Ultrastructure of bone marrow in patients with visceral leishmaniasis

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SUMMARY Ultrastructural studies were performed on bone marrow aspirates from three patients with visceral leishmaniasis. The patients were moderately anaemic but showed a suboptimal increase in the absolute reticulocyte count. Serum and red cell folate concentrations and serum vitamin B₁₂ concentrations were normal in all three cases, and serum ferritin concentrations were normal or increased. The bone marrows were hypercellular and showed erythroid hyperplasia; a high proportion of the erythroblasts showed dyserythropoietic changes. Amastigote forms of Leishmania donovani were found within bone marrow macrophages and within occasional neutrophil and eosinophil granulocytes. Electron microscopy showed the presence of many abnormal cells, which probably represented immature erythroblasts with giant lysosomes. These cells were often large, usually contained immature nuclei with relatively little condensed chromatin, had 1–20 electron dense cytoplasmic granules with an average diameter of 0.5 μm, and regularly displayed substantial lymphocytic activity. A few abnormal cells and intermediate and late erythroblasts appeared to have been phagocytosed by macrophages. The data indicate that dyserythropoiesis and ineffective erythropoiesis have a role in the pathogenesis of the anaemia of at least some cases of kala-azar.

Visceral leishmaniasis (kala-azar) is a chronic disease caused by the intracellular parasitic organism Leishmania donovani. It is characterised by severe splenomegaly and varying degrees of pancytopenia. The pancytopenia seems to be mainly related to the splenomegal that results in increased pooling of blood cells within the spleen, an increase in plasma volume (haemodilution), and some reduction in the life span of blood cells (due to "hypersplenism"). Thus in a large series of cases Cartwright et al. showed that the severity of pancytopenia correlated both with the size of the spleen and the duration of symptoms. In addition, as in other conditions with substantial splenomegal, both an increase in plasma volume, and a shortening of the life span of red cells and platelets and of the half life of circulating granulocytes has been shown in kala-azar.² -- ⁵

Additional mechanisms have also been proposed for the anaemia in kala-azar. These include dyserythropoiesis and ineffective erythropoiesis, attributed to the action of toxins generated by the organisms, and haematmic deficiencies.⁶ Although several studies have shown the presence of complement components³,⁷ or IgG plus complement components on the red cells of patients with kala-azar,⁸ there is no information as to the part played by these immunoprofes in the pathogenesis of the anaemia.

Little attention has been paid to the ultrastructure of the bone marrow in kala-azar; a survey of the published reports showed only electron microscopic data on a single patient who was studied days after antimony treatment had been started.⁹ In view of the possibility that marrow dysfunction may have some role in the pathogenesis of the pancytopenia in kala-azar we made a detailed ultrastructural study of the bone marrows of one previously treated and two untreated cases. The present paper describes our findings.

Material and methods

The study was carried out in 1981 during an outbreak of kala-azar in the Kitui district of Kenya. The subjects were three children who were referred to the Clinical Research Centre, Nairobi, with a diagnosis of kala-azar. The splenic aspirates of all subjects were positive for L donovani.

Soon after admission the patients were examined
and blood was taken into edetic acid and into a plain tube for haematological studies. Bone marrow was aspirated from the posterior superior iliac crest. Smears were made at the bedside, and the remainder of the aspirate was mixed with 2 ml of Hanks’s balanced salt solution containing 20 units of preservative free heparin. Patients were treated with daily intramuscular injections of sodium stibogluconate and the haematological studies were repeated two weeks after treatment was started.

HAEMATOLOGICAL STUDIES

A full blood count was obtained using a Coulter Model S electronic cell counter. Platelet counts were performed using a Neubauer counting chamber. Serum and red cell folate concentrations were measured by a microbiological assay based on chloramphenicol resistant Lactobacillus casei. Serum vitamin B12 and serum ferritin concentrations were determined using the Becton Dickinson B12 radioassay kit (57Co) and the Becton Dickinson ferritin (125I) radioimmunoassay kit, respectively. The direct antiglobulin test was performed using the microtitre method outlined previously. The reagents used were a specific rabbit antihuman-IgG, anti-C3, and anti-C4. These were kindly supplied by Mr T Hunt of the Blood Transfusion Centre in Oxford. All reagents were absorbed to remove non-specific activity and tested for specificity by using cells coated with IgG, C3, and C4, before use.

The bone marrow smears were fixed in methanol and stained using the May-Grunwald-Giems-sa stain and Perl’s stain for haemosiderin. A differential cell count was carried out on 1000 consecutive nucleated cells. In addition, the severity of dyserythropoiesis was assessed by counting the numbers of cells that showed dyserythropoietic features among 200 erythroblasts. The dyserythropoietic features scored included cytoplasmic vacuolation or stippling, intererythroblastic cytoplasmic bridges, irregularly shaped nuclei, karyorrhexis, binuclearity and multinuclearity, and intercellular chromatin bridges. The number of individual intracellular and extracellular L donovani per 1000 nucleated bone marrow cells was also quantitated.

ELECTRON MICROSCOPIC STUDIES

Only the pretreatment marrow samples were used for ultrastructural studies. Bone marrow fragments were removed from the mixture of marrow plus heparinised Hanks’s solution and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) at room temperature. The fixed fragments were processed for transmission electron microscopy, as described by Wickramasinghe et al., except that the sections were cut on a Reichert Om U4-Ultracut ultramicrotome.

Table 1  Essential clinical and haematological features of three patients studied with kala-azar before (a) and after (b) leishmaniacidal treatment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Case 1 (a)</th>
<th>Case 2 (a)</th>
<th>Case 3 (a)</th>
<th>Case 1 (b)</th>
<th>Case 2 (b)</th>
<th>Case 3 (b)</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>10</td>
<td>7</td>
<td>9</td>
<td>10</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>Sex</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>Spleen size†</td>
<td>25</td>
<td>15</td>
<td>15</td>
<td>25</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Liver size†</td>
<td>8</td>
<td>6</td>
<td>3</td>
<td>8</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Blood count:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>7.0</td>
<td>5.8</td>
<td>7.9</td>
<td>8.5</td>
<td>7.0</td>
<td>6.8</td>
</tr>
<tr>
<td>Red blood cells (× 1012/l)</td>
<td>2.13</td>
<td>1.97</td>
<td>3.52</td>
<td>3.42</td>
<td>2.68</td>
<td>2.77</td>
</tr>
<tr>
<td>Mean corpuscular volume (fl)</td>
<td>78</td>
<td>72</td>
<td>65</td>
<td>72</td>
<td>79</td>
<td>85</td>
</tr>
<tr>
<td>Reticulocytes (× 1012/l)</td>
<td>0.52</td>
<td>0.34</td>
<td>0.28</td>
<td>0.21</td>
<td>0.25</td>
<td>0.22</td>
</tr>
<tr>
<td>White cell count (× 109/l)</td>
<td>4.4</td>
<td>7.3</td>
<td>14.7</td>
<td>3.4</td>
<td>3.6</td>
<td>4.5</td>
</tr>
<tr>
<td>Neutrophils (× 109/l)</td>
<td>1.5</td>
<td>2.3</td>
<td>3.7</td>
<td>0.9</td>
<td>0.9</td>
<td>1.9</td>
</tr>
<tr>
<td>Lymphocytes (× 109/l)</td>
<td>2.7</td>
<td>4.7</td>
<td>10.0</td>
<td>2.1</td>
<td>2.3</td>
<td>1.8</td>
</tr>
<tr>
<td>Monocytes (× 109/l)</td>
<td>0.2</td>
<td>0.4</td>
<td>1.0</td>
<td>0.4</td>
<td>0.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Eosinophils (× 109/l)</td>
<td>0</td>
<td>0</td>
<td>0.03</td>
<td>0.04</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>Platelets (× 109/l)</td>
<td>120</td>
<td>96</td>
<td>125</td>
<td></td>
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</tr>
</tbody>
</table>

DAGT:

- Anti-IgG
- Anti-C3
- Anti-C4
- Serum vitamin B12 (ng/l)†† 563 241 204
- Serum folate (μg/l)†† 42 48 93
- Red cell folate (μg/l)†† 270 270 327
- Serum ferritin (μg/l)†† 2237 42 45

*Had been treated previously with three 30 day courses of stibogluconate (10 mg/kg/day); †cm below the mid point of the costal margin; †Normal ranges for children between 4 and 10 years: Hb 9.2–15.6 g/dl, MCV 72–97 fL; ††Normal ranges: serum vitamin B12 165–684 ng/l, serum folate 2.1–28.0 μg/l, red cell folate 160–640 μg/l, serum ferritin 7–142 μg/l.
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Table 2  Characteristics of bone marrow smears from three patients with kala-azar before (a) and after (b) leishmaniacidal treatment

<table>
<thead>
<tr>
<th>Variable</th>
<th>Case 1 (a)</th>
<th>Case 1 (b)</th>
<th>Case 2 (a)</th>
<th>Case 2 (b)</th>
<th>Case 3 (a)</th>
<th>Case 3 (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythroblasts (%)</td>
<td>13-28</td>
<td>58</td>
<td>49</td>
<td>45.5</td>
<td>43</td>
<td>40</td>
</tr>
<tr>
<td>Neutrophils and precursors (%)</td>
<td>45-77</td>
<td>32</td>
<td>34</td>
<td>35</td>
<td>38</td>
<td>39</td>
</tr>
<tr>
<td>Eosinophils and precursors (%)</td>
<td>1.0</td>
<td>1.2</td>
<td>1.2</td>
<td>2.4</td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>5-36</td>
<td>8.6</td>
<td>12.8</td>
<td>13.8</td>
<td>11.6</td>
<td>14.8</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>1.8</td>
<td>1.2</td>
<td>0.4</td>
<td>1.4</td>
<td>2.4</td>
<td>3.2</td>
</tr>
<tr>
<td>Plasma cells (%)</td>
<td>0.2-0.6</td>
<td>0.4</td>
<td>1.2</td>
<td>1.6</td>
<td>3.6</td>
<td>2.8</td>
</tr>
<tr>
<td>M:E ratio</td>
<td>1.2-5.2</td>
<td>0.55</td>
<td>0.70</td>
<td>0.68</td>
<td>0.88</td>
<td>0.98</td>
</tr>
<tr>
<td>Organisms per 1000 cells</td>
<td>6</td>
<td>5</td>
<td>14.4</td>
<td>4</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Erythroblasts with dyserythropoietic (%)</td>
<td>2-7</td>
<td>30</td>
<td>17</td>
<td>20</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>Sideroblasts (%)</td>
<td>20-90</td>
<td>51</td>
<td>50</td>
<td>15</td>
<td>32</td>
<td>22</td>
</tr>
</tbody>
</table>

Results

Table 1 summarises the main clinical and laboratory data prior to and two weeks after the start of treatment. At presentation all three patients had massive splenomegaly and moderately severe anaemia. The absolute reticulocyte counts were raised to between about two and five times higher than normal. Platelet counts were only slightly reduced. Mild neutropenia was seen in one of the cases. There was little change in the blood picture at the end of two weeks of treatment with antimony.

The pretreatment marrow fragments were grossly hypercellular with a virtually complete absence of fat cells. Table 2 summarises data on the cellular composition and other features of the marrow before treatment. All three patients showed reduced myeloid:erythroid (M:E) ratios due to erythroblast hyperplasia, and increased numbers of megakaryocytes. They also showed an increased proportion of erythroblasts with dyserythropoietic features. Most of the morphologically abnormal erythroblasts were early and late polychromatic cells; the most common erythroblast abnormalities were cytoplasmic stippling and irregularly shaped nuclei or karyorrhexis. Cytoplasmic stippling was seen in 6-14% and irregularly shaped nuclei or karyorrhexis in 7-13% of the erythroblasts. There were 1-4% of binucleate or multinucleate erythroblasts. Occasional giant metamyelocytes and several megaloblasts were found in case 2. Stainable iron was present in the marrow fragments of all three cases. A noticeable reduction in the proportion of morphologically abnormal erythroblasts occurred two weeks after the start of leishmaniacidal treatment (table 2).

Prior to treatment the number of intracellular and extracellular *L. donovani* seen per 1000 nucleated marrow cells was lowest in case 1 and highest in case 2. The prevalence of *L. donovani* in the marrow smears, however, did not correlate with counts of the organism in splenic aspirates or with the activity of the disease, as judged by the spleen size.

Electron Microscopy

Macrophages were prominent in the bone marrows of all three patients. Amastigote forms of *L donovani* were found within a high proportion of the macrophage profiles in cases 2 and 3 and in only a rare macrophage profile in case 1. The organisms seemed to be rounded or oval in outline and were limited by a trilaminar periplast consisting of an outer unit membrane of host cell origin, a middle unit membrane of parasite origin, and an inner layer of subpellicular hollow microtubules. Other cellular structures recognised included the nucleus, the kinetoplast, a flagellar pocket, the flagellum, mitochondria, a Golgi apparatus, multivesicular bodies, electron lucent vacuoles—sometimes containing varying quantities of electron dense granular material, large rounded homogeneously electron dense inclusions, and lipid droplets. Between one and 11 organisms were usually seen within a single macrophage profile and were found singly or in groups of two or four. In some sections, and particularly when the parasites were present in groups, the two unit membranes of the periplast, which are usually closely opposed to each other were separated to varying extents by granular electron dense material.

Parasitic organisms, some of which showed various degrees of degeneration or degradation, were also found within occasional neutrophil and eosinophil granulocytes (fig 1). The granulocytes containing the organisms were usually located near a broken up parasitised macrophage. Furthermore, a few parasites were occasionally found extracellularly attached to the cell membrane of the granulocytes, and these were surrounded by a narrow fluffy rim of residual macrophage cytoplasm.

A proportion of the profiles of intermediate and late erythroblasts, particularly of cases 1 and 2, displayed various ultrastructural abnormalities including irregularly shaped nuclei, abnormally long or multiple intranuclear clefts, and binuclearity and multinuclearity (figs 2a-c). Occasionally erythroblast profiles contained abnormally large autophagic
vacuoles (fig 2d). Erythroblast profiles in contact with heavily infected macrophages did not necessarily show ultrastructural abnormalities.

In cases 1, 2, and 3, respectively, 23·1, 10·8, and 11·9% of the profiles of nucleated cells belonged to an abnormal cell type (fig 3). Such cells displayed more or less rounded or oval outlines, were generally large, being up to 11 μm in their long axis, and contained a single large nucleus and moderate quantities of cytoplasm. The outlines of the nuclei were roughly circular or oval and, occasionally, quite irregular. The nuclei usually contained only very small or small quantities of heterochromatin and displayed prominent nucleoli. The cell membrane regularly showed evidence of substantial rhopheocytotic activity (fig 4). The electron density of the cytoplasmic matrix was usually low, being similar to that in pronormoblasts, but was occasionally considerably higher (fig 3). The cytoplasm contained many ribosomes, often arranged as polysomes, several mitochondria, and a few to several strands of rough endoplasmic reticulum. The cytoplasm of the abnormal cell profiles characteristically contained one to 20 (usually one to 10) large membrane bound, moderately or strongly, electron dense granules, which were rounded or oval in outline and which had average diameters of 0·2–0·8 μm (mean 0·5 μm). The granules were sometimes scattered throughout the cytoplasm but, more often, were concentrated in a fairly restricted area near the Golgi apparatus and centrioles. Some granules were homogeneous in appearance; some showed one or more rounded areas of increased density in their centre; and others contained a variable number of irregularly shaped, more or less well defined electron lucent areas (fig 4). Occasional granules contained ferritin molecules that were either diffusely distributed or localised to one region within the granule matrix. In addition to the large electron dense granules described above, some cell profiles displayed much smaller granules of similar electron density. Several abnormal cells were sometimes associated with a single infected macrophage and the erythroblasts surrounding it; the abnormal cells were in intimate contact with the macrophage or its cytoplasmic processes, other abnormal cells, or erythroblasts. Abnormal cells were also found apparently unassociated with a macrophage profile or associated with macrophage profiles not including sectioned organisms. Some of the abnormal cells interdigitated with adjacent normal looking erythroblasts in a manner similar to the inter-
Fig 2  Examples of ultrastructural abnormalities affecting erythroblasts in kala-azar. (a) Binucleate erythroblast; (b) trinucleate erythroblast in which nuclear masses are stuck together; (c) erythroblast with irregularly shaped nucleus displaying three intranuclear clefts; (d) erythroblast with irregularly shaped nucleus, large intracytoplasmic granule, and large presumably autophagic intracytoplasmic vacuoles. Case 1. (a) × 10950; (b) × 13250; (c) × 14000; (d) × 11650.
digitation observable between normal erythroblasts, and a few contained siderosomes. Occasional abnormal cells contained moderate or large quantities of heterochromatin and moderately electron dense cytoplasm and resembled intermediate or late erythroblasts, except for the presence of the characteristic large cytoplasmic granules (fig 3b). The abnormal cells did not display evidence of phagocytic activity and did not contain secondary lysosomes; nor did they protrude cytoplasmic processes at their periphery.

In occasional sections of macrophages there were presumably phagocytic vacuoles containing neutrophils, extruded erythroblast nuclei, intermediate or late erythroblasts, or cells containing granules of the type described in the preceding paragraph (fig 5).

Fig 3  Examples of abnormal cells containing granules found in bone marrows of patients with kala-azar. (a) Three large cells with nuclei containing small amounts of heterochromatin and prominent nucleoli. Electron density of cytoplasm of two upper cells is similar to that of proerythroblast, and that of third cell (arrow) is considerably greater. (b) Smaller cell with moderate quantities of condensed chromatin, moderately electron dense cytoplasm, and intranuclear cleft. All four cell profiles contained large electron dense granules and showed rhopheocytotic activity. Case 1. (a) × 9200; (b) × 10000.
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Fig 4  Prominent rhopheocytotic activity (small arrows) in abnormal cell containing granules. Some profiles of cytoplasmic granules (large arrow) display electron lucent areas. Case 2. × 33 550.

Fig 5  Abnormal cell (large arrow) within cytoplasm of macrophage; except for presence of cytoplasmic granules, abnormal cell resembles intermediate erythroblast. Three organisms (curved arrows) are seen inside macrophage. Case 2. × 11 850.
Discussion

In these three patients with kala-azar the distribution of intracellular organisms within the bone marrow was similar to that reported previously in a single patient studied three days after treatment with antimony had been started. Most of the parasites were situated within macrophages, and a few were found inside neutrophil and eosinophil granulocytes. Most of the neutrophil and eosinophil granulocytes containing the organisms were observed near disrupted infected macrophages, suggesting that the organisms located within granulocytes were derived from damaged macrophages. The damage to macrophages had probably occurred in vivo as a consequence of their invasion. The possibility cannot be excluded, however, that at least some of the intragranulocytic parasites had been released from macrophages which had been damaged during the marrow aspiration and had entered the granulocytes during the short period (less than 30 minutes) between marrow aspiration and the fixation of marrow fragments for electron microscopy. The ultrastructural features of the organisms within both the bone marrow macrophages and the bone marrow granulocytes of our patients were similar to those described previously for organisms found within the splenic macrophages of hamsters in vivo and laryngeal macrophages of a patient from central Italy.

The physiological reserve of normal bone marrow is such that this tissue can increase the rate of delivery of new red cells into the circulation (effective erythropoiesis) by a factor of 6–8. If the absolute reticulocyte count is taken as a rough index of effective erythropoiesis the three patients with kala-azar we studied showed only two to five-fold increases in the rate of effective erythropoiesis and thus seemed to have mounted a suboptimal marrow response despite persistent moderately severe anaemia. As the three patients showed severe erythroid hyperplasia in the marrow and a substantial increase in the prevalence of morphologically abnormal erythroblasts, it seems likely that the suboptimal marrow response resulted from increased dyserythropoiesis and ineffective erythropoiesis. The possibility that erythropoiesis was ineffective was supported by the finding that occasional intermediate and late erythroblasts seemed to have been phagocytosed by macrophages. Thus the haematological data in our patients support the previously published finding that although the anaemia of kala-azar may be primarily a consequence of splenomegaly, dyserythropoiesis and ineffective erythropoiesis also play a part in its pathogenesis. As there was no evidence that the dyserythropoiesis was related to iron, vitamin B₁₂, or folate deficiency (table 1) it seems likely that the abnormality of erythropoiesis was some consequence of the infection. This view is supported by our finding of improved erythroblast morphology two weeks after antimony treatment had been started. Macrophages are in intimate contact with haemopoietic cells and seem to participate in the generation of various substances which influence haemopoiesis, such as colony simulating factors, erythropoietin, prostaglandin E, interferons and interleukin I, either by producing them or by releasing factors which promote their production by other cells. Infection of bone marrow macrophages in visceral leishmaniasis may interfere with the generation of such substances and thereby impair haemopoiesis. In addition, infected macrophages may release cytotoxic factors, such as superoxide or parasite products, which may adversely affect haemopoietic cells. In view of the present light and electron microscopic evidence, indicative of dyserythropoiesis and increased ineffective erythropoiesis in kala-azar, it would be of interest to make a proper quantitative assessment of total, effective, and ineffective erythropoiesis in this disorder from detailed ferrokinetic and erythrogenic studies. It should also be pointed out that absolute reticulocyte counts might provide an underestimate of the rate of effective erythropoiesis in our patients because of a preferential sequestration of reticulocytes within the enlarged spleen.

An important finding of this study was that 11–23% of the nucleated marrow cells consisted of an abnormal cell type. The profiles of the abnormal cells were often large, usually included immature looking nuclei, and contained between one and 20 large electron dense cytoplasmic granules of variable appearance. On morphological grounds we consider it to be very unlikely that these large granular cells were natural killer cells; T suppressor cells; or abnormally developing early precursors of basophils, mast cells, or neutrophils. The abnormal cells found in our patients differed from circulating human natural killer and T suppressor cells in that they were larger and had much less heterochromatin, less rough endoplasmic reticulum, and a larger number of cytoplasmic granules. The abnormal cells also differed from normal neutrophil promyelocytes and early basophil precursors in having little rough endoplasmic reticulum and showing considerable rhabdomyotic activity.

Several of our findings strongly suggest that the abnormal cells represented immature erythroblasts with giant lysosomes. These findings are: (i) extensive rhabdomyotic activity at the surface of the abnormal cells; (ii) the presence of ferritin molecules within some of the intracytoplasmic granules; (iii) the occurrence of siderosomes in occasional abnormal cells; and (iv) the presence of large granules of the type seen in the abnormal cells in several cells that otherwise resembled intermediate or late erythroblasts. The for-
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formation of giant lysosomes may be a manifestation of damage to early erythroblasts, possibly caused by a locally generated or circulating parasite product or macrophage product, and could well be associated with a high mortality among the affected cells. In this respect it is noteworthy that the prevalence of abnormal cells with large granules was greatest in case 1, with the highest percentage of mature erythroblasts with dyserythropoietic features, and that occasional abnormal cells seemed to have been phagocytosed by macrophages. Electron microscopic studies using gold labelled cell lineage specific monoclonal antibodies and electron microscopic autoradiographic studies of iron (\(^{55}\)Fe) uptake would be required for a definitive biochemically based identification of the abnormal cells.

This project was supported by a grant from the Rockefeller Foundation. We thank Professor Mutuma Mugambi, director of the Kenya Medical Research Institute, for giving us the opportunity to investigate patients attending the Clinical Research Centre, Nairobi. We are grateful to Ms Madeleine Hughes for her invaluable assistance with the electron microscopic studies.

References


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