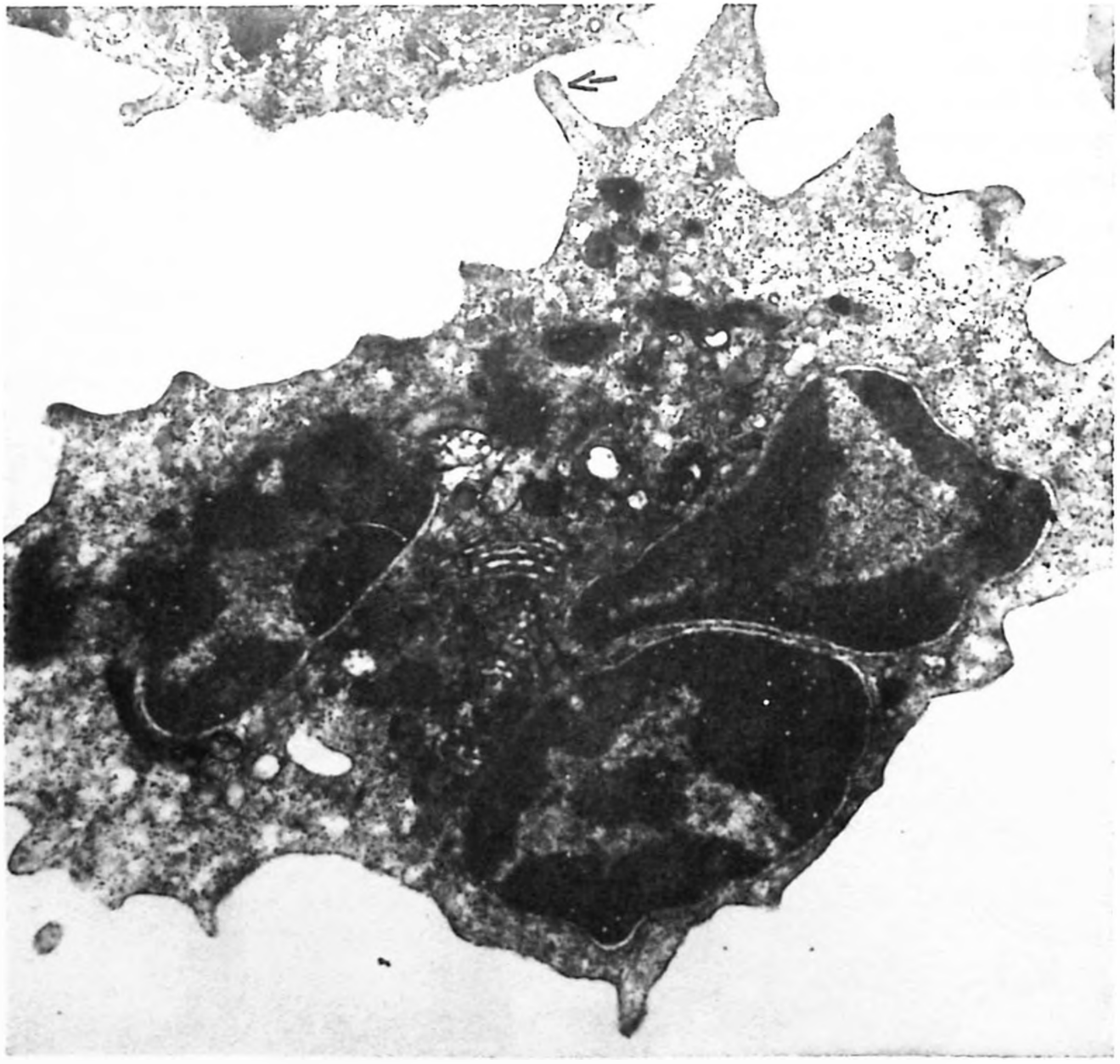

Fig. 2a: Electron micrograph of PMN from the patient showing short, narrow pseudopodia (arrow), and an atypical body (double arrow), the Golgi complex (g) (x 25000).

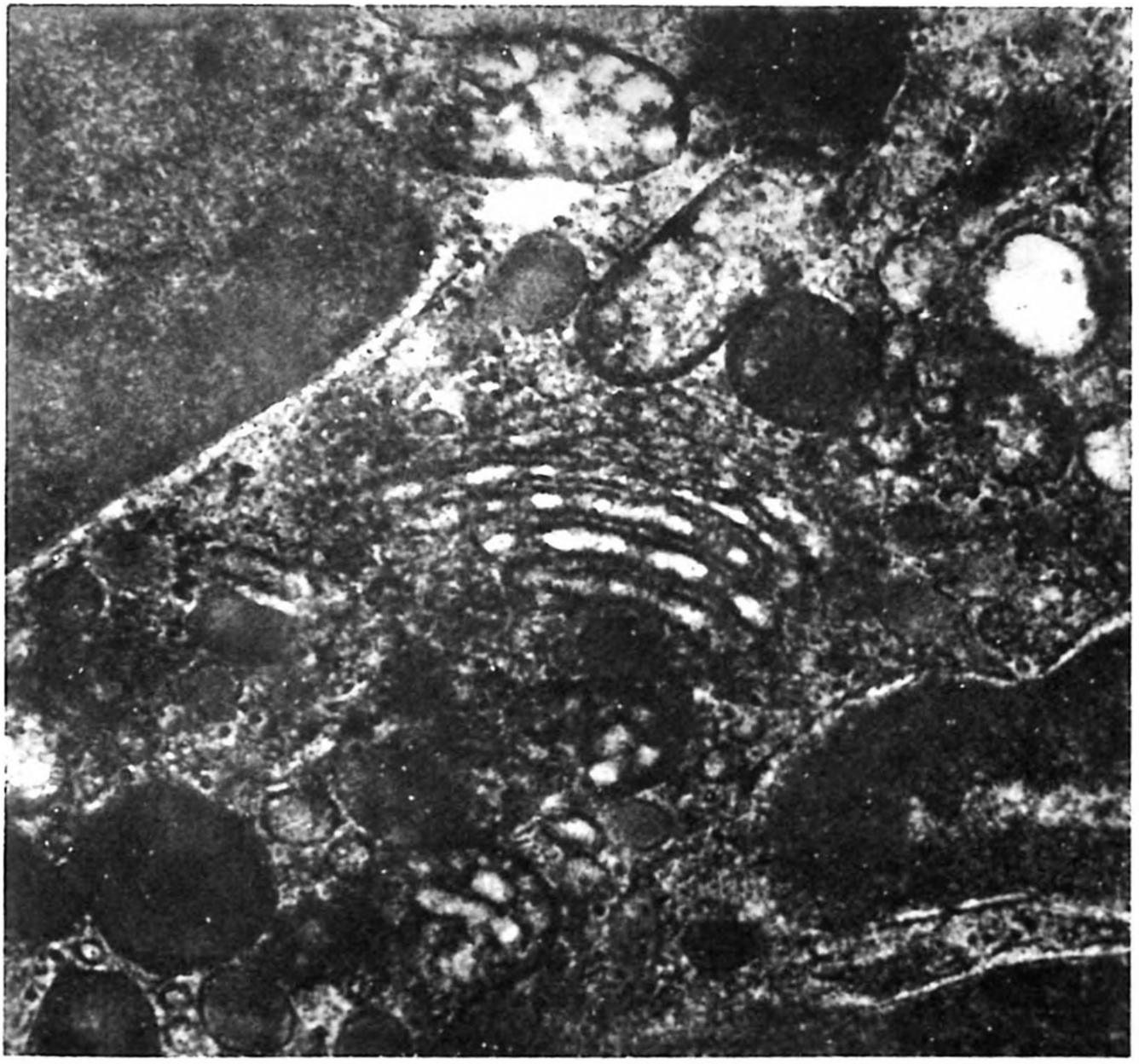

Fig. 2b: Higher magnification of Fig. 2 showing the relationship between the atypical body and the Golgi complex (x 31000).

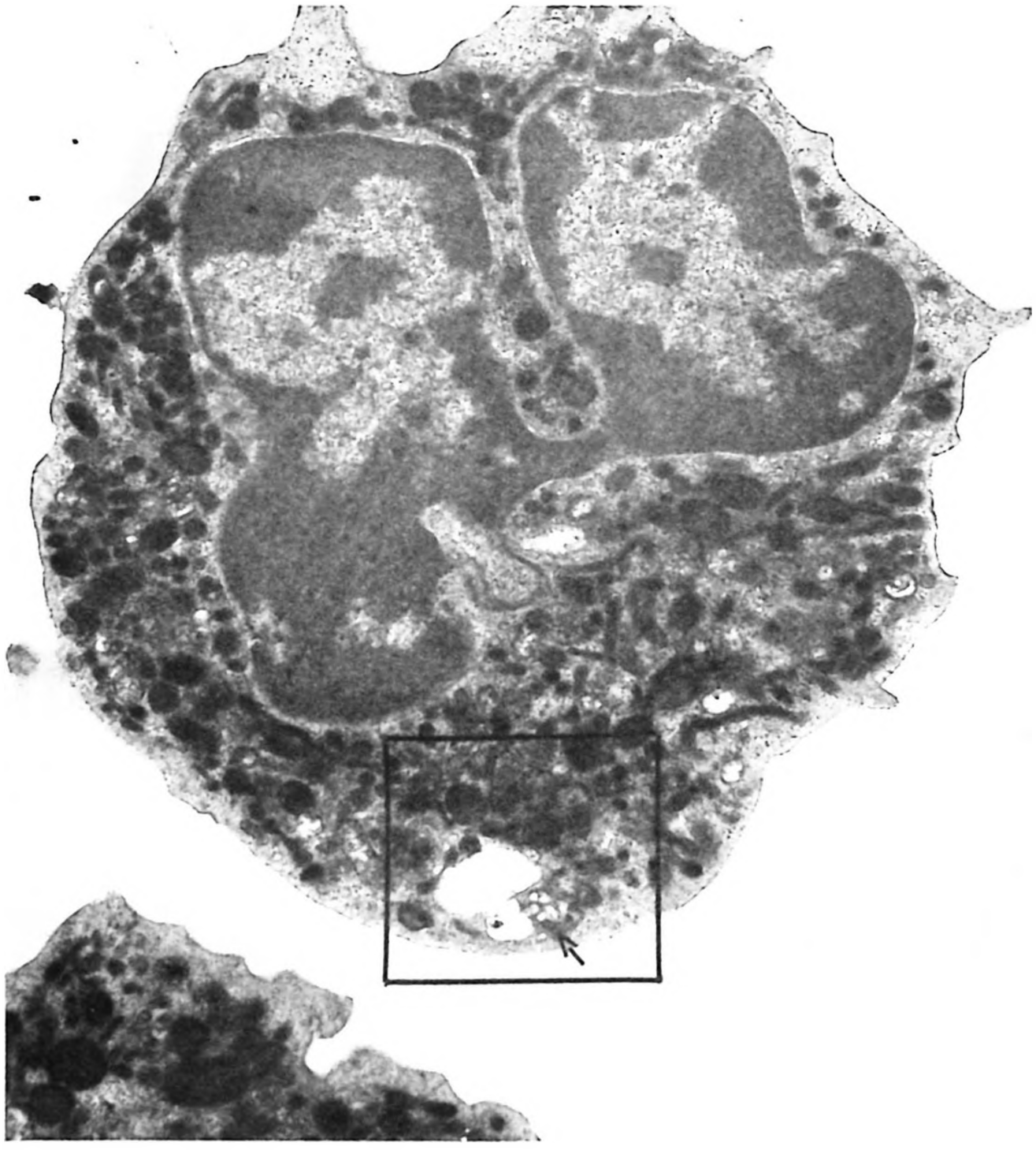

Fig. 3a: Another of the patient's PMN. At the lower part of the PMN one atypical body and a few secondary granules fused with a phagocytic vacuole (arrow) are seen (x 25000).

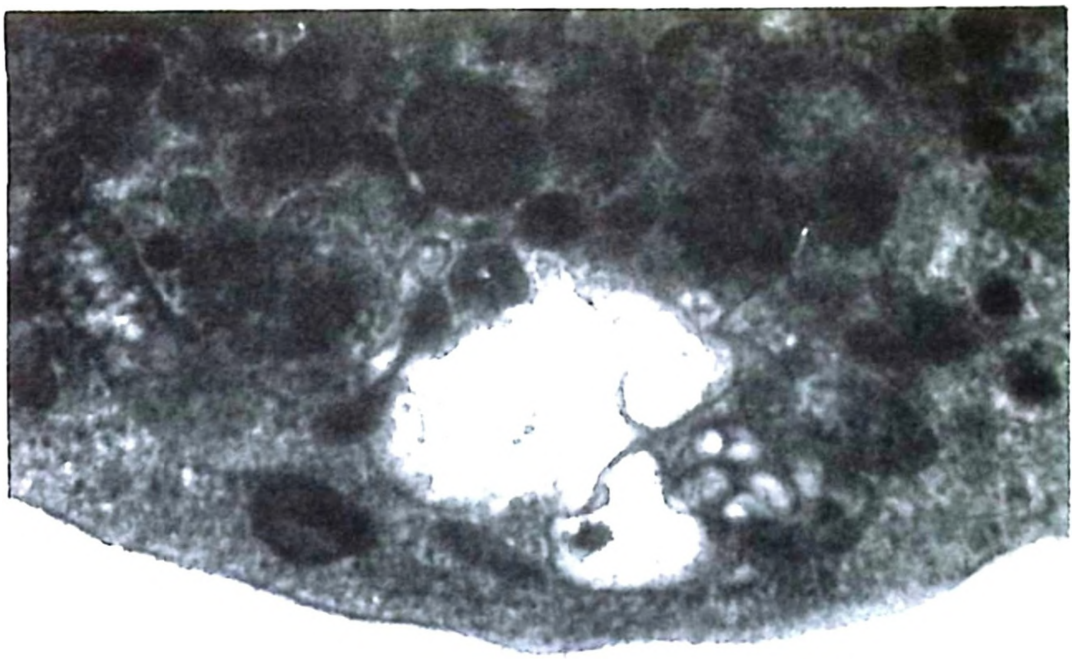

Fig. 3b: Higher magnification of Fig. 3. (x 62000).

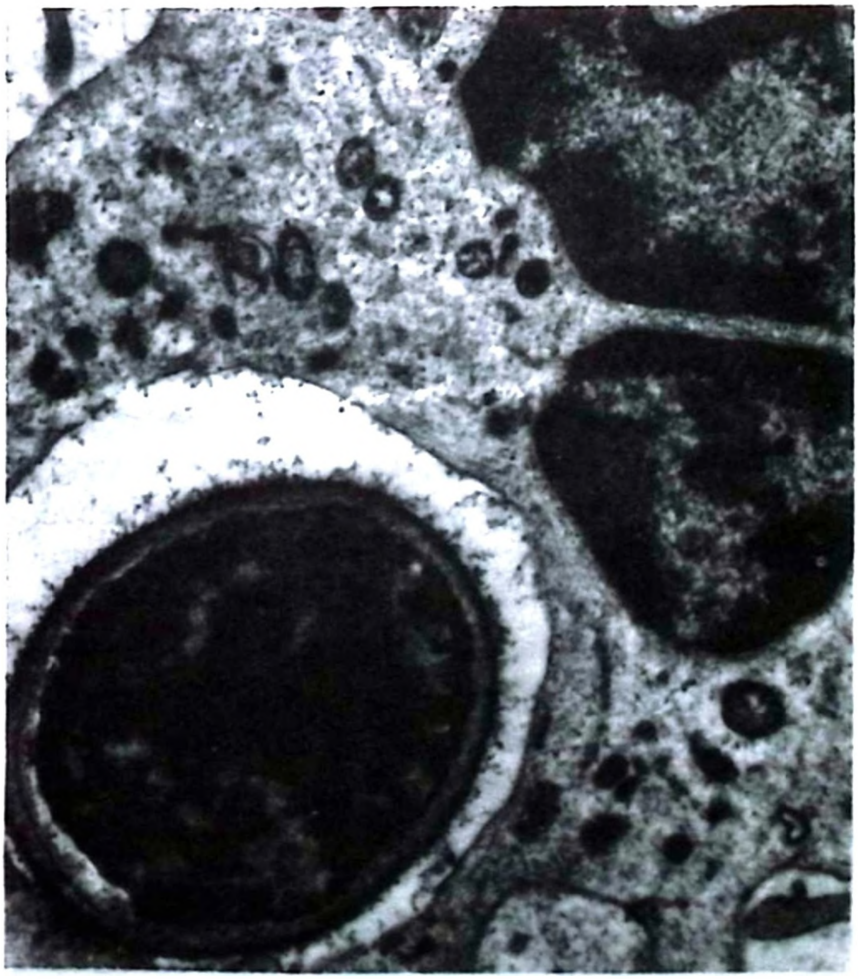

Fig. 4: The phagocytosis of *Candida albicans* by the PMN. Phagocytic vacuole containing a candida is partly surrounded by a microfilamentous network. Secondary granules are diminished and atypical ones have disappeared.

Discussion

The patient described above was unusually susceptible to infection. Tests for the evaluation of humoral, cellular immunity and complement system were found to be within normal limits. He was able to produce PMNs, generate chemotactic activity and ingest candida. But his PMNs exhibited profound impairment in locomotion and intracellular killing. No inhibitor of chemotaxis or bactericidal activity could be detected in the patient's serum or plasma. These in vitro studies suggest that the PMN abnormalities observed in our case were cellular in nature rather than secondary to serum factors. Although the patient's PMN locomotion was defective, they apparently had no difficulty in leaving the bone marrow. During a period of six months, our patient never had neutropenia and responded to active infection normally with neutrophilia as high as 20300 PMN/µl.

It has been clearly shown that normal PMNs contain two basic types of granules namely, primary (azurophil) and secondary (specific). The important role of these granule types in the normal PMN function is well established[17-20].

The most striking ultrastructural abnormality observed in our patient's PMNs was these atypical granule-like structures. Close relation to the Golgi complex, fusion with phagocytic vacuoles and rapid disappearance after phagocytosis suggest that these atypical structures were related to the granules. PMN alkaline phosphatase activity, as a marker of secondary granules was found to be normal and increased in two separate determinations. Myeloperoxidase staining and lysosomal enzyme levels of primary granules were found to be within normal limits. Both primary and secondary granules were present in the patient's PMNs ultrastructurally. Abnormalities of cytoplasmic granules have been shown in acute, chronic myelogenous leukemias[19,21,22] and the Chédiak-Higashi syndrome[23], but the clinical and laboratory findings in our patient were not indicative of leukemia or Chédiak-Higashi syndrome. A varying degree of PMN dysfunction has been reported in association with qualitative or quantitative primary or secondary granule abnormalities[19,20]. Depressed chemotactic response and bactericidal activity of PMNs have been clearly shown in patients with secondary granule deficiency[24-27]. Our findings do not indicate primary or secondary granule deficiency. Furthermore, atypical bodies in our patient were not observed in the cases reported. Although ultrastructural studies were not routinely performed in patients with PMN dysfunction, none of the reported cases which showed ultrastructural PMN abnormalities associated with impaired function match our case as far as we know[19,24,26-29]. The exact nature of these atypical bodies observed in the patient's PMNs, the mechanism of increased surface ruffling and rudimentary pseudopodia formation remains to be elucidated. These ultrastructural abnormalities may be responsible for defective chemotaxis and bactericidal, fungicidal inefficiencies. It is unlikely that these PMN abnormaliti-

es could be a consequence of the infection. Most of the PMN function studies were done during periods of well-being in order to avoid the possible effects of infection and medication. Furthermore, we studied the PMN chemotaxis of many children with pulmonary infection including five cases with bronchiectasis and the chemotactic activity was found to be increased in these cases as reported by Hill et al[30,31]. Clinical course and degree of PMN functional abnormalities e.g., candicidal, staphylocidal defect, observed in our case were milder than patients with chronic granulomatous disease. Recurrent abscesses and lymphadenitis were not observed during our two-year observation period but we can not exclude the effects of close follow-up and appropriate chemotherapy according to culture results in addition to trimethoprim with sulfamethoxazole prophylaxis which was given for the last one and a half years. However, these measures failed to control the progress of bronchiectasis. These clinical findings, normal NBT dye reduction and ultrastructural observations exclude the possibility of chronic granulomatous disease in this patient. Approximately 15 primary defects of PMN function associated with recurrent infections have been described to date but the molecular basis for most of these disorders is not known[18,32]. We believe that our case represents a new example of these abnormalities. Continued study of such patients is necessary for delineating and understanding the role of PMN function in the host resistance to infection.

Summary

Polymorphonuclear neutrophils (PMN) from a patient with recurrent infections were found to have ultrastructural abnormalities and impaired functions. The PMNs had atypical cytoplasmic granule-like structures, increased surface ruffling and rudimentary pseudopodia formation. Close relation to the Golgi complex, fusion with phagocytic vacuoles and rapid disappearance after phagocytosis suggest that these atypical structures are related to the granules. Ultrastructurally, both the primary and secondary granules were present in the patient's PMNs. Myeloperoxidase staining, lysosomal enzyme levels of primary granules and alkaline phosphatase activity of PMNs were found to be normal. The PMNs were impaired in chemotaxis and bactericidal capacity. This data indicates a new instrinsic PMN defect which may be responsible for the increased susceptibility of our patient to pyogenic infections.

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