STUDIES ON THE PANCYTOPENIA OF KALA-AZAR

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It has long been recognized that visceral leishmaniasis (kala-azar) is accompanied by marked changes in the peripheral blood, namely; pronounced leukopenia, anemia and thrombocytopenia. Pathologically, the disease is characterized by a marked hyperplasia of the reticulo-endothelial system, particularly of the spleen, liver, bone marrow and lymph nodes. As a result of this reticulo-endothelial hyperplasia there is a marked enlargement of the spleen, and a moderate enlargement of the liver and lymph nodes. The bone marrow is hyperplastic and infiltrated with reticulo-endothelial cells, many of which are parasitized.

The anemia in kala-azar is moderately severe. Kuroya, Young, Tang and Hong in a study of 151 cases in Kiangsu Province, China, found an average value of 2,930,000 red cells per cu. mm. The lowest count was 700,000. Keefer, Khaw and Yang in a study of 191 cases in Peiping found the red count in the majority of patients to be between 2 and 4 million per cu. mm. Similar values have been obtained by Young in Peiping, Botzaris in Greece, Knowles, Napier and Sharma, Bramachari, and Rogers in India, and Mo in Manchuria. In the majority of patients it has been reported that the hemoglobin is reduced in proportion to the erythrocyte count. Kuroya et al. reported the mean color index in their 150 cases to be 0.9 with a range of 0.63 to 1.0. Similar results were obtained by Mo. Napier and Sharma have reported a mean of 0.885 in 47 Indian cases. Botzaris found the color index to be low in 5 cases, normal in 2 and high in 2.

The size of the red cells in kala-azar has not been studied extensively. Napier and Sharma, using an Eve hemometer, report a mean diameter of 7.359 μ in 22 cases prior to treatment and a mean diameter of 7.375 μ following treatment. Kuroya, Young, Tang and Hong studied the mean cell diameter in 35 cases. The values varied from 7.03 μ to 8.25 μ with an average of 7.81 μ which they considered normal. In 28.6 per cent of the cases the mean diameters were increased, in 17.2 per cent reduced, and in 54.3 per cent normal. The Price-Jones curves showed a spreading of the base and a flattening of the peak. Curves having a tendency to shift to the right were comparatively numerous. The variation in width of the erythrocyte diameters was 5.2. Therefore, there was an abnormal amount of anisocytosis in most of the cases. There are no reports in the literature concerning mean corpuscular volume or mean corpuscular hemoglobin concentration.

Most authors agree that in kala-azar there is a slight reticulocytosis. In a series of 141 cases values less than 1.0 per cent were recorded in 23 cases (16.3 per cent), 1.1 - 3.0 per cent in 39 cases (28.9 per cent), 3.1 - 5.0 per cent in 34 cases (24.1 per

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cent), and above 5.1 per cent in 15 cases (10.6 per cent). The mean value in this
group was 2.8 per cent. In another series of 47 cases\(^9\) the mean reticulocyte per-
centage was reported to be 2.07. Keefer, Khaw and Yang\(^6\) state in reference to
their 191 cases: "The reticulocytes are slightly increased, they are seldom above
four per cent."

Normoblasts in the peripheral blood in kala-azar are not a constant or prominent
feature. Napier and Sharma\(^9\) report that in 47 cases with anemia no nucleated
red blood cells were observed. Kuroya, Young, Tang and Hong\(^4\) found normoblasts
in the peripheral blood in only 26 of 151 cases examined. In 19 of these the normo-
blasts numbered 1 to 2 per 100 white cells, in 6 cases 3 to 6 per 100 white cells,
while in one case the number reached 19. Keefer, Khaw and Yang\(^4\) in a study of
191 cases state: "Signs of blood regeneration are usually lacking when one ex-
amines the peripheral blood. We have never seen nucleated red blood cells . . . ."

Several authors have stated that normoblasts frequently appear in the blood but
not in great numbers.\(^6\) â€“ \(^8\)\(^,\)\(^11\) It must be remembered that iron deficiency, a condition
in which normoblasts appear in the peripheral blood, may have been a complicating factor in some of the patients.

Polychromatophilia, stippling and poikilocytosis are rarely observed in kala-
zar.\(^1\)\(^,\)\(^7\) Polychromatophilia has been reported in 2.8 out of 151 cases but then only
in small numbers.\(^4\) Stippling was seen in only one case. Knowles\(^8\) and Botzaris\(^7\)
reported a moderate degree of poikilocytosis but Kuroya et al.\(^4\) were unable to
confirm this.

The characteristic leukopenia in kala-azar was first described by Rogers in 1904.\(^10\)
He stated that if the proportion of leukocytes to red corpuscles is less than 1:1500,
the patient is probably suffering from kala-azar. Manson-Bahr\(^12\) states that the
reduction in the number of leukocytes is persistent and that usually the proportion
of leukocytes to red blood corpuscles is reduced to 1:2000 â€“ 4000. The leukopenia
has subsequently been confirmed by many authors.\(^6\) â€“ \(^8\)\(^,\)\(^11\) Kuroya, Young, Tang
and Hong\(^4\) in their cases found the total leukocyte count below 1000 in 2.7 per
cent, 1000 â€“ 2000 in 11.9 per cent, 2000 â€“ 3000 in 34.4 per cent, 3000 â€“ 4000 in 19.2
per cent, 4000 â€“ 5000 in 13.2 per cent and in only 8.6 per cent was the leukocyte
count above 5000. The lowest count was 740 and the highest 11,740. The average
leukocyte count was 3052 per cu. mm. This is slightly lower than that reported
by Young,\(^6\) Knowles\(^6\) and Mo.\(^11\)

The leukopenia of kala-azar is due mainly to a neutropenia. Both the percentage
and the absolute number of neutrophilic leukocytes are markedly reduced. There
is a distinct shift to the left as far as the number of juvenile neutrophils is concerned
but in one series of 151 cases myelocytes were seen in only one case.\(^4\) Toxic granules
are very few. The eosinophils generally decrease in numbers or disappear entirely
from the blood. Basophils occur in normal numbers. Plasma cells are occasionally
observed. The percentage of lymphocytes is generally increased to about 50. The
absolute number of lymphocytes is moderately reduced. Several authors have
reported that both the percentage and the absolute number of monocytes show an
increase.\(^6\) â€“ \(^8\) On the other hand, other authors state that the monocytes show an
increased percentage, but the absolute count is normal or less than normal.\(^10\) â€“ \(^12\)
Kuroya, Young, Tang and Hong found that the monocytes were less than 10 per cent in 71 per cent of their cases. In only 8.6 per cent was the monocyte count above 15 per cent. Similar findings have been reported by Botzaris.

From the differential white cell studies reported it can be concluded that the characteristic leukopenia of kala-azar is mainly due to the great reduction of neutrophilic leukocytes, the other classes of leukocytes playing only a minor role. Acute agranulocytosis has been reported to occur in patients with kala-azar.

One of the outstanding clinical features of kala-azar is a hemorrhagic tendency. Epistaxis and bleeding from the mucous membranes are frequent although frank purpura is rare. The hemorrhagic tendency resembles purpura hemorrhagica in that there is a prolonged bleeding time, a normal coagulation time, a nonretractile clot, and in some instances a positive tourniquet test. Darling was the first to comment on the change in blood platelets when he stated: "Judging from films from the peripheral blood and spleen, there is a very marked reduction in the number of platelets." In 1930 Yang and Ch'en confirmed Darling's observation. The platelets in their 23 cases varied from 50,000 to 100,000 per cu. mm. Kuroya, Young, Tang and Hong performed platelet counts on 117 patients. The count was below 40,000 per cu. mm. in 2.3 per cent, between 40,000 and 80,000 in 42 per cent, between 80,000 and 120,000 in 18 per cent, between 120,000 and 160,000 in 11 per cent, and above 160,000 in 6 per cent with an average for all cases studied of 76,763 per cu. mm.

Detailed studies of the bone marrow in kala-azar with differential cell counts have not been reported. Most of the studies are only gross estimates obtained from postmortem specimens stained with hematoxylin-eosin. These studies for the most part have been made on patients in the advanced stage of the disease. Differential cell studies done on thin smears stained with Wright's stain and taken in the early stages of the disease are lacking. Consequently, there is considerable discrepancy in the literature as to the changes in the marrow. Chatterjee, on the basis of the postmortem femoral bone marrow studies of hematoxylin-eosin preparations in 6 patients, divides the marrow into three stages. In the first and earliest stage a differential count gave the following proportion of cells: myeloid, 23 per cent; erythroid, 25 per cent; clasmatocytes, 45 per cent; other cells, 7 per cent. About 30 per cent of the clasmatocytes were parasitized. In the second stage the total number of cells had become fewer and the differential count of one case was as follows: myeloid, 30 per cent; erythroid, 40 per cent; clasmatocytes, 5 per cent; other cells, 25 per cent. Almost all of the clasmatocytes were parasitized. In the third, or more advanced stage, the total number of cells had become even fewer, the cells of the myeloid series being diminished. A differential count from one case was as follows: myeloid cells, 11 per cent; erythroid, 50 per cent; clasmatocytes, 10 per cent; other cells, 29 per cent. Many of the erythropoietic cells, it is stated, were megaloblasts. This finding led the author to state: "The appearance of megaloblasts and a large number of erythroblasts may be associated with the increasing anemia which occurs in this condition and which might ultimately turn into an anemia of the macrocytic type." Piney, on the other hand, states that the erythroid cells are
markedly decreased to less than 8 per cent and that there is a marked increase of the lymphocytes and monocytes in the marrow to about 35 per cent. Meleney\textsuperscript{19} studied the rib marrow in one case of kala-azar in man and reported: "This marrow contains no fat, and approximately 80 per cent of the space is occupied by clasmatoocytes, packed closely together into definite clasmocyte tissue. . . . The normal hematopoiesis is almost entirely absent, especially erythropoiesis. Most of the cells aside from the clasmocytes seem to be lymphocytes. There are a few myelocytes and megakaryocytes. ... Hu\textsuperscript{2}, in a somewhat more extensive, although still not a detailed study of the bone marrow in human kala-azar, reported similar changes. The marrows were hyperplastic and contained many reticulo-endothelial cells. The number of myelocytes was increased, but the metamyelocytes and leukocytes were either greatly reduced in number or entirely absent. The number of normoblasts was moderately increased in a few cases, but in most instances the number was either normal or decreased. Plasma cells were prominent. The megakaryocytes were reduced from 3 to 4 per high power field to an average of about 2 per high power field. Hu and Cash\textsuperscript{21} studied the bone marrow of two patients coming to autopsy and report: "The bone marrow of the femur consisted in a solid mass of firm, dark, grayish red tissue, which microscopically was found to consist mainly of all forms of myeloid cells and nucleated red blood corpuscles. Such a degree of hematopoietic activity is rarely seen other than in cases of pernicious anemia. Many megakaryocytes and a number of large phagocytic cells similar to those seen in the spleen were present."

In hamsters\textsuperscript{22}, squirrels\textsuperscript{24} experimentally infected with kala-azar the bone marrow is hyperplastic due to an increase in the number of reticulo-endothelial cells. Chung and Ch'in\textsuperscript{24} state: "In squirrels infected with kala-azar the bone marrow shows the most marked hyperplasia of the reticulo-endothelial cells, as compared with other organs. About three-fourths or more of the marrow space is occupied by these cells. It is true that all of the blood elements of an active marrow are present. But when one considers the preponderance of the reticulo-endothelial cells, one cannot get away from the impression that the myelogenic and erythro-rogenic elements are actually being crowded out by the reticulo-endothelial cells. In fact, these cells show such an active proliferation that they come to resemble a malignant tumor growth invading or crowding out the marrow tissue."

The pathogenesis of the pancytopenia is poorly understood. Most students of the disease have offered the theory,\textsuperscript{1, 5, 11, 19, 20, 22, 23, 24} and it has been generally accepted, that the hematologic changes are the result of impairment of the hematopoietic function of the bone marrow which is destroyed mechanically by the overgrowth of parasitized reticulo-endothelial cells; that is, myelophthisic anemia. The evidence for this rests solely in the demonstration of numerous reticulo-endothelial cells in the marrow and much of this evidence has been obtained from the animal experiments mentioned above. Napier and Sharma\textsuperscript{9} suggested that the anemia is due to excessive hemolysis. They based their conclusions on the slight reticulocytosis, the presence of an indirect blood Van den Bergh reaction, and the presence of "urobilinuria." It has since been suggested that the urobilinuria
is due to hepatic insufficiency. Mo has pointed out that the erythrocyte fragility is normal. Furthermore, the hemolytic theory does not explain the leukopenia and thrombocytopenia.

It is now recognized that in many conditions in which there is enlargement of the spleen due to hyperplasia of the reticulo-endothelial system there is an accompanying anemia, leukopenia, thrombocytopenia and hyperplasia of the bone marrow. Following splenectomy the cellular elements of the blood and bone marrow rapidly return to normal. These manifestations have been grouped together under the term "hypersplenism." Any one (thrombocytopenic purpura, congenital hemolytic icterus, primary splenic neutropenia) or all (primary splenic pancytopenia) of the cellular elements of the blood may be affected. The pathogenesis of the hematologic changes is in dispute. One group holds that a hormonal relation exists between the spleen and the bone marrow. The other fosters the "phagocytic hypothesis," in which the anemia, leukopenia and thrombocytopenia are thought to be due to increased phagocytic activities of the enlarged spleen. Since in kala-azar there is marked proliferation of the reticulo-endothelial system and consequent splenomegaly, the possibility that the blood changes are due entirely or at least in part, to hyperfunction of the spleen must be seriously considered. Splenectomy has been reported to be beneficial in kala-azar but unfortunately the blood changes were not followed closely and the patients received antimony prior to splenectomy. A careful study of the effect of this procedure alone on the blood has not been reported.

Materials

The case material for this study consisted of 143 patients with kala-azar. Twenty-five of these have been previously reported by one of us (H. L. C.) All patients gave a history characteristic of the disease. None had received specific therapy prior to study. The diagnosis in each case was proved by the demonstration of Leishmania donovani in material from either sternal marrow puncture or splenic puncture. One-hundred and twenty-four cases were uncomplicated.

Twenty-seven patients were studied in detail and this group comprises the main body of this report. All of these except 5 were hospitalized for study. The significant clinical data for this group are presented in table 1. Eighteen of the patients had uncomplicated kala-azar. In general, the majority of these patients were in the intermediate stage of the disease. Follow-up studies after the onset of specific therapy were available in 12 patients.

Methods

Blood Counts. These were performed in the usual manner according to Wintrobe.

Hemoglobin. For these determinations the Sahli hemoglobinometer was used.

Peripheral Blood Smears. Smears were prepared by the cover-glass technic and stained with Wright's stain. Differential white cell counts were performed on 100 cells, and the morphology of the red cells noted.

Peripheral Platelet Counts. The counts were made indirectly by determining the
number of platelets per 1000 red cells in the blood smear. The number of platelets per cu. mm. of blood was then calculated from the red count which was done at the same time. In normal Chinese we found the platelet count to vary between 288,000 and 384,000 with an average of 330,000 per cu. mm.

**Globulin Test.** From an ordinary skin puncture 20 cu. mm. of blood was taken into a hemoglobin pipet and transferred immediately into a clean test tube (7–8 mm. in diameter) containing 0.6 cc. of distilled water. The contents of the tube were mixed by whirling in the hand. The tube was then allowed to stand vertically without further shaking and examined at intervals of 5, 15, 30 and 60 minutes. A haziness at the end of 5 minutes was taken to indicate a positive test. A definite settling of the precipitate within 15 minutes was recorded as 4 plus; definite settling of the precipitate within 15 to 30 minutes as 3 plus; definite settling between 30 and 60 minutes as 2 plus; and a haziness with a fine precipitate but no sediment after one hour as one plus.

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* Measured in cm. below costal margin in the mid-clavicular line.

F—Female; M—Male.

TABLE I.—Clinical Data
Sternal Puncture. This was performed by the introduction of a large lumbar-puncture needle into the marrow space. A small amount of marrow fluid, usually less than 0.3 cc., was withdrawn into a clean dry syringe and thin cover-glass preparations drawn and stained with Wright's stain. The cover-glasses were then fixed to glass slides with neutral Canada balsam. After examining three such preparations the percentage of reticulo-endothelial cells was estimated. Because of the tendency of these cells to clump in the thick sections of the preparations and because of their extreme fragility in the thin sections precise counts were not possible. The percentage of these cells parasitized was also estimated. Differential
counts on the bone marrow cells were done on 500 to 1000 cells, exclusive of reticulo-endothelial cells and megakaryocytes, and the percentage of each accurately determined. The total number of megakaryocytes per million nucleated cells was determined by pasting a 10 x 10 mm. square cut out of paper over each of three cover-glass preparations. This area represented approximately 8000 oil immersion fields. The number of nucleated cells in each area was estimated by accurately enumerating the nucleated cells, including the reticulo-endothelial cells, in three oil immersion fields in each corner and the center. The megakaryocytes in each of the three 10 x 10 mm. squares were counted and the number per million nucleated cells calculated and averaged. To determine the percentage of megakaryocytes forming platelets at least 50 megakaryocytes were counted.

The values for the relative numbers of nucleated cells in the normal bone marrow were taken from Wintrobe. These values agree closely with those found by us in normal marrow. The total number of megakaryocytes per million nucleated cells was determined in 10 normal marrows. The values were found to range from 85 to 200 with an average of 142. The percentage of megakaryocytes forming platelets in normal marrow was found to vary from 55 to 87. The average percentage was 75.

Fig. 3. Showing the relation between the duration of symptoms and the hemoglobin (116 patients) leukocyte count (108 patients) and platelet count (36 patients) in uncomplicated kala-azar.
In attempting to interpret the correlation between the duration of the symptoms and the blood counts (fig. 3), the size of the spleen and the blood counts (fig. 4) and the duration of symptoms and the size of the spleen (fig. 5) certain considerations must be kept in mind. Many patients were unable to remember clearly the exact time of onset of symptoms. The size of the spleen was measured in one diameter only and the size of the spleen in relation to the size of the patient was not considered. Characteristic of kala-azar are remissions and relapses rather than
a gradually progressive course. The curves are plotted from a large body of data. They are shown to represent the general trend. A particular patient might not be expected to fit exactly in this scheme.

**Observations**

**A. PERIPHERAL BLOOD**

1. **Anemia.** The distribution of the hemoglobin values in 134 uncomplicated cases is shown in figure 1. The average value for the entire group was 8.3 grams per cent. The anemia at times was severe. In 33 per cent of the patients the hemoglobin was less than 7 grams per cent. As shown in figure 3 significant anemia (10.6 Gm. per cent) was usually present shortly after the onset of symptoms and thereafter gradually progressive, reaching an average value of about 5 grams per cent in 2.4 months if the disease remained untreated. In patients with kala-azar and an associated bacterial infection the anemia was more severe (average for 19 patients, 7.2 grams per cent). The correlation between the size of the spleen and the degree of anemia is demonstrated in figure 4. By the time the spleen had become palpable there was a significant degree of anemia (11.2 Gm. per cent hemoglobin) and thereafter the anemia increased in proportion to the splenic enlargement.

2. **Red Cell Morphology.** The red cell morphology was studied in 27 cases (table 2). Facilities were not available for determining the mean corpuscular volume. Judging from the appearance of the red cells in the thin smears the anemia was normocytic. The average color index for the group was 1.0. A significant degree of macrocytosis was observed in only two smears and complications were present in both patients. Neither microcytosis nor hypochromia were observed. A moderate amount of anisocytosis was generally present but poikilocytosis of significant degree was observed in only 4 of the uncomplicated cases. Polychromatophilia and stippling were not seen in uncomplicated kala-azar whereas a significant degree of polychromatophilia was seen in 3 of the 9 patients with complications. Nucleated red cells were observed in the peripheral blood in only 5 of the 27 patients and then never in large numbers. Three of the 5 patients with nucleated red cells in the peripheral blood presented complications.

3. **Leukopenia.** As demonstrated in table 2 and figure 2, leukopenia was an almost constant feature of uncomplicated kala-azar. The average white cell count for the group of 134 patients was 2855 per cu. mm. The highest white count observed was 7150. Only 16 of the patients (12.9 per cent) had a white count above 5000 per cu. mm., all of these being early cases. Leukopenia was invariably present in late uncomplicated kala-azar. The leukopenia developed early, about the time of the onset of symptoms, and thereafter became only slightly more severe (fig. 3). The reduction in the white count appears to correspond closely to the degree of splenic enlargement (fig. 4). Leukopenia was usually present at the time the spleen was first palpable.

The white count in the patients with bacterial infections in addition to kala-azar was variable (table 2). Leukopenia was present in 4, a normal leukocyte count in 3 and a leukocytosis in 2. The latter response is demonstrated in figure 6.
Following treatment of the severe noma with penicillin the white count fell from 18,000 to 4000 per cu. mm. and after specific antileishmanial therapy returned to 6200.

Table 2.—Peripheral Blood Studies

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<th>Patient</th>
<th>RBC Million c.mm.</th>
<th>Hemoglobin Gm./100 cc.</th>
<th>W.B.C. per c.mm.</th>
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</table>

Aver. 3.22 9.5 2786 154 0 5.9 32.7 0.2 0.1 32.2 8.8 0.2 N 1+ 0 o 0

* N—Normocytic; M—Macrocytic

4. Differential Leukocyte Count. The most conspicuous change in the differential count was a reduction in the neutrophils (table 2). In uncomplicated cases the neutrophils were reduced to an average of 32.7 per cent. This was a reduction in the absolute number from about 4000 to 910 per cu. mm. A significant 'shift' of the nuclei, either to the left or to the right, was not noted. The proportion of
metamyelocytes was not increased significantly and the absolute number was reduced by less than one-half. A single myelocyte was seen in two smears. Toxic granules in the neutrophils were not evident. Plasma cells were occasionally seen. Although the percentage of lymphocytes was increased to an average of 52.1 per cent, the absolute number of lymphocytes was reduced from the normal of about 2100 to 1450 per cu. mm. The percentage of monocytes was slightly above normal but again the absolute number was reduced (245 per cu. mm. average). Eosinophils were seen in only 3 smears and then not above 2 per cent. Basophils were also rarely seen.

5. Platelets. The platelet count in the majority of the patients was reduced (table 2). In the uncomplicated cases the average platelet count was 103,000 per cu. mm., as compared with the normal average value of 330,000 per cu. mm. obtained by us under the conditions of this study. As seen in figure 3 the thrombocytopenia developed somewhat later than the anemia and leukopenia. Significant thrombocytopenia was generally not present until about two months following the onset of symptoms. After this time the platelet count fell rapidly. As the spleen enlarged the platelet count became progressively lower (fig. 4). A prolongation of the bleeding time and in most cases a positive tourniquet test were associated with the thrombocytopenia, although frank purpura was present in only one of the 143 patients. When complicating infections were present the thrombocytopenia was generally more severe. In one patient (no. 21), critically ill with lobar pneumonia, the platelet count was reduced to 51,000 per cu. mm. and the bleeding time was increased to 30 minutes. Another patient (no. 13), with severe noma, had a platelet count of only 34,000 and a bleeding time of seven minutes. However, in one patient with complicating diphtheria the platelet count was 306,000 per cu. mm. even though symptoms of kala-azar had been present for six months.

B. BONE MARROW

1. Hyperplasia. The bone marrow in kala-azar was consistently hyperplastic. Thin smears and imprints of sternal marrow were exceedingly cellular. Globules of fat were rarely seen. Indeed it was even difficult to obtain a thin smear because of the cellularity of the marrow. Biopsy specimens of the sternal marrow stained with hematoxylin-eosin contained little fat and showed hyperplasia of the reticulo-endothelial tissue as well as blood forming tissue.

2. Reticulo-endothelial Cells. Much of the hyperplasia in the sternal marrow could be accounted for by the increase in reticulo-endothelial cells. These cells, it was estimated, constituted from 3 to 50 per cent of all the cells in the sternal marrow (table 3). In about half the cases less than 1 per cent of the reticuloendothelial cells were parasitized but in one patient (no. 20) as many as 60 per cent were estimated to be parasitized. Even in the marrows with 40 to 50 per cent reticulo-endothelial cells, large numbers of bone marrow cells were present. In no case did there appear to be growth of reticulo-endothelial cells at the expense of the normal blood forming tissue. There was a tendency for the proportion of reticulo-endothelial cells to increase with the duration of the disease but there was a very poor correlation between the proportion of these cells and the severity
of the anemia, leukopenia and thrombocytopenia. Leukopenia appears to precede any great growth of reticulo-endothelial cells in the marrow (patients nos. 4, 11, 21, 26). Photomicrographs of parasitized reticulo-endothelial cells are presented in figure 7.

3. Leukocytes. The most striking alteration of the leukocytes was in the polymorphonuclear neutrophils. These cells were reduced to less than 8 per cent from the normal of about 20 per cent. This reduction appeared to take place early. The

<table>
<thead>
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<th>Table 3. Sternal Marrow Studies</th>
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<td>19</td>
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<tr>
<td>20</td>
</tr>
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Patients with complications:

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<th>Reticulo-endothelial</th>
<th>R.E. Cells</th>
<th>Polymorphs</th>
<th>Neutrophils</th>
<th>Monocytes</th>
<th>Plasma Cells</th>
<th>Protoerythroblasts</th>
<th>Metamyelocytes</th>
<th>Eosinophils</th>
<th>Myelocytes</th>
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average value for the 6 uncomplicated cases of approximately one month's duration was 6.4 per cent whereas the average value for the group was 5.4 per cent. This diminution in the adult neutrophils appears to be the first change to take place in the bone marrow. This coincides with the finding of leukopenia in the peripheral blood early in the course of the disease. The percentage of myeloblasts, promyelocytes, neutrophilic myelocytes and metamyelocytes was not significantly altered in most patients, although the metamyelocytes as well as the adult neutrophils were markedly diminished in the one case of extremely long duration (no. 25). The eosinophilic myelocytes and adult eosinophils were reduced in numbers, especially in the late cases where they were rarely if at all encountered during the examination of 500 to 1000 cells. Basophilic cells were more infrequently seen than normally. Lymphocytes and monocytes were present in normal numbers. The granulocyte-nongranulocyte ratio was reduced from approximately 5:1 to 1.6:1. The proportion of plasma cells was increased from 0.4 per cent to an average of 2.8 per cent.

4. Erythroid Cells. Erythroid cells were numerous in the bone marrow in kala-azar, constituting about 36 per cent (normal, 22 per cent) of all cells, exclusive of
Fig. 7. Showing parasitized reticulo-endothelial cells in the sternal marrow.

Fig. 8. Showing the reduction in the myeloid (leukocyte)-erythroid ratio with progressive enlargement of the spleen.
reticulo-endothelial cells. The majority of nucleated red cells were polychromatic normoblasts. Megaloblasts were not encountered and there was no increase in early forms of nucleated red cells. The leukocyte-erythroid ratio was shifted from the normal of approximately 3.5:1 to an average of 1.8:1 (table 3). This ratio decreased as the size of the spleen increased, as shown in figure 8. The granulocyte-erythroid ratio was diminished from 3:1 to 1.2:1.

Fig. 9. Typical megakaryocytes from the bone marrow in untreated kala-azar. Note the absence of platelet formation around the periphery of the cytoplasm of the cells.

5. Megakaryocytes. The relative number of megakaryocytes in the sternal marrow of the uncomplicated cases varied considerably. The number was less than normal in 6, normal in 11 and slightly greater than normal in one. The number of megakaryocytes per million nucleated cells (including reticulo-endothelial cells) for the entire group of uncomplicated cases averaged 122, as compared with the normal of 142. In view of the fact that the reticulo-endothelial cells were increased in numbers, the ratio of megakaryocytes to nucleated blood forming cells was somewhat higher. The relative number of megakaryocytes in the
patients complicated by other diseases was less than normal in 8 and normal in only one.

Staining abnormalities were noted in many of the adult megakaryocytes. Their cytoplasm was less granular and took on a purplish hue. The nuclei of many of these cells appeared somewhat degenerated. No striking abnormalities were noted in the differential counts of the megakaryocytes although this was not studied in detail. All forms from megakaryoblasts through adult megakaryocytes were present. The latter were more frequently seen than any other form.

Platelet production from the megakaryocytes was found to be markedly reduced. In normal marrow approximately 75 per cent (55 to 87) of the megakaryocytes were found to contain platelets or platelet-like bodies at the peripheries of their cytoplasms, whereas in kala-azar platelet production was obvious in only about 32 per cent and then never to any great extent (fig. 9). In normal marrow as many as 60 or more platelets could be seen attached to the periphery of the cytoplasm of
the megakaryocytes and it was not unusual to find between 10 and 50 (fig. 10).
In the kala-azar marrows there generally were less than 10 platelets surrounding
the relatively few platelet-producing megakaryocytes. In only 4 of the marrows
was platelet production from megakaryocytes normal and all of these were ex-

<table>
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<th>TABLE 4. Blood and Sternal Marrow Studies Following Specific Therapy</th>
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<td>------------------</td>
</tr>
<tr>
<td><strong>Patient</strong></td>
</tr>
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<td>------------------</td>
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</table>
| 6 | 0.11.0 | 2350 | 187 | 7 | 6 | 245.7 | 0.0 | 2.7 | 4.7 | 0.2 | 0.4 | 6.7 | 4.9 | 1.6 | 2.7 | 3.0 | 0.4 | 12.0 | 1.8 | 1.3 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | 1.1 | 1.8 | 19.3 | 0.1 | 0.1 | 0.8 | 2.0 | 5.0 | 6.2 | 4.3 | 19.0 | 8.2 | 1.8 | extremely early cases (nos. 9, 11, 23, 27). Free platelets were scarce in the bone marrow preparations. Large groups of platelets, such as are noted in normal marrow, were almost never seen.

6. Changes in the Sternal Marrow During Therapy. Follow-up studies were available in 12 patients under treatment. These studies are presented in table 4. Due to
existing circumstances a variety of antileishmanial drugs, American, German, British, and Japanese, was used. Some of these were more efficacious than others. The response of 8 of the patients (nos. 4-6, 9, 13-15, 18) was good, in 4 (nos. 7, 8, 16, 20) the response was poor. The changes in the peripheral blood of one patient (no. 18) during treatment are presented in figure 6.

During effective therapy parasitized reticulo-endothelial cells rapidly disappeared and the percentage of reticulo-endothelial cells gradually diminished. There was a "shift" of the leukocytes of the bone marrow to the right. The proportion of neutrophilic myelocytes diminished and the polymorphonuclear neutrophils increased. The metamyelocytes temporarily increased, decreased or remained unchanged. At the same time there was a significant increase in eosinophilic myelocytes and adult eosinophils. Basophilic myelocytes were more frequently seen. Lymphocytes became more numerous. The relative number of plasma cells diminished to normal. Nucleated red cells became less numerous as the leukocyte-erythroid ratio increased to normal. As this change took place the proportion of polychromatic normoblasts to orthochromatic normoblasts decreased from 2.9 to 0.7. These changes are shown graphically in figure 11.

The number of megakaryocytes per million nucleated cells increased during effective therapy. The only exception to this was patient no. 6. In this patient the number of megakaryocytes per million nucleated cells was slightly greater than normal before therapy and the number decreased to within the normal range during therapy.

The percentage of megakaryocytes forming platelets increased to within the normal range in 6 of the 8 patients responding to therapy (table 4). Not only did the percentage of megakaryocytes forming platelets increase but the average number of platelets attached to each megakaryocyte was greatly increased, even beyond the normal. This is illustrated in figure 12. The number of platelets attached to each of 100 megakaryocytes in the marrow of the same patient (no. 6) was counted as accurately as possible before and after treatment and compared with a normal.

Photomicrographs of megakaryocytes taken from the same preparations as used in preparing figure 12 are presented in figures 9, 10, and 13. These photographs again illustrate the absence of platelet formation in untreated kala-azar (fig. 9) and the greatly increased platelet formation in the marrow following treatment (fig. 13) as compared with the normal (fig. 10).

Following the production of platelets by the megakaryocytes, huge groups of platelets were frequently seen in the marrow smears. These were larger and more numerous than those seen in smears made from normal marrow and were in sharp contrast to the very few platelets seen scattered about the marrow in the preparations made from untreated cases. The platelet count in the peripheral blood then approached normal as a rule, although no increase was noted in patients 13 and 15. However, the platelet counts were just at the lower limit of normal and it would be expected that the platelet rise in the peripheral blood would occur later than the bone marrow change.

Two patients (nos. 14, 18), in whom there was no increase in platelet production
STUDIES ON PANCYTOPENIA OF KALA-AZAR

Fig. 11. Bone marrow changes in a patient (no. 14) with kala-azar before and during specific anti-leishmanial therapy (solumebosan).

Fig. 12. Number of platelets attached to the cytoplasm of 100 megakaryocytes in normal marrow, in a patient (no. 16) with kala-azar prior to specific therapy and in the same patient after specific therapy.

in the bone marrow, were both complicated in that severe infections existed. As expected, no rise in the platelets in the peripheral blood was noted.
From table 4 it is evident that the first change which took place in the platelet forming tissue was an increase in the number of megakaryocytes. Following this, platelet production from the megakaryocytes increased and later there was a rise in the number of platelets in the peripheral blood. This took place after there had
been a significant rise in the number of leukocytes and the quantity of hemoglobin in the blood. Thus the three cellular elements of the blood were restored to normal in the same order as their reductions from normal had taken place. The increase in platelets in the peripheral blood was not as dramatically rapid as occurs following splenectomy in primary thrombocytopenic purpura. This is natural since the reduction in the size of the spleen in kala-azar in response to therapy is very gradual over the course of months.

Four patients (nos. 7, 8, 16, 20) responded poorly to therapy (table 4). There was no detectable clinical improvement, no diminution in the size of the spleen, and little or no change in the peripheral blood. Leishmania were present in the bone marrows twenty-nine days after treatment was commenced in one patient (no. 20), after thirty-seven days in another (no. 16) and after seventy days in another (no. 8). Leishmania were found in the spleen in the last named patient on the one hundredth day of therapy. The marrows remained normoblastic and a 'shift' to the right in the myeloid series did not occur. There was, however, an increase in the number of megakaryocytes per million nucleated cells in each case and in 2 of the patients (nos. 7, 20) platelet production by the megakaryocytes had commenced. Three of the patients (nos. 7, 8, 16) were treated initially with a Japanese preparation, solunesbosan. Two of the patients (nos. 16, 20) had complicating infections and 2 (nos. 8, 16) developed agranulocytosis during therapy. All 4 of these patients were heavily parasitized at the onset of therapy and none were in the early stage of the disease. Patient no. 20 was given 50 mg. of pteroyl-glutamic acid orally daily for 10 days without any apparent effect on the leucopenia.

**Discussion**

Differential counts on the sternal marrow from cases of kala-azar have thus revealed a rather characteristic pattern. Myeloblasts, promyelocytes, neutrophilic myelocytes and metamyelocytes were present in approximately normal proportions but polymorphonuclear neutrophils were markedly reduced and eosinophilic cells were rarely seen. In the patient with disease of two years duration, the metamyelocytes were also reduced in number and in one patient with kala-azar and advanced bilateral pulmonary tuberculosis approximately 62 per cent of the myeloid cells were myelocytes or younger forms even though the leucocyte-erythroid ratio was 18:1. This would seem to indicate either an inability of the leucocytes to mature fully or rapid dissolution of the adult cells. Study of the bone marrow has shown, however, that this defect can, under certain circumstances, be overcome since occasionally patients with secondary bacterial infections develop leucocytosis in spite of the existence of severe kala-azar with marked granulocytopenia.

The presence of staining abnormalities, the reduction in total numbers of megakaryocytes in some cases, and the absence of a normal degree of platelet production suggest an abnormality in the development of the megakaryocytes which results in a failure to produce platelets. This offers an explanation for the thrombocytopenia seen in kala-azar. If platelets were being destroyed rapidly in the peripheral blood one would expect to find hypertrophy of the megakaryocytic
tissue in the marrow with each megakaryocyte producing a maximal number of platelets.

Nucleated red cells, especially polychromatophilic normoblasts were numerous in the marrow. It is impossible to say from our data whether the increase was absolute or only relative to a decrease in the leucocytic tissue. One might postulate that since there are many normoblasts in the marrow there is a failure of these cells to make the transition to erythrocytes. Such a situation does exist in the bone marrow in patients with anemia due to iron deficiency and in patients with disturbed hemoglobin synthesis due to lead intoxication. On the other hand, it is also true that in patients with active erythropoiesis, such as occurs in anemia due to hemorrhage or hemolysis, there is an increase in normoblastic activity in the marrow.

None of our patients were jaundiced. Rachmilewitz, Braun and De Vries have reported a single patient with kala-azar, macrocytic anemia, reticulocytosis, normoblastic bone marrow, jaundice and a significantly increased excretion of urobinogen in the urine and feces. They state: "The type of anemia, the hyperplastic bone marrow, the increased excretion of urobinogen before treatment, and the subsequent changes following treatment strongly suggest increased red cell destruction (most probably by phagocytosis) as the cause of the anemia." From our studies we cannot rule out this possibility but it should be noted that patients with chronic liver disease (cirrhosis) and jaundice may have an increased excretion of both urinary and fecal urobinogen in the absence of increased hemolysis. The fact that the patient reported by Rachmilewitz, Braun and De Vries had macrocytic anemia and jaundice suggests that their patient varied somewhat from the usual patient with kala-azar.

As pointed out in the introduction it has been generally accepted that the hematologic changes seen in kala-azar are the result of impairment of the hematopoietic function of the bone marrow which is destroyed mechanically by the overgrowth of parasitized reticulo-endothelial cells; that is, the anemia is classed with the myelophthisic or leuko-erythroblastic anemias. However, the hematologic changes in kala-azar differ in several respects from those seen in patients with carcinoma, osteosclerosis, multiple myeloma, myelosclerosis and marble bone disease. In such examples of so-called "myelophthisic anemia" the most significant abnormality of the erythrocyte series in the peripheral blood is the presence of nucleated red cells in numbers quite out of proportion to the degree of anemia. As many as 53 nucleated red cells per 100 leucocytes have been observed in cases in which there was little anemia. Polychromatophilia and stippling are frequently seen. Myelocytes in the peripheral blood are almost a constant feature and myeloblasts are occasionally seen. The leukocyte count is usually normal, occasionally increased, and only rarely reduced and the differential white cell formula usually maintains its normal proportions. In kala-azar nucleated red cells are rarely seen in the peripheral blood. In our own series of patients they were seen in only 5 patients and then in small numbers. Napier and Sharma and Keefer, Khaw and Yang did not observe nucleated red cells in the peripheral blood of any of their patients even when severe anemia was present.
Kuroya et al.4 recognized normoblasts in only 17 per cent of their cases and then never in very great numbers. Polychromatophilia and stippling are also rarely seen. A single myelocyte was seen in two smears in the series reported here. Kuroya et al.4 observed myelocytes in only one case out of 151. In uncomplicated kala-azar there is almost invariably a leukopenia. In the differential white cell formula in kala-azar the lymphocytes, in contrast to what occurs in myelophthisic anemia, predominate.

Vaughan32, 35 has reported that leuko-erythroblastic anemia (myelophthisic anemia) is associated with an increase rather than a decrease of skeletal red marrow and is not dependent upon mechanical limitation of the marrow. She was able to find little correlation between the degree of mechanical blocking and the degree of anemia. In some of her cases with great degrees of occlusion of the marrow there was little anemia. In the series of patients presented in this study infiltration of the sternal marrow by reticulo-endothelial cells varied from 5 to 50 per cent and there was little correlation between the degree of infiltration and the severity of the blood changes. Leukopenia frequently appeared before there was significant infiltration of the marrow. This has also been observed by others.34, 35 Furthermore, evidence of an extension of the functioning bone marrow in kala-azar has been observed repeatedly.11, 19 In normal adults only approximately half of the marrow is in an active state. Thus, if all of the marrow is made available and if 50 per cent of this is invaded by reticulo-endothelial cells there may still be no reduction from the normal in the total amount of functioning tissue.

The hematologic changes in kala-azar are similar in many respects to those seen in Gaucher’s disease, Still-Chauffard-Felty syndrome, tuberculous splenomegaly and ‘primary splenic pancytopenia.’30 In these so-called ‘hypersplenic states’ there is anemia with little evidence of blood regeneration, neutropenia with a relative lymphocytosis and thrombocytopenia. Hirschboeck37 has reported differential counts of sternal marrow smears in two cases of Still-Chauffard-Felty syndrome. In both cases the marrow was hyperplastic and normoblastic. There was a marked shift to the left of the myeloid elements and the majority of the cells were myelocytes and metamyelocytes. Similar findings have been reported by Doan and Wright25 in 3 cases of ‘splenic pancytopenia.’ In 2 of these cases the myeloid–erythroid ratio was reversed, 1:4. In Banti’s syndrome and in other splenomegalic states Limarzi and his co-workers37 have reported myeloid hyperplasia in the earliest stage and ‘maturation arrest’ of the myeloid and megakaryocyte tissue later. In the last stage of the condition marked erythroid immaturity was found as well.

In kala-azar the reduction in the numbers of erythrocytes, leukocytes and platelets is, in general, proportional to the degree of splenic enlargement.

One apparent point of difference between the bone marrow in kala-azar and that in symptomatic ‘hypersplenic’ thrombocytopenic purpura is that Dameshek and Miller38 in 5 cases of the latter (Gaucher’s disease, infectious splenomegaly, cirrhosis of liver, splenic vein thrombosis, and Felty’s syndrome) found the number of megakaryocytes increased and a normal proportion of platelet-producing cells. Their results in this group of cases differed from their findings in idiopathic
thrombocytopenic purpura (which they consider a form of hypersplenism) in that the proportion of platelet producing cells was markedly reduced in the idiopathic group.*

Although there is, thus, a certain amount of evidence suggesting that the blood changes associated with kala-azar are due to hyperfunction of the spleen, it is clear that no direct evidence has been given in this paper for the hypersplenic theory. Such evidence could come from the demonstration that the anemia, leukopenia and thrombocytopenia are alleviated by splenectomy.† This was considered but for various reasons was not performed. Experiments with splenectomy are now under way in animals experimentally infected with kala-azar and it is anticipated that this will be the subject of another communication.‡

**Summary**

1. The peripheral blood changes in uncomplicated kala-azar are those of pancytopenia; namely, anemia, leukopenia and thrombocytopenia. The red blood cell morphology is normal and there is very little evidence of increased erythrogenic activity. The leukopenia is due to a reduction in all types of cells, especially neutrophils.

2. When the disease is complicated by other infections the anemia is more severe and anisocytosis, poikilocytosis, and polychromatophilia may appear and normoblasts may occasionally be seen in the peripheral blood. Leukocytosis may develop, the leukopenia may persist or the syndrome of agranulocytosis may intervene.

3. As the duration of the disease increases, the spleen tends to become larger and the anemia, leukopenia and thrombocytopenia become progressively more severe. Leukopenia generally appears first, followed by anemia and finally thrombocytopenia. The degree of leukopenia, anemia and thrombocytopenia follow closely the degree of splenic enlargement.

4. The bone marrow in kala-azar is hyperplastic and infiltrated by reticuloendothelial cells. In spite of this there appears to be an abundance of blood forming tissue, especially erythropoietic tissue.

5. Differential cell studies on preparations of sternal marrow reveal a marked

* More recent studies by Dameshek and Estren indicate a very definite diminution in platelet production by megakaryocytes in various types of hypersplenism, both due to known cause (i.e. symptomatic or secondary) and in idiopathic cases, the findings being similar to those in idiopathic thrombocytopenic purpura. Ed.

† Since this paper was submitted for publication a report has appeared by Burchenal, Powers and Haedicke (Am. J. Trop. Med. 27: 699, 1947) of a case of leishmaniasis with severe anemia and leukopenia which was refractory to antileishmanial therapy. Following splenectomy there was a rapid and sustained increase in leukocytes and hemoglobin. Reference is made in this paper to two other case reports of leishmaniasis in the literature in which splenectomy was followed by a rapid rise in leukocytes.

‡ Preliminary results indicate that hamsters experimentally infected with *Leishmania donovani* develop a leukopenia (5,615 cu. mm.) as compared with normal noninfected hamster (10,615 cu. mm.). Two months following splenectomy the leukocyte count had risen to 14,437 cu. mm. in the infected group, whereas there was only a slight increase (11,475 cu. mm.) in the normal noninfected group following splenectomy.
reduction in the polymorphonuclear neutrophils and eosinophils. Myeloblasts, promyelocytes, neutrophilic myelocytes and metamyelocytes are present in approximately normal proportions as are the lymphocytes and monocytes. Plasma cells are somewhat increased. Erythroid cells, especially polychromatic normoblasts are numerous and the leukocyte–erythroid ratio is altered, more than the normal proportion of normoblasts being found. Megakaryocytes are present in normal or slightly reduced numbers. Staining abnormalities are noted in these cells and there is a striking reduction in platelet production.

6. During effective anti-leishmanial therapy parasitized reticulo-endothelial cells disappear and the percentage of reticulo-endothelial cells gradually diminishes as the polymorphonuclear neutrophils increase. There is a significant increase in the eosinophilic cells. Lymphocytes become more numerous and the plasma cells diminish in number. Nucleated red cells become less numerous and the leukocyte–erythroid ratio returns to normal. At the same time the proportion of orthochromatic normoblasts to polychromatic normoblasts increases. The relative number of megakaryocytes increases and platelet formation from the megakaryocytes is accelerated even beyond the normal. Huge groups of platelets are frequently seen in the marrow smears. A rise in platelets in the peripheral blood takes place late, after there has been a significant rise in hemoglobin and leukocytes. The three cellular elements are restored to normal in the peripheral blood in the same order as their reduction from normal.

7. Evidence is presented which contradicts the view that the pancytopenia is due to a crowding out of the bone marrow by reticulo-endothelial cells.

8. Certain similarities between the hematologic changes in this disease and those accompanying the hypersplenic syndromes are noted.

REFERENCES

GEORGE E. CARTWRIGHT, HUI-LAN CHUNG AND AN CHANG

STUDIES ON THE PANCYTOPENIA OF KALA-AZAR

GEORGE E. CARTWRIGHT, HUI-LAN CHUNG and AN CHANG

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