

**Zeitschrift:** Acta Tropica  
**Herausgeber:** Schweizerisches Tropeninstitut (Basel)  
**Band:** 35 (1978)  
**Heft:** 2

**Artikel:** Leukokinetic studies in mediterranean Kala Azar  
**Autor:** Musumeci, S. / D'Agata, A. / Schilirò, G.  
**DOI:** <https://doi.org/10.5169/seals-312380>

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Department of Pediatrics, University of Catania, Italy

## Leukokinetic studies in mediterranean Kala Azar

S. MUSUMECI, A. D'AGATA, G. SCHILIRÒ, A. FISCHER

### Summary

Two patients with acute Kala Azar were studied with  $DF^{32}P$  (diisopropyl-fluorophosphate) and three patients with  $^{51}Cr$  (chromate) in an attempt to delineate the mechanism producing neutropenia in this disease. The granulocyte life span was found to be reduced in all the patients with exception of one who was studied during Glucantim treatment. The surface radioactivity counts showed that the reduced granulocyte life span was due to pooling and probable destruction of granulocytes in the spleen and to a lesser degree in the liver. Bone marrow neutrophil reserve, evaluated by the response to the intravenous hydrocortisone hemisuccinate, was found to be markedly reduced in all patients. An enlarged marginal granulocyte pool indicated also that the neutropenia may be due to altered intravascular granulocyte distribution.

*Key words:* Kala Azar; leukocyte kinetics.

### Introduction

Hematological aspects of acute Kala Azar are characteristically represented by anemia, thrombocytopenia and leukopenia (Cartwright et al., 1948).

Anemia and thrombocytopenia are attributable to splenic sequestration and shortened red cell survival (Knight et al., 1967; Musumeci and Mattina, 1969; Woodruff et al., 1972) and platelet survival (Musumeci et al., 1974). But the mechanism of leukopenia is not completely known. Leukopenia particularly relates to the granulocytes and eosinophils while the number of lymphocytes and of monocytes are relatively increased or normal (Sen Gupta et al., 1970).

Studies of neutrophil kinetics in neutropenia from various causes have suggested that in Kala Azar three basic mechanisms or a combination of these could induce a reduction of the number of neutrophils in blood: 1. failure of production or of release of neutrophils from the storage pool to peripheral

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Correspondence: Dr. S. Musumeci, Department of Pediatrics, University of Catania, Viale A. Doria, 95125 Catania, Italy

blood as a consequence of RES (reticuloendothelial system) hyperplasia and of a depressant action by parasite toxins; 2. alteration of neutrophil distribution between the circulating and the marginal pool so that the marginal pool becomes expanded at the expense of the circulating pool; 3. reduced granulocyte survival in a manner analogous to the mechanism responsible for anemia and thrombocytopenia.

In acute malaria (Dale and Wolff, 1973) only one of these mechanisms has been recognized as responsible for neutropenia (alteration of distribution between marginal and circulating pool) while the granulocyte survival was found to be astonishingly prolonged. In an attempt to delineate the mechanism of the neutropenia in Kala Azar we investigated neutrophil kinetics in five consecutive patients with acute Kala Azar at the Infectious Section of our Pediatric Clinic.

## Materials and methods

### *Hematological data*

The routine hematological tests were performed by the usual methods (Dacie and Lewis, 1968) at the beginning of the study.

Bone marrow samples were obtained by aspiration of sternal (patients 1, 3, 4 and 5) and tibial (patient 2) bone marrow. The smears were stained with May-Grunwald-Giemsa and a differential count made and bone marrow histology examined.

### *Bone marrow neutrophil reserve*

Bone marrow neutrophil reserve was evaluated by a total granulocyte count obtained immediately before and every hour up to 6 h after the intravenous injection of hydrocortisone hemisuccinate (3 mg/kg) in patients 1, 2, 4 and 5.

### *Diisopropylfluorophosphate (DF<sup>32</sup>P) leukokinetic study*

This method was applied in two patients (1 and 5). 150 ml of blood were collected in a siliconized glass vessel containing 20 ml of ACD (formula A) as anticoagulant. Then 50  $\mu$ Ci of DF<sup>32</sup>P (Radiochemical Center, Amersham, specific activity 200–400  $\mu$ Ci/mg) were added to each glass, the blood was gently mixed for 1 h at room temperature and rapidly reinfused over 15 min into the same subjects. Ten ml of blood were kept in heparinized tubes and further 10 ml samples were taken 15 min, 1, 3, 6, 12 and 24 h after reinfusion. Leukocytes were concentrated from the samples by dextran sedimentation and the granulocytes were collected by differential centrifugation for removal of platelets and lymphocytes. Contaminating red blood cells (RBC) were hemolyzed with cold hypotonic solution and saponin, according the original technique described by Athens et al. (1959). From the neutrophil percentage (>90%) of the final preparation and leukocyte specific activity (cpm/mg leukocyte protein, determined by the method of Lowry et al., 1951) of the reinfused blood and the first post-infusion sample, the size of total blood granulocyte pool (TBGP) was calculated, by means of the isotope dilution principle (Athens et al., 1961).

From the other blood samples following infusion the disappearance curve and  $T_{1/2}$  of blood neutrophil specific activity was determined. Circulating granulocyte pool (CGP), marginal granulocyte pool (MGP) and granulocyte turnover rate (GTR) were calculated by the formulae of Athens et al. (1961).

### *<sup>51</sup>Cr leukokinetic study*

Approximately 250 ml of blood from a normal compatible donor with a high percentage of neutrophils at the differential count were collected in siliconized glass containing 40 ml of ACD

(formula A) and the leukocytes isolated after dextran sedimentation of RBC and differential centrifugation of the supernatant plasma to reduce platelets and RBC contamination. Then 50  $\mu$ Ci of  $^{51}\text{Cr}$  (Radiochemical Center of Amersham, specific activity 100–300  $\mu$ Ci/mg) were added to the cell suspension and gently mixed for 30 min at 37° C. The residual  $^{51}\text{Cr}$  chromate was reduced with 50 mg of ascorbic acid and the cells washed three times with homologous plasma-dextran. After the resuspension of the labelled cells in 50 ml of plasma-dextran a sample was taken to determine the leukocyte specific activity (cpm/mg of leukocyte protein) and neutrophil percentage. The remaining labelled cells were infused over 15 min into patients 2, 3 and 4. Five ml of heparinized blood were collected at 5 min, 30 min, 1, 2, 3, 6, 12, 24 h after infusion, and at the same intervals surface counts using a pediatric collimator of 2 inches were performed over precordium, liver, spleen and lung. The curves of surface radioactivity were expressed in organ ratio by the method of Jandl et al. (1956). The granulocytes were isolated from the blood samples by the Ficoll-sodium diatrizoate method of Boyum (1968) and the contaminating erythrocytes were lysed with cold hypotonic solution and saponin. The percentage of neutrophils in the final preparation was >95%. From the specific activity in the periodic blood samples the disappearance curve of injected neutrophils and  $T_{1/2}$  were determined. Other leukokinetic data were calculated according the formula of Athens et al. (1961).

### Clinical features of the patients studied

*Case 1:* A seven-year-old girl, from Catania, was admitted to the Pediatric Clinic two months after the symptoms had begun. She was feverish, pale and asthenic. Her weight was 16.5 kg. The spleen was palpable 6 cm below the left costal margin, while the liver was also enlarged to 3½ cm below right costal margin. Hematological data obtained on admission are shown in Table 1. The bone marrow biopsy (sternal) showed numerous amastigotes of *Leishmania*. Leukokinetic study with  $\text{DF}^{32}\text{P}$  was performed two days before a twenty day course of Glucantim treatment was started. The Glucantim treatment was started increasing doses up to 0.1 g/kg/die on the third day. This 20 day course of treatment was repeated after a pause of 10 days.

*Case 2:* A five-month-old child was admitted about one month after the beginning of fever. His weight was 7.220 kg. He was pale, feverish, but lively. His spleen was palpable 2½ cm below the left costal margin, and liver 2 cm below the right costal margin. Hematological data are reported in Table 1. Tibial bone marrow aspirate showed numerous amastigotes. Also in this case the leukokinetic study with leukocytes, collected from a normal donor and labelled with  $^{51}\text{Cr}$ , was initiated two days before Glucantim treatment was started.

*Case 3:* An eight-month-old baby girl, from Catania, was admitted at the Infectious Disease Section, with a diagnosis of *Salmonella* gastroenteritis. A week after admission a diagnosis of Kala Azar was made on the basis of hematological (see Table 1) and clinical data. Her weight was 5.3 kg. She was feverish, pale and asthenic. On her back there were bed-sores. The liver, soft, was palpable at 4 cm below the right costal margin, and the hard spleen 4 cm below the left costal margin. Tibial bone marrow aspirate showed a good number of amastigotes. In this case the leukokinetic study transfusing leukocytes labelled with  $^{51}\text{Cr}$  from the normal donor was started after the first week of treatment with Glucantim.

*Case 4:* A five-year-old girl, living in Catania, was admitted one month after the beginning of symptoms. Her weight was 21 kg. Clinically she was pale, and continuously feverish. On admission her abdomen was expanded with the umbilical cicatrix protruding and with evident venous reticulum. Her liver was palpable 7 cm below the right costal margin, while her spleen was palpable in the iliac fossa. Hematological data are reported in Table 1. The sternal bone marrow biopsy showed numerous amastigotes collected in groups. The radioisotopic studies with  $^{51}\text{Cr}$  were performed nine days after the beginning of treatment with Glucantim, when blood examination revealed continuous alteration in RBC and WBC count with a relative prevalence of lymphocytes (80%) over neutrophils (20%) and the fever was still irregular and persistent.

*Case 5:* A 15-month-old baby girl, from Syracuse, was admitted 15 days after the beginning of

Table 1. Hematological data in five patients with Kala Azar

Laboratory findings*	Patient				
	1	2	3	4	5
Hb (g/100 ml) . . . . .	7	6.2	6.4	6	7
R.B.C. ( $10^6/\text{mm}^3$ ) . . . . .	3.4	3.6	3.0	2.5	3.4
M.C.H. ( $\mu\text{g}$ ) . . . . .	20.5	19.0	21.3	24	20.5
M.C.H.C. (%) . . . . .	25.9	26.0	25.6	30	25.9
M.C.V. ( $\mu^3$ ) . . . . .	78	80	83.3	80	79.4
Reticulocytes (%) . . . . .	24	10	18	34	32
W.B.C. ( $10^3/\text{mm}^3$ ) . . . . .	3.4	5.6	6.8	5.5	5.3
Polymorphs:					
N. (%) . . . . .	50	16	44	11	25
E. (%) . . . . .	1	—	—	—	—
Lymphocytes (%) . . . . .	46	84	56	86	75
Monocytes (%) . . . . .	3	—	—	3	
Platelets ( $10^4/\text{mm}^3$ ) . . . . .	18	20	16	2.7	17.3
Plasma protein (g/100 ml) . . .	8.13	7.06	10.9	8.8	6.63
Alb./glob. ratio . . . . .	0.58	0.69	0.35	0.3	0.58
Protein fractions:					
$\alpha_1$ . . . . .	8	7	5	6	8
$\alpha_2$ . . . . .	7	10	13	12	5
$\beta$ . . . . .	14	17	13	12	19
$\gamma$ . . . . .	43	26	43	46	39
Serum iron ( $\mu\text{g}/100\text{ ml}$ ) . . . .	52	70	223	90	195

\* Abbreviations according to Dacie and Lewis (1968)

symptoms. She was pale, feverish and asthenic. Her weight was 9 kg. The liver was palpable 4 cm below the right costal margin and the spleen was palpable 5 cm below the left costal margin. The diagnosis was made when amastigotes were found in the tibial bone marrow smears. The leukokinetic studies with  $\text{DF}^{32}\text{P}$  were started two days before the Glucantim treatment.

## Results

Bone marrow smears in all patients showed a hypercellular tissue with erythroid hyperplasia; differential bone marrow count, compared with normal data given by Dacie and Lewis (1968) (Table 2), showed an inverted myeloid-erythroid ratio. The percentage of myeloid precursors (myeloblasts and promyelocytes) was normal or increased, while the percentage of myelocytes was found at a lower limit of the normal. Early form and stab form metamyelocytes and polymorphonuclears were also decreased. The maturation ratio was normal.

Table 2. Bone marrow examination in five patients with Kala Azar. Maturation ratio is calculated by the percentage of metamyelocytes (early and stab forms) to the percentage of myeloblasts, promyelocytes and myelocytes

Cells	Normal*	Patient				
		1	2	3	4	5
Hemohistioblasts . . . . .	0.1–2%	—	—	—	—	—
Hemocytoblasts . . . . .	0.1–1%	—	—	—	—	—
Myeloblasts . . . . .	0.1–3.5%	0.5	1.8	—	—	—
Promyelocytes . . . . .	0.5–5%	4.0	6.0	2.0	2.0	2.0
Myelocytes:						
N. . . . .	5 –20%	5.5	4.6	6.0	4.0	6.0
E. . . . .	0.1–3%	—	0.2	—	—	—
B. . . . .	0 –0.5%	—	—	—	—	—
Metamyelocytes:						
early forms . . . . .	10 –30%	19.0	21.2	8.0	9.0	10.0
stab forms . . . . .	—	—	—	—	—	—
Polymorphs:						
N. . . . .	7 –25%	8.0	5.0	13.0	13.0	13.0
E. . . . .	0.2–3%	—	0.4	—	—	—
B. . . . .	0 –0.5%	—	—	—	—	—
Lymphocytes . . . . .	5 –20%	7.0	18.4	14.0	17.0	13.0
Monocytes . . . . .	0 –0.2%	1.0	0.8	4.0	2.0	1.0
Megakaryocytes . . . . .	0.1–0.5%	1.0	1.0	1.0	—	1.0
Plasma cells . . . . .	0.1–3.5%	—	—	1.0	—	—
Pronormoblasts . . . . .	0.5–5%	4.0	4.0	2.0	5.0	1.0
Normoblasts:						
early and intermediate forms . . . . .	2 –20%	15.0	15.2	19.0	16.0	7.0
late forms . . . . .	2 –10%	35.0	21.4	30.0	32.0	46.0
Myeloid-erythroid ratio . .	2.5–15:1	0.68	0.96	0.92	0.88	0.83
Maturation ratio . . . . .	2.2	2.0	1.68	1.0	1.5	1.25
Interpretation . . . . .		Hypercellular, erythroid hyperplasia				

\* Data from Dacie and Lewis (1968)

The bone marrow reserves as measured by the response to the hydrocortisone hemisuccinate were diminished in all the patients in the acute phase of the disease with a maximum increase in polymorphonuclears of 100, 600, 700 and 300 granulocytes/mm<sup>3</sup> respectively. In the first two patients, several months after recovery the bone marrow reserve responses were normal with a maximum increase of 5,201 granulocytes/mm<sup>3</sup> and 2,734 granulocytes/mm<sup>3</sup> respectively (Fig. 1).

In patients 1 and 5 the leukokinetics with DF<sup>32</sup>P showed a faster disap-



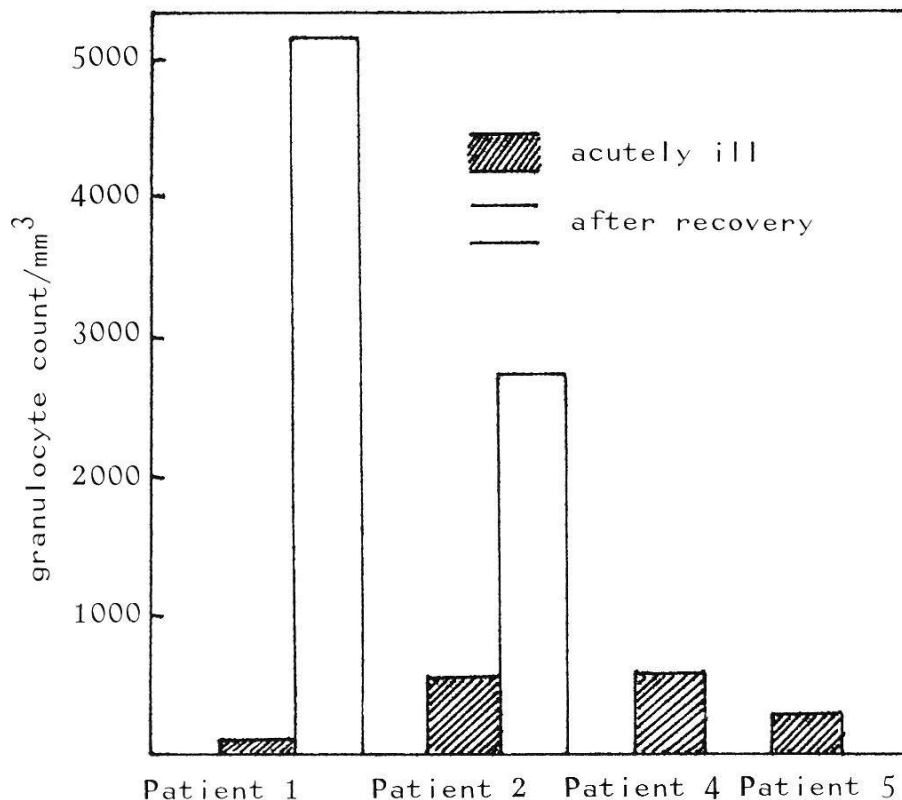


Fig. 1. Bone marrow granulocyte reserves in four patients with Kala Azar. Maximum granulocyte increase from baseline following hydrocortisone hemisuccinate (3 mg/kg) in acute phase of disease and after recovery.

pearance of their own labelled neutrophils from the circulation with a  $T_{1/2}$  of 3 h and 10 min in the first patient and 2 h and 5 min in the fifth (Fig. 2). In patient 1, examined after one year after acute illness, the value of  $T_{1/2}$  was 6 h and 10 min. The total granulocyte pool was normal in both patients; while the distribution of neutrophils between circulating and marginal pool was markedly abnormal. The marginal granulocyte pool was expanded at expense of the circulating granulocyte pool. Also the granulocyte turnover rate was many times greater than normal (Table 3). In patients 2, 3, 4 where leukokinetics were studied by transfusing labelled granulocytes from normal donors, the neutrophil survival was found to be markedly reduced with the exception of patient 3 who was studied after a week of treatment. However, he was not neutropenic ( $2.992 \times 10^9/l$ , Wintrobe mean  $1.83 \times 10^9/l$ ). In patient 4 also studied during Glucantim treatment, the neutrophil survival was found to be shortened. It is notable that in this patient the Glucantim treatment was followed by a slow decrease of fever. The distribution of neutrophils between circulating and marginal pool was also found to be altered in these cases. The surface count showed in the first day a rapid increase of  $^{51}Cr$  radioactivity over the spleen coincidentally with the disappearance of leukocytes from the circulation. A similar increase but of a lesser degree was observed over the liver. The radioactivity over the lung remained unaltered with respect to precordium. The spleen liver ratio clearly showed greater sequestration of neutrophils in the spleen than in the liver. In

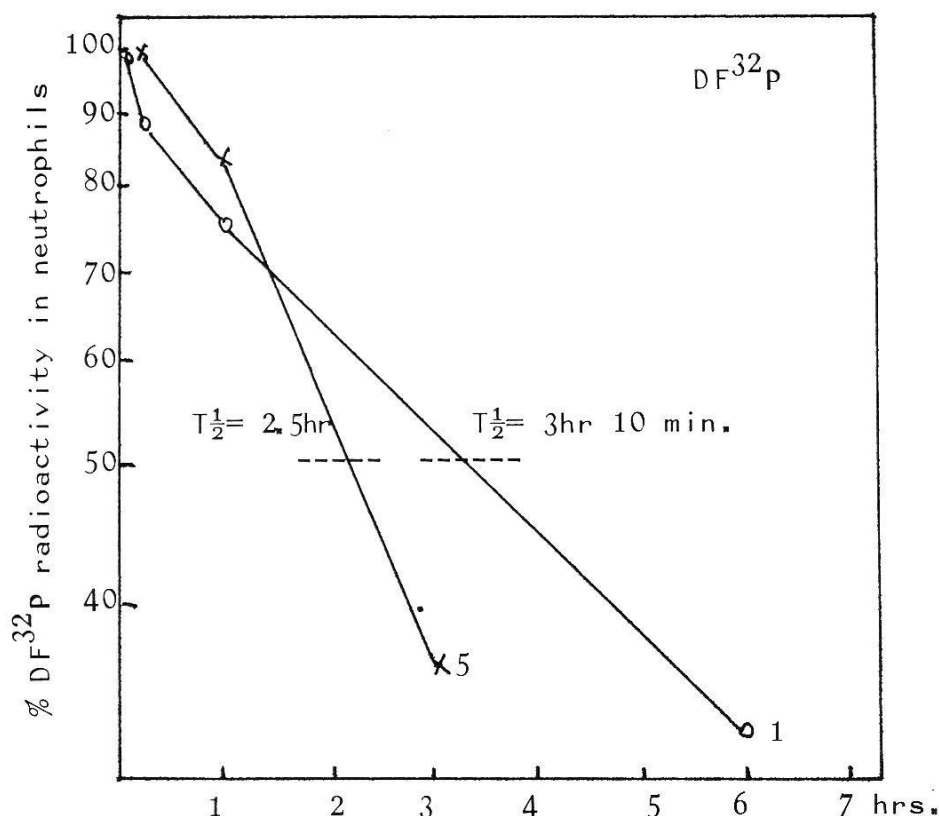


Fig. 2. Neutrophil kinetics with DF<sup>32</sup>P in patients 1 and 5.

patient 2 when the leukokinetics study with <sup>51</sup>Cr was repeated several months after recovery, the neutrophil half disappearance curve had returned to normal ( $T_{1/2}$  10.5 h).

## Discussion

This study shows that the reduced neutrophil survival and the shifting of neutrophils from the circulating pool to an enlarged marginal pool are the principal mechanisms responsible for the reduced neutrophil count seen in children with Kala Azar. The reduction of neutrophil survival is due to an extracorporeal factor; the reduction of neutrophil survival was also observed when neutrophils of normal donors were transfused into the patients. The study of granulocyte function carried out by our group (Schilirò et al., 1976) in another group of patients with Kala Azar has demonstrated that the NBT (Nitroblue-tetrazolium) test showed inactivation as a consequence of protozoal infection while the bactericidal activity, determined as staphylococci killed in 60 min, was normal.

The bone marrow was found to be hypercellular and no sign of myeloid maturation blocking was observed in any of the cases. The altered myeloid/erythroid ratio is in our experience only the consequence of an erythroid hyperplasia due to hyperhemolysis characteristic of Kala Azar (Roccuzzo, 1946; Aversa and Crosca, 1947).



Table 3. Leukokinetic studies: half time disappearance of neutrophils (PMN), total blood granulocyte pool (TBGP), circulating granulocyte pool (CGP), marginal granulocyte pool (MGP) and granulocyte turnover (GTR) in five patients with Kala Azar

Patient	PMN (T½ h)	TBGP	CGP (cells 10 <sup>7</sup> /kg)	MGP	GTR (cells 10 <sup>7</sup> /kg/day)
1 .....	3.16	75.9	9.62	66.28	399.47
2 .....	2.00	67.44	11.46	55.98	560.83
3 .....	7.00	172.50	28.87	146.67	409.86
4 .....	3.00	45.60	6.84	38.76	252.80
5 .....	2.50	99.90	4.53	95.37	664.61
Mean when acutely ill. ....	3.53 ± 1.9	92.26 ± 48.8	11.66 ± 8.3	80.61 ± 42.2	457.51 ± 158.9
Normal .....	6.6 ± 1.64	65.0 ± 22.4	31.7 ± 11.1	33.3 ± 16	179.0 ± 74.3

Normal values derived from Wintrobe (1967)

Reduction of the bone marrow reserve (after hydrocortisone hemisuccinate) was observed in all the patients we studied and it could be explained by the same mechanism responsible for neutropenia: 1. an increased peripheral disappearance of granulocytes as a consequence of infection; or 2. as the bone marrow reserve is measured indirectly by the increase of granulocytes in the circulating compartment (Vogel et al., 1967) and the cells released from the bone marrow are not counted because they immediately disappear into the marginal pool, the reduction may be only apparent. This last hypothesis is sustained by our observation that in all the patients the marginal pool was found markedly enlarged. Further demonstration of an enlarged marginal pool in Kala Azar derives from the measurement of the marginal pool calculated by the response of the leukocyte count to parenteral administration of adrenalin (Cartwright et al., 1964). The adrenalin test was performed only on patient 4 but a former study on 26 patients affected by indian Kala Azar has shown an evident increase of leukocytes and neutrophils after adrenalin administration, which reached a maximum at 15 min and a reduction of spleen size of 3–5 cm (Sen Gupta and Bhattacharyya, 1953).

These results, in accordance with Wintrobe (1967), are sufficient to demonstrate a shift of cells from an enlarged marginal pool to the reduced circulating pool.

The neutrophil kinetic study with <sup>51</sup>Cr showed a marked reduction of granulocyte survival with pooling and probably destruction occurring mostly in the spleen and less in the liver. It could be objected that in all splenomegalies 30–40% uptake represents cells entering the slow circulating pool rather than cell destruction. However, the behaviour of case 2 clearly shows accumulation of <sup>51</sup>Cr after this initial equilibration phase. These results are similar to those

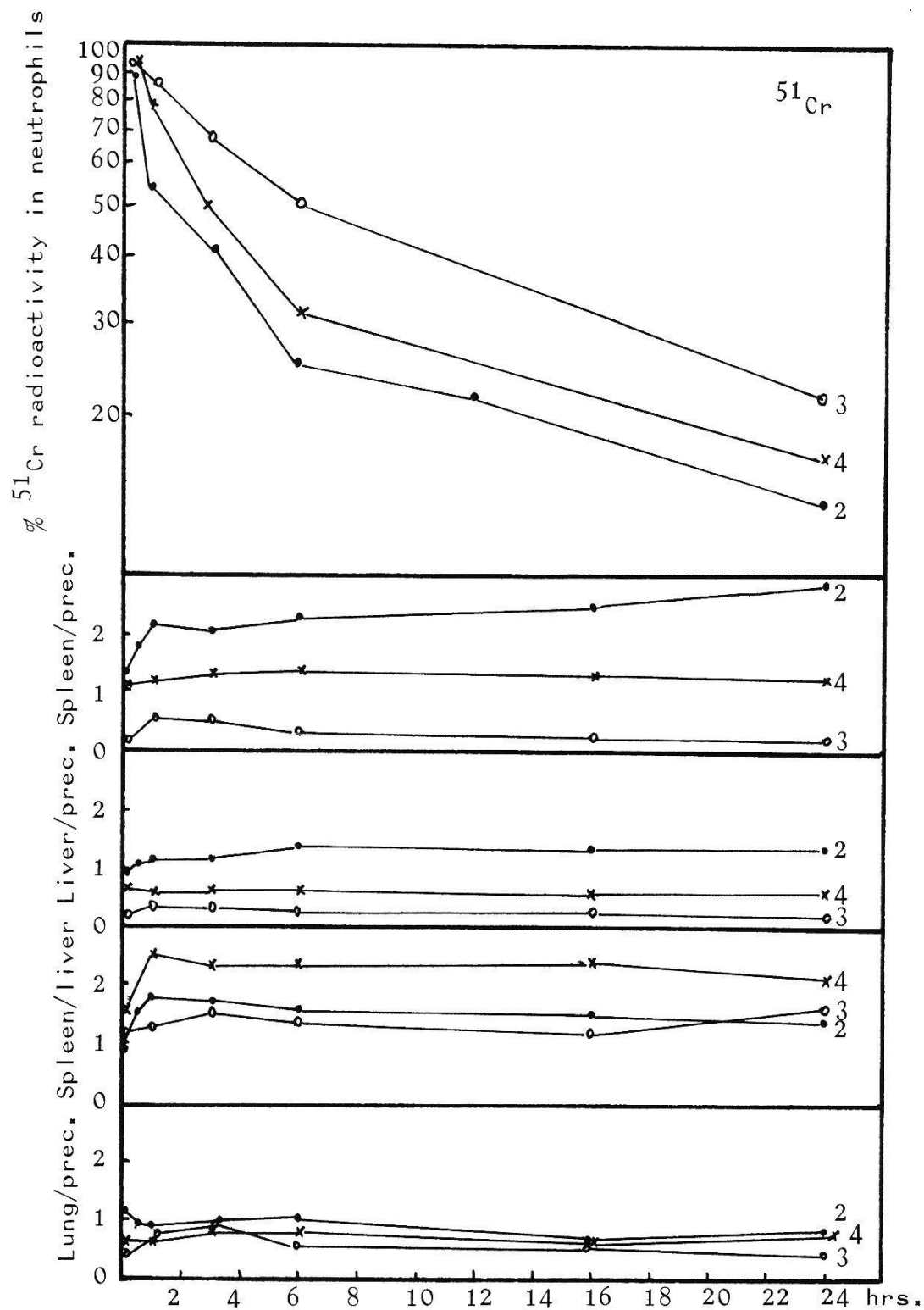


Fig. 3. Disappearance curve of homologous  $^{51}\text{Cr}$  labelled granulocytes in patients 2, 3 and 4. Surface radioactivity curves are expressed as spleen/precordium, liver/precordium, lung/precordium and spleen/liver ratio.

obtained by studying the red cell and platelet survival with  $^{51}\text{Cr}$  (Musumeci and Mattina, 1969; Musumeci et al., 1974).

The role of the spleen in the destruction of neutrophils and in altering the distribution of neutrophils between marginal and circulating pool is not completely clear. However, the hypothesis of an essential role for the spleen in the mechanism of neutropenia is supported by the absence of anemia and leucopenia in a patient affected by Kala Azar who was submitted to splenectomy a year earlier (Woodruff et al., 1972).

Studies of neutrophil kinetics (Bishop et al., 1971) report conditions in which splenomegaly is associated with shortened granulocyte survival and increased marginal pool, suggesting that the leukocytes as well as erythrocytes and platelets may be sequestered and consequently destroyed by the enlarged spleen.

The effect of antimony treatment on neutrophil survival is so rapid that in patient 3, studied after eight days of treatment, the neutrophil survival was quite normal. When the leukokinetic study was made the baby had no fever, the white cell count had become normal as well as the platelet count. This rapid response to antimony was also observed while studying red cell and platelet survival and was attributed to a block of the RES (Musumeci et al., 1974).

In case 4 the neutrophil survival was found to be decreased even though the treatment with Glucantim had been started nine days before. It is notable that when leukokinetic studies were made this patient was feverish and leukopenic and the adrenalin test was not followed by a reduction of spleen volume. The reason for this delayed response to Glucantim is not clear. It should also be noted that after three cycles of antimony treatment this patient still remained leukopenic with dysproteinemia and a spleen palpable  $2\frac{1}{2}$  cm below the umbilical transversal line. The fever, however, had disappeared with the first cycle of treatment.

It is still not established whether it will be necessary to carry out a splenectomy on this patient as the last therapeutic remedy for Kala Azar as recommended by Sen Gupta (1970).

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