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Mémoire.

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FALCIPARUM MALARIA IN NATURALLY INFECTED HUMAN PATIENTS : IV-ULTRASTRUCTURAL CHANGES IN PERIPHERAL WHITE BLOOD CELLS

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SUMMARY

Ultrastructure of white blood cells (WBC) were studied in peripheral venous blood from Saudi patients with acute falciparum malaria (AFM) and compared with their counterparts in same patients 2 weeks after chloroquine treatment and full recovery. A counting system was incorporated to determine the rate of abnormal to normal cell type in plastic thick sections during the course of the disease. Neutrophilia, monocytosis, eosinopenia and lymphocytosis were associated with various ultrastructural abnormalities including: (1) Knobby phagocytic polymorphnuclear neutrophils (PMN) and promyelocytes, and PMN with highly vacuolated cytoplasm. (2) Irregularly outlined electron-dense nuclei in non-functional monocytes. (3) Unusual distribution of nuclear chromatin in resting B-lymphocytes, while others possess highly vacuolated cytoplasm and knobby surfaces. (4) Absence of granules in granular lymphocytes containing the known diagnostic paratubular crystalline arrays. (5) Plasmablasts containing electron-dense granules and swollen mitochondria. These abnormalities were suggested to be due to the high level of parasitaemia producing some toxic soluble products. They may also be attributed to alteration of bone marrow macrophages as a sequence of their interaction with soluble parasite products or their phagocytic parasitized red cells and debris released during the rupture of schizonts. This study showed that the number of abnormal WBC increases in patients with high level of parasitaemia; plasmablasts have the lowest rate of abnormalities, while monocytes have the highest; old patients present with lower degree of parasitaemia than young patients due to a less mature immune system; and the AFM may have independent effects on the structure of human WBC.

RÉSUMÉ : **Paludisme à *P. falciparum*. IV-Altérations ultrastructurales des globules blancs périphériques.**

L'ultrastructure des globules blancs est étudiée dans le sang veineux périphérique chez des malades d'Arabie Saoudite atteints de paludisme aigu à *falciparum*. Elle est comparée à celle des globules blancs chez les mêmes malades deux semaines après traitement à la chloroquine et guérison totale.

Des comptages ont été réalisés sur coupes semi-fines pour déterminer le pourcentage d'anomalies cellulaires pendant le cours de la maladie. Neutrophilie, monocytose, éosinopénie et lymphocytose étaient associées à diverses anomalies ultrastructurales : 1) protubérances de l'enveloppe évoquant des knobs chez les promyélocytes, parfois associées à un cytoplasme très vacuolisé chez les polynucléaires neutrophiles; 2) contour nucléaire irrégulièrement dense chez les monocytes non fonctionnels; 3) distribution anormale de la chromatine nucléaire dans certains lymphocytes B au repos, tandis que d'autres ont un cytoplasme vacuolisé et une surface rugueuse; 4) absence de granules chez les lymphocytes granuleux contenant les structures cristallines paratubulaires; 5) granules denses et mitochondries gonflées dans les plasmoblastes.

Cette étude montre que, le nombre de globules blancs anormaux augmente chez les patients ayant de fortes parasitémies, les monocytes ont plus d'anomalies que les plasmoblastes, les malades âgés ont une parasitémie plus faible que les jeunes ce qui est dû à un système immunitaire plus mûr, et que, les effets du paludisme aigu à *falciparum* sur les globules blancs peuvent être sous la dépendance de mécanismes variés.

INTRODUCTION

Acute falciparum malaria (AFM) is known to induce several peripheral blood changes. These include anaemia and thrombocytopenia in addition to neutrophilia, monocytosis, eosinopenia, plasmacytosis and lymphocytosis (Facer and Jenkins, 1989), with atypical lymphocytes (Kueh and Yeo, 1982). Peripheral blood neutropenia has also been reported in some (Dale and Wolff, 1973) but not other (Martello *et al.*, 1969) studies.

Phagocytosis of pigment and of parasites by monocytes/macrophages, and less frequently by neutrophils has been observed in the peripheral blood of patients with malaria (Vernes, 1980).

To date, no observations on the ultrastructural changes of WBC have been reported in peripheral blood of patients with AFM. The following work represents a brief summary of the most significant ultrastructural changes observed in WBC of Saudi patients who were suffering from AFM. In separate papers, the ultrastructural morphological changes of the red blood cells and blood platelets in addition to malaria pigments are presented (El-Shoura, 1993; El-Shoura and Al-Amari, 1993*a, b*).

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MATERIALS AND METHODS

Twenty eight Saudi patients (males) aged 5-56 were admitted to Asir Central Hospital, in the South West Region in Saudi Arabia, during 1989-1992 with a diagnosis of AFM. All patients were febrile (median duration of fever, 4 days) and 19 showed splenomegaly. Eight patients had had previous attacks of malaria (species unspecified) and 20 had no history of previous infection. No other physical abnormality was recorded. None of the patients had any other infectious disease, bleeding diathesis or any clinical features known to complicate malaria.

Except for thrombocytopenia (platelet less than 150×10^9/L) which was found in 23 (82.14 %) patients, haemoglobin and haematocrit were within normal limits in all patients. The mean of the total and differential nucleated WBC count/10^9L was as follows: WBC, 12.9; neutrophils, 6.8; eosinophils, 0.03; monocytes, 1.45; and lymphocytes, 4.5. Brucella serology was negative in all patients. *Plasmodium falciparum* parasites were seen in peripheral blood film in all patients. Parasitaemia, or parasite count (PC), was expressed as absolute number of parasitized erythrocytes per microliters (μL) blood. The mean of parasitaemia was 152.375 PC/μL in seven patients who were aged between 5-12, and was 65.850 PC/μL in 21 patients who were aged between 15-56.

Five milliliter venous blood samples (VBS) were collected from each patient before treatment and 2 weeks after treatment (median duration of treatment, 3 days), and full recovery. Each sample was immediately transferred into plastic tubes containing EDTA. The buffy coat obtained after centrifugation at 1,500 rpm was fixed in 1.5 glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 1 h at room temperature. Each VBS was post-fixed in 1 % osmium tetroxide for 1 h, and washed twice in the phosphate buffer. After recentrifugation, each pellet was preembedded in 1 % Agar, cut into 2-3 mm^3 cubes, dehydrated in an ascending series of ethanol, and embedded into Spurr's resin. Five different blocks were randomely selected from each sample. To determine the rate (mean) of each abnormal to normal cell type of all patients, 100 serial thick sections (0.5 μm) were cut from each block, stained with toluidine blue and examined by light microscopy (LM). The count was carried out in 4 groups of 25 sections each. To study the ultrastructural abnormality(s) of each cell type in each patient, and to compare them with those obtained from same patients after treatment, 100 serial ultrathin sections were cut from each block, stained with uranyl acetate and lead citrate, and examined in Jeol 1200 EX transmission electron microscopy (TEM) at 80 kV. Examination of ultrathin sections was carried out in 2 groups of 50 sections each.

RESULTS

At the LM level, several structural abnormalities were detected in certain types of WBC obtained from all patients before treatment. These cells included polymorphnuclear neutrophils (PMN), monocytes (M), lymphocytes (Ly), and plasmablasts (Pb). Analysis of thick sections showed that the rate (mean) of abnormal to normal cells was as follow: PMN 2:5, M 3:4, Ly 2:5, Pb 1:7. In general, the number of abnormal cells was higher in patients with high level of parasitaemia. All patients responded well to treatment with oral chloroquine alone. Two weeks after treatment, their blood films were negative for malaria parasites and their thick sections were free of abnormal WBC.

At the TEM level, abnormal WBCs were detected and structurally compared with their normal counterparts obtained from the same patients after treatment. Identification of normal WBCs was based on a previous description by Weiss (1977).

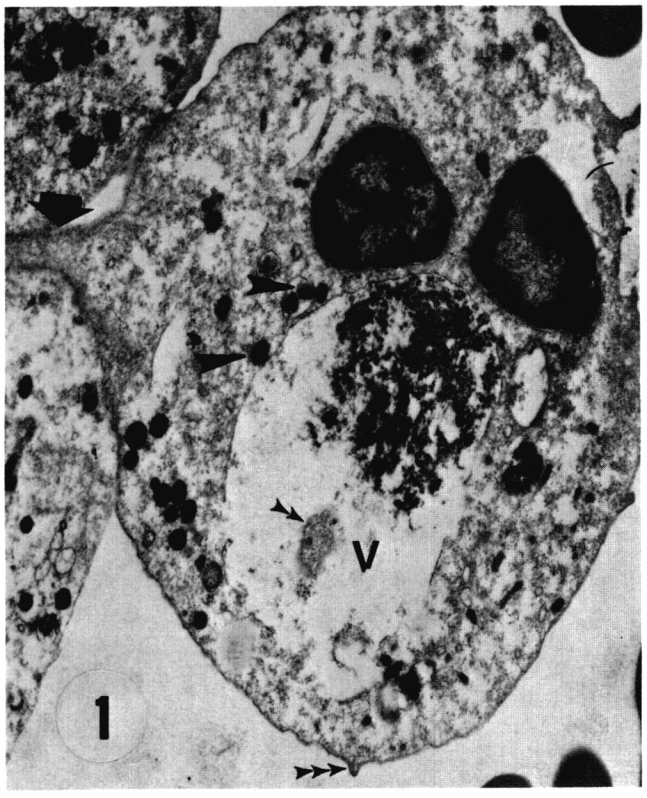
Various mature neutrophils with a few scattered, electron-dense, small granules and large phagocytic vacuoles containing digested material, presumably of pigment or parasites, were observed *(fig. 1)*. The other less dense, small « specific » granules seen in normal cells *(fig. 2)*, were absent. Some of the dense granules were attached to the vacuole membrane, while contents of others were released into the vacuole lumen. The cell has two to three nuclear lobes. In some other neutrophils, the cytoplasm appeared dramatically vacuolated and partially necrotic, while the nucleus appeared disintegrated *(fig. 3)*. In addition to the mature neutrophils, immature cells, or promyelocytes, which were larger in diameter than that of the neutrophils, were also recognized *(fig. 4)*. Some cytoplasmic areas appeared degenerated while the non degenerated ones contained electron-dense granules and small vacuoles. Unlike normal cells *(fig. 2)*, abnormal neutrophils and promyelocytes possessed surface knobs *(fig. 3, 4)*, while cytoplasmic pseudopodia-like extensions were protruded only from the neutrophils *(fig. 1)*.

FIG. 1. — An abnormal neutrophil obtained before treatment showing knobs on the cell surface (triple arrowheads) and a large phagocytic vacuole (v) containing electron-dense digested material (compare with Fig. 2). Note the few scattered electron-dense granules, some of which are in close vicinity to the vacuole membrane (arrowheads), while contents of some others have already been released into the vacuole lumen (double arrowheads). Arrow points to a cytoplasmic extension (x 10,000).

FIG. 2. — A normal neutrophil obtained after treatment from the same patient from whom the cell in Fig. 1 was obtained. Note the presence of many surface pseudopodia-like structures, and the less dense small « specific » granules which were absent in Fig. 1 (× 15.00).

FIG. 3. — As in Fig. 1 but showing a large perinuclear vacuole (*). Arrow points to a damaged cytoplasmic region. Arrowheads represent surface knobs (× 20,000).

FIG. 4. — A large promyelocyte obtained after treatment showing membrane-bound vacuoles (small *), some of which enclose parasites (arrowheads). Large (*) indicates damaged cytoplasmic areas. Triple arrowheads point to a surface knob (× 10,000).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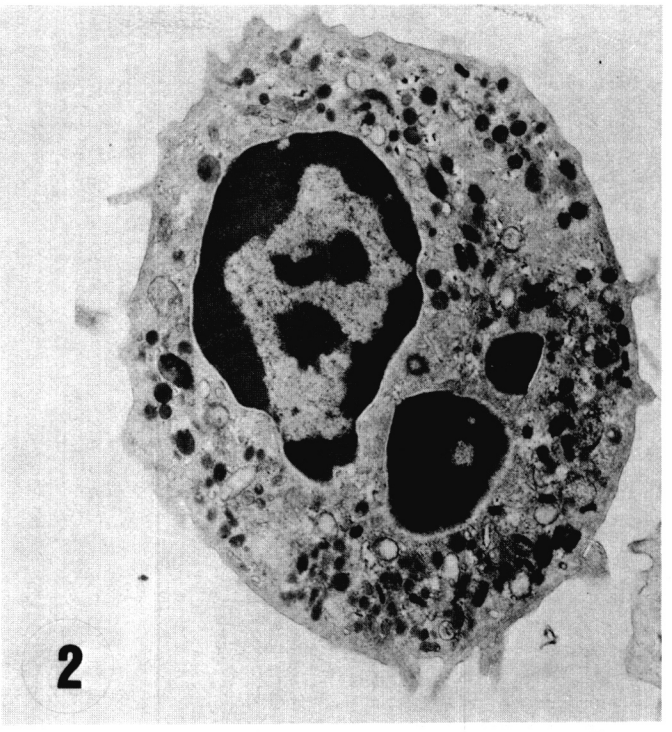
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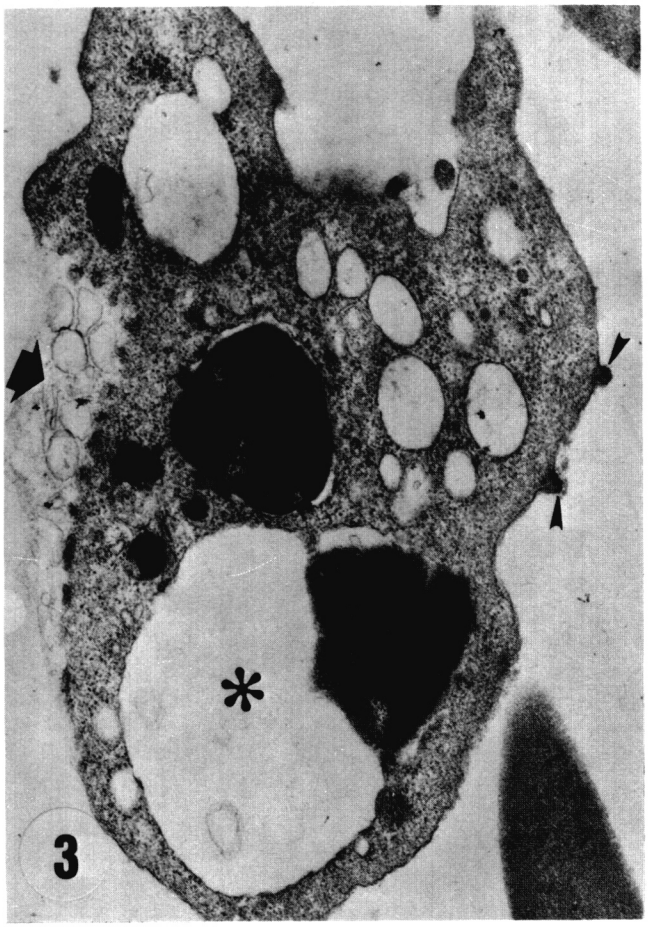
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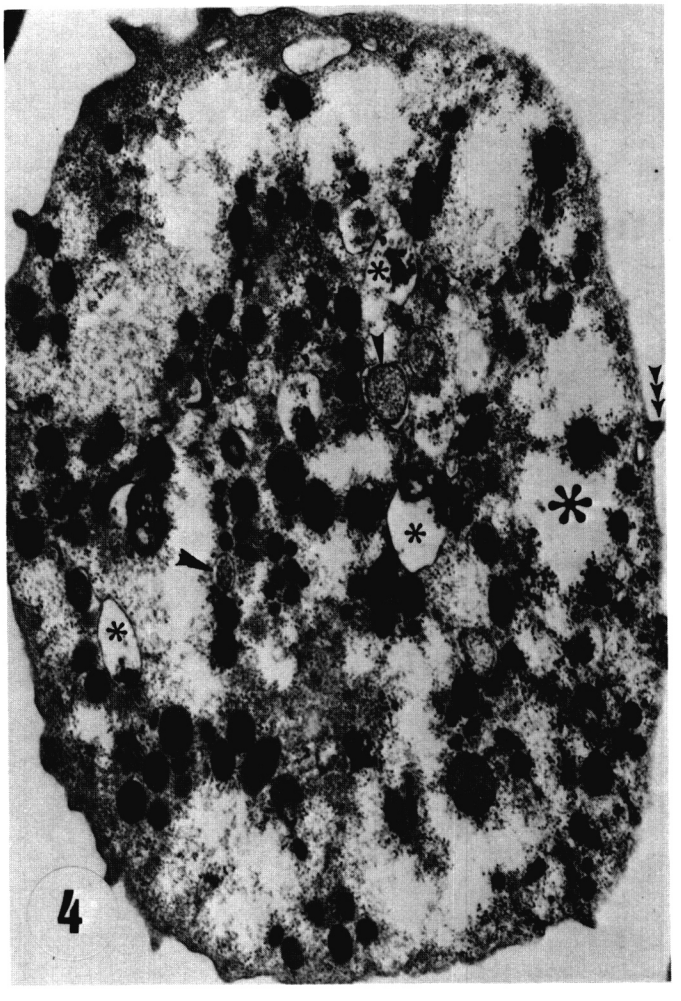
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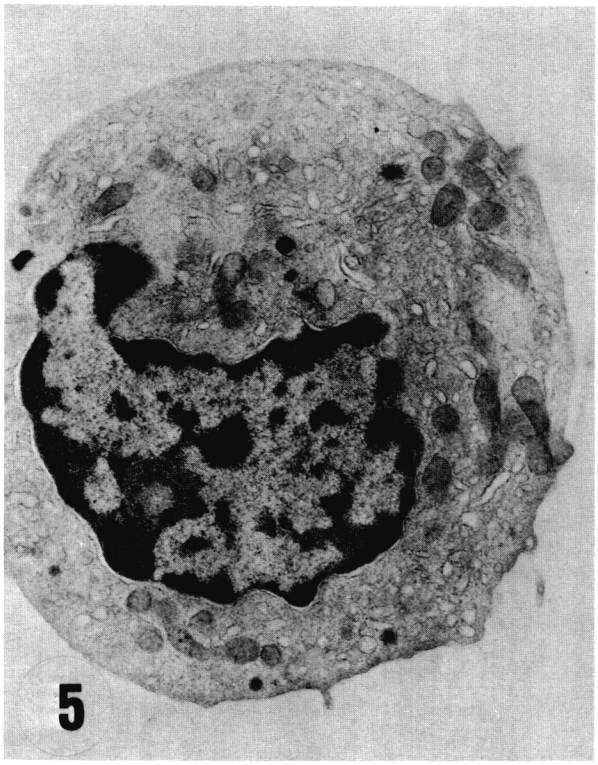
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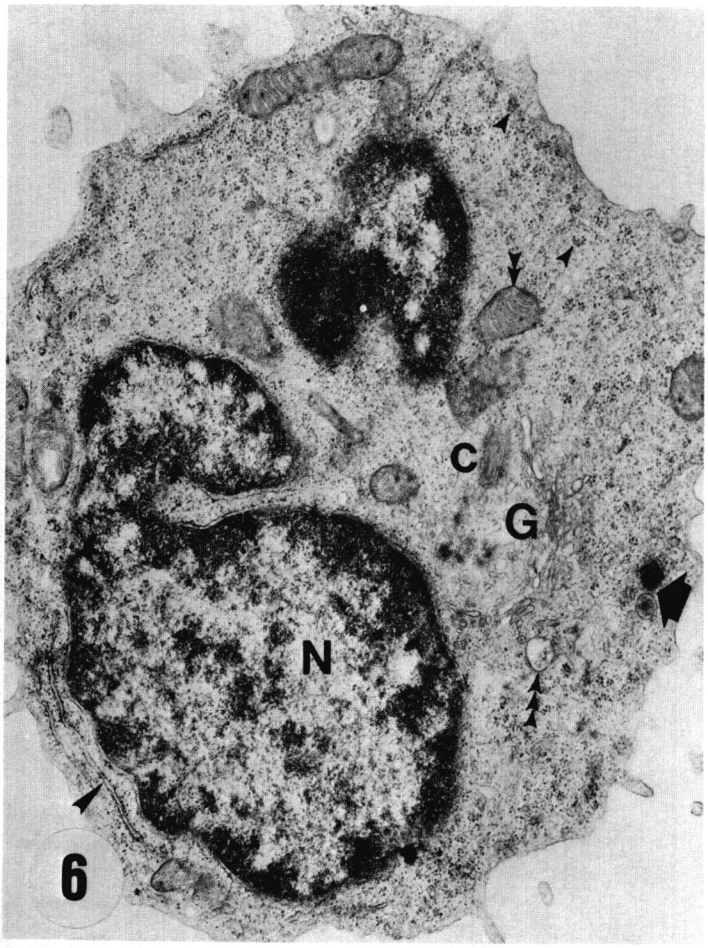
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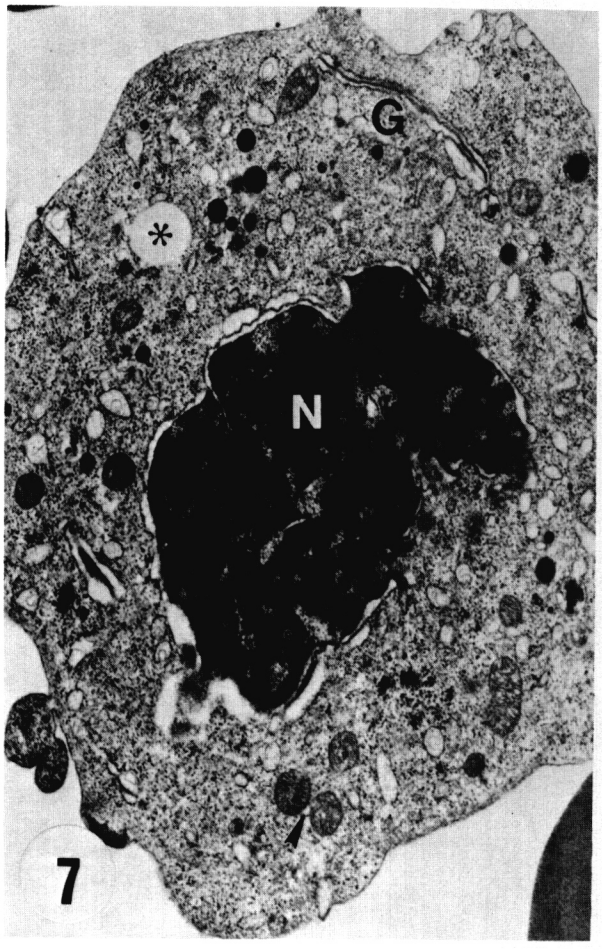
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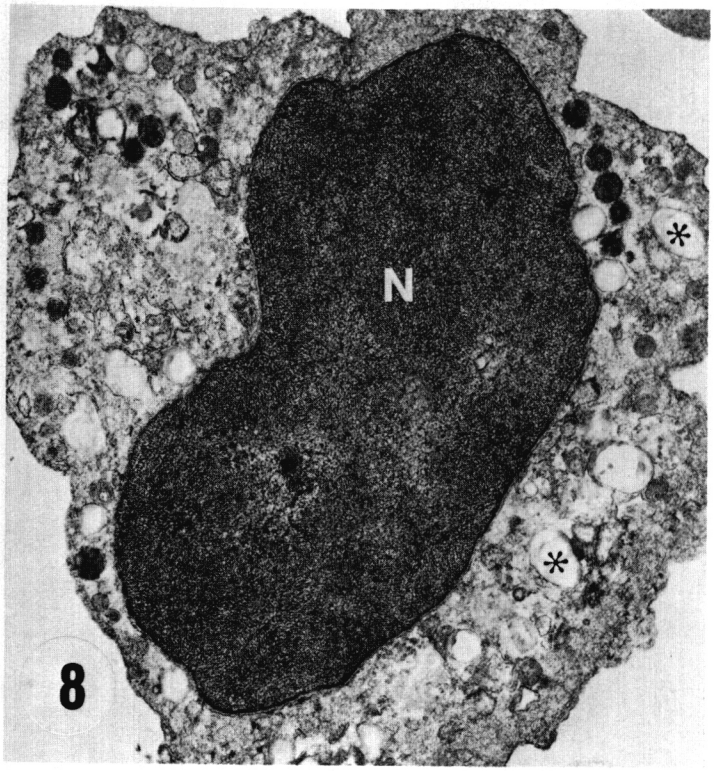
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In comparison to normal monocytes *(fig. 5, 6),* abnormal cells showed nuclei displaying an irregular outline with large quantities of electron-dense heterochromatin containing many small, electron-lucent areas *(fig. 5);* other nuclei showed homogeneously condensed chromatin *(fig. 6).* Despite these nuclear abnormalities, cell organelles including Golgi bodies, vacuoles, electron-dense, small granules and mitochondria similar to those found in normal cells *(fig. 5, 6),* could be easily identified in the cell cytoplasm *(fig. 7, 8).*

Unlike normal lymphocytes *(fig. 9),* minor ultrastructural changes were displayed by abnormal cells *(fig. 10-12).* Various lymphocytes with high nuclear/cytoplasmic ratio were observed *(fig. 10).* Ribosomes, mitochondria and peripheral vacuoles accounted for the majority of cytoplasmic organelles. The round, abnormal looking nucleus displayed thick, electron-dense, peripheral heterochromatin surrounding a large zone of less dense central nucleoplasm *(fig. 10).* In addition, lymphocytes containing parallel tubular arrays (PTA's) of membrane-bound tubules and organized in a paracrystalline structure were detected *(fig. 11).* Moreover, lymphocytes with highly vacuolated cytoplasm and surface knobs, similar to those found in the mature and immature neutrophils *(fig. 1, 3, 4),* were also observed *(fig. 12).*

Abnormal plasmablasts displayed swollen mitochondria and numerous dense granules, these were connected to long parallel cisternae of rough endoplasmic reticulum *(fig. 13).* The eccentrically situated large nucleus possessed heterochromatin organized in a patchy, wheel-like pattern, and electron-dense, compact nucleoli. These aberrated organelles have not been observed in normal plasmablasts *(fig. 14).*

DISCUSSION

The findings presented show the first description of the ultrastructural changes of the peripheral WBC in patients with AFM. The small mature neutrophils and the large immature promyelocytes with membrane-bound vacuoles, enclosing digested material in the former and parasite in the latter, are apparently phagocytic cells. In bone marrow, phagocytic neutrophil granulocytes (Wickramasinghe *et al.,* 1987), and neutrophil metamyelocytes (Abdalla, 1989) containing merozoites and pigment were reported. The electron-dense granules attached to the vacuole membrane and released into the vacuole were considered to be lysosomes containing several hydrolytic enzymes (Baggiolini, 1972; Ohlsson *et al.,* 1977). On the other hand, knobby neutrophils and promyelocytes have not been reported in the literature. It is well documented that parasitized mature erythrocytes are the only knobby blood cells which appear during infection with human malaria (Aikawa, 1988; El-Shoura and Al-Amari, 1993*a*). Recently, knobby non-parasitized immature and mature erythrocytes were demonstrated in the peripheral blood in patients with AFM (El-Shoura, submitted). However, knobs found on the surface of neutrophils and promyelocytes are structurally related to those described for parasitized and non-parasitized red blood cells and are known to play a role in adhesion to endothelial cells (Wickramasinghe *et al.,* 1987).

During infection with malaria parasites, active phagocytic monocytes and macrophages were usually detected in both the peripheral blood (Facer and Brown, 1981) and bone marrow (Wickramasinghe *et al.,* 1989). In the present study, neither phagocytic monocytes nor macrophages were observed but only monocytes with ultrastructurally abnormal nuclei and cytoplasm. The most foremost reported functions of monocytes are those activities related to defense against infection, especially infection due to intracellular parasites (Sharma and Remington, 1981).

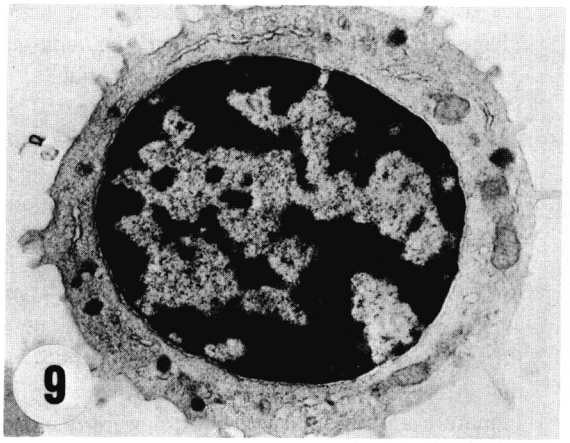
The lymphocytes containing paratubular arrays are similar to granular lymphocytes which constitute up to 20 % of peripheral blood lymphocytes (Herberman, 1982). These cells are comprised of cell types including those mediating natural immunity. In healthy subjects, the paratubular arrays are almost found with electron-dense granules positive for acid phosphatase (Grossi *et al.,* 1982), whereas in the membrane-bounded paratubular arrays the acid phosphatase reaction is detected in the translucent space which separates the tubular structures from the limiting mem-

FIG. 5. — A normal monocyte showing « healthy » looking nucleus and cytoplasm packed with small cisternae of RER and mitochondria in addition to a few scattered electron-dense granules (× 30,000).

FIG. 6. — As in Fig. 5 but showing advanced developmental stage containing Golgi bodies (G), centriole (C), rough endoplasmic reticulum (long arrowhead), membrane-bound granules (arrow), polyribosomes (small arrowheads), mitochondria (double arrowheads) and small membrane-bound vacuole (triple arrowheads). N. nucleus (× 25,000).

FIG. 7. — An abnormal monocyte showing irregularly outlined nucleus (N) with large quantities of electron-dense heterochromatin. The cytoplasm contains Golgi bodies (G), mitochondria (arrowhead) and vacuoles (*) (× 15,000).

FIG. 8. — As in Fig. 7 but showing a nucleus (N) with homogeneously condensed chromatin. (*) points to membrane-bound vacuoles (× 20,000).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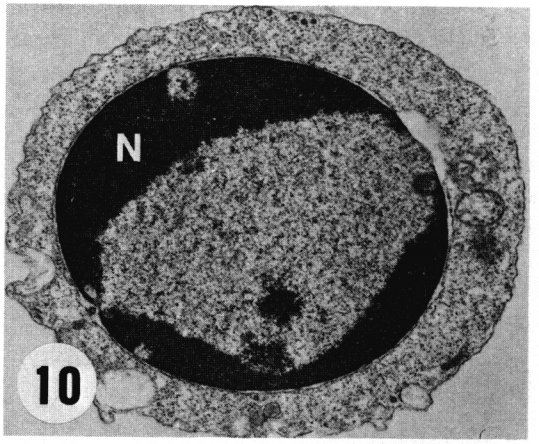
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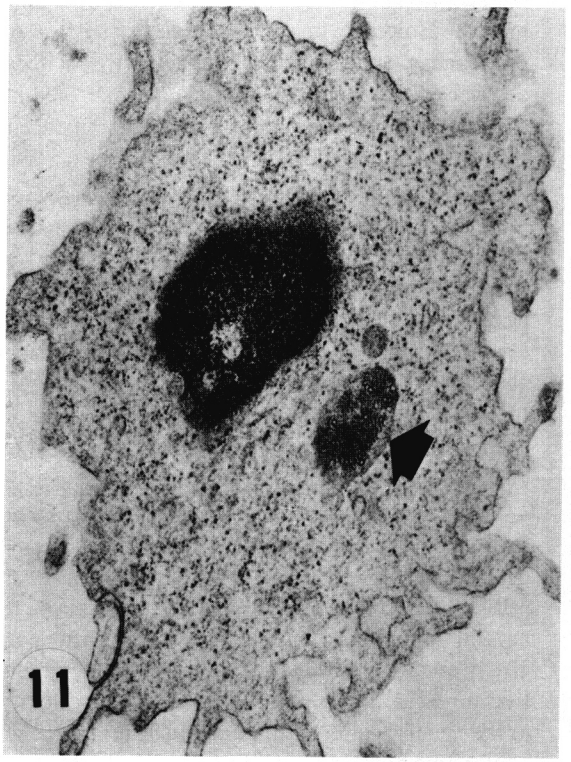
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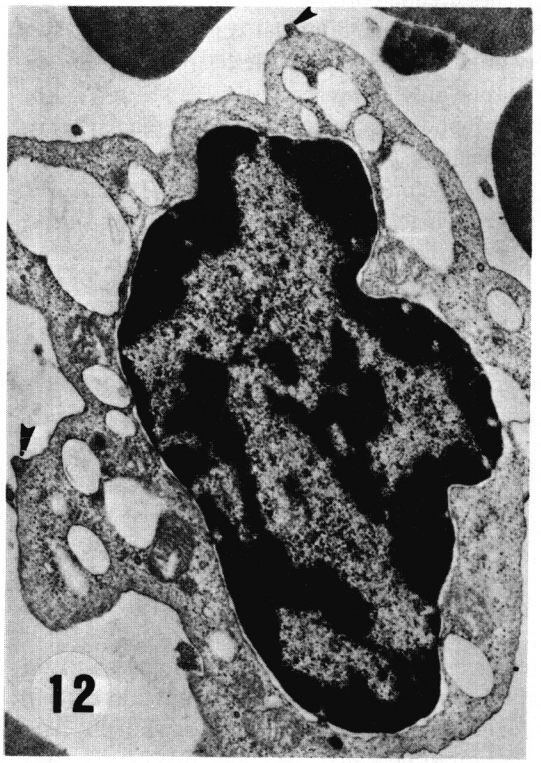
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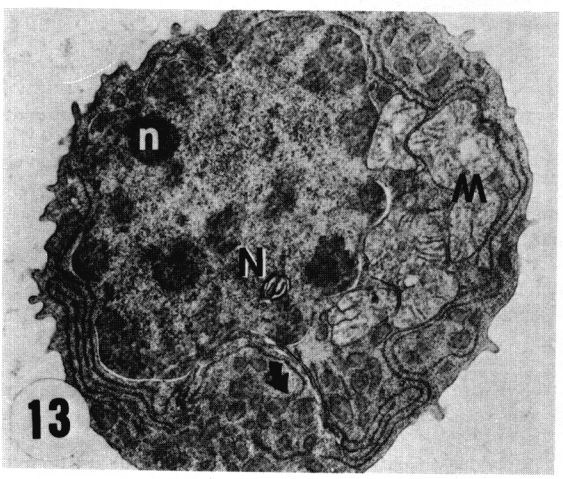
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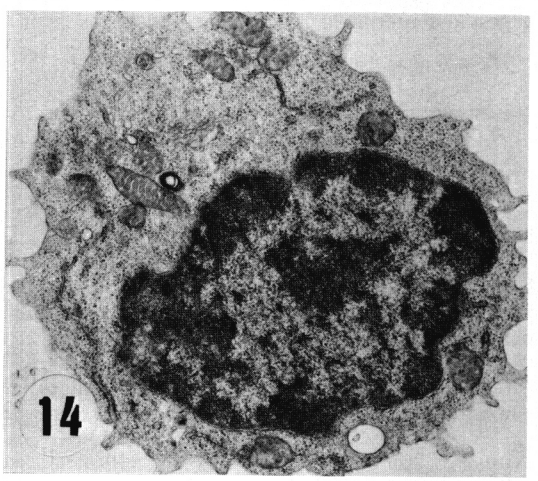
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FIG. 9. — A normal B-lymphocyte showing high nucleus/cytoplasm ratio (× 25,000).

FIG. 10. — As in Fig. 9 but showing an abnormal cell with unusual distribution of the nuclear heterochromatin (N) (× 15,000).

FIG. 11. — An aberrated Lymphocyte containing transversally sectioned paratubular crystalline arrays (arrow) (× 30,000).

FIG. 12. — An abnormal lymphocyte with highly vacuolated cytoplasm and surface knobs (arrowheads) (× 20,000).

FIG. 13. — An abnormal plasmablast with numerous granules connected to long cisternae of rough endoplasmic reticulum (arrow). Note that the heterochromatin is organized in a patchy, wheel-like pattern. M, swollen mitochondria (× 20,000).

FIG. 14. — A normal plasmablast showing a « healthy » looking nucleus and cytoplasm containing mitochondria and cisternae of RER (× 30,000).

brane. Absence of the cell granules may be due to their consumption during infection with the AFM. However, knobby lymphocytes are demonstrated here for the first time, and these knobs seem to be related to those previously reported for parasitized and non-parasitized red blood cells.

Altered WBC are apparently characteristic features during infection with AFM. Wickramasinghe *et al.* (1989), suggested that some toxic soluble parasite product and alteration of bone marrow (BM) macrophages as a sequence of their interaction with soluble parasite products or their phagocytosing parasitized red cells and debris released during the rupture of schizonts are the cause of erythropoiesis abnormalities. However, these toxic products and alterations of BM macrophages could also be the cause of ultrastructural aberrations of WBC during AFM. In addition, an unbalanced metabolism could also be a suggestive cause of these cellular aberrations since they are being overproduced in response to infection with AFM.

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