Zeitschrift: Acta Tropica
Herausgeber: Schweizerisches Tropeninstitut (Basel)
Band: 37 (1980)
Heft: 4

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DOI: https://doi.org/10.5169/seals-312671

Acta Tropica *37*, 351–365 (1980)

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Hematological and serological aspects of Mediterranean Kala-Azar in infancy and childhood

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Summary

Some hematological aspects of Mediterranean Kala-Azar were studied with radioisotopical methods. The results showed that pancytopenia is due to increased destruction of circulating elements in the spleen, less in the liver, and not to a defect in production. The serological alterations are characterized by an increase of immunoglobulins, and in particular of IgG, IgA and IgM, which is recorded not only in the acute phase of the disease but also many years after recovery. An hypothesis to explain the persistent alterations of serum proteins is suggested.

Key words: Kala-Azar; pancytopenia; serological alterations.

Introduction

Mediterranean Kala-Azar (K. A.) is characterized by irregular fever, liver and spleen enlargement and severe blood alterations. Anemia and neutropenia are always present. Usually anemia is of hypochromic microcytic type but, sometimes, hyperchromic macrocytic anemia can be found (Napier, 1946; Cartwright et al., 1948; Chatterryea and Sen Gupta, 1970). Reduction of platelets completes the picture of the progressive pancytopenia which is typical of K. A. (Musumeci et al., 1976; Bada et al., 1979). Other features of this disease are: serum alterations, characterized by a decrease of albumin and by an increase of globulin components (Gerbasi, 1929), gamma fraction in particular, and by the presence of a slowly migrating fraction called "gamma atypical" (Cooper et al., 1945); increase of immunoglobulins, especially IgM (Silver et al., 1961) and IgG (McKelvey and Fahey, 1965); increase of serum iron due to rise of erythrocyte destruction (Patanè and Musumeci, 1968).

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The purpose of this paper is to report the results of our researches carried out in the last ten years in the Department of Pediatrics of the University of Catania.

Materials and methods

We studied 56 patients (29 males and 27 females), whose age ranged from 6 months to 12 years. The diagnosis of K. A. was confirmed by the finding of *Leishmania donovani* in bone marrow and/or spleen aspirates. For the hematological standard tests we used in all patients the methods reported by Dacie and Lewis (1968); for particular examinations (red cell survival in 10 patients, iron kinetics in 5 patients, neutrophil kinetics in 5 patients, etc.) we employed the methods reported in the original papers, previously published.

All patients were treated with Glucantime i.m. after demonstrating the parasite in bone marrow or in spleen aspirate, by increasing doses up to therapeutic one in the third day of 0.10 g/kg/day, for two cycles of 15 days each. The treatment has never caused relapses, and only seldom in the acute phase of the disease we had to apply other therapeutic agents (blood transfusions, iron).

Results

1. A. *The bone marrow.* The bone marrow aspiration was performed in all patients for diagnostic purpose. It always showed a hypercellular tissue with erythroid hyperplasia and altered leuko-erythroid ratio (<1). No signs of medullar dyserythropoiesis were found in all smears examined. Megacaryocytes were present in normal percentage.

B. *Hemoglobin and erythrocyte count.* The values of hemoglobin and red cell counts in all subjects, studied before the treatment, are reported in Table 1. We divided our patients according to the age but without distinction of sex. Anemia was more marked in the children of 0–3 years of age. We found a significant difference ($p<0.005$) in RBC and Hb levels between the first and the second group, while no significant difference was found between the second and the third one. Red cell morphology was characterized by poikylocytosis of mild

Table 1. Hemoglobin and RBC count values

Range age (years)	Number of cases	Hemoglobin (g/100 ml)		RBC (10^6/mm^3)	
0–3	44	5.9 ± 1.95	$p<0.05$*	2.6 ± 0.65	$p<0.005$*
4–7	10	7.1 ± 1.15		3.2 ± 0.53	
8–12	2	7.5 ± 0.55		3.1 ± 0.76	
Total	56				

* Student's t test

Table 2. Survival of RBC marked with ^{51}Cr

Case Nr.	T½ ^{51}Cr (days)	
	before Glucantime	after Glucantime
1	15	–
2	15.5	26
3	13.2	26.5
4	15	22.5
5*	14.2	28.5
6	15	27
7	16	22.5
8*	11	11
9	21	30
10	22	30
	15.8 ± 3.3	24.9 ± 5.9

* Patients transfused with compatible red cells of normal donor (mother)

degree. Anemia was more evident and precocious in the youngest children and it was usually of the hypochromic type.

C. *Red blood cell survival.* RBC survival labelling erythrocytes with ^{51}Cr was studied in 10 patients. Two patients (nr. 5 and 8) were transfused with labelled compatible red cells of normal donor (mother). RBC survival was found markedly reduced in all patients, before Glucantime treatment but it usually tended to become normal (Table 2) immediately after Glucantime treatment was started (Musumeci, 1971). In Fig. 1 we report the curves of surface radioactivity over spleen and liver. As can be seen, the sequestration and destruction of RBC was found more pronounced in the spleen than in liver.

D. *Hb F and Hb* A_2. In 40 patients the average fetal hemoglobin has been found in the acute phase of disease significantly higher (3.4 ± 2.3%) than in normal controls of the same age (0.9 ± 1.8%) (Schilirò et al., 1980). A relationship was found between circulating Hb and fetal Hb levels. In 2 patients we studied the gamma chain synthesis and found that the ratio gamma-glycine/gamma-alanine was of fetal type. Hb A_2 levels were always within normal range (2.2 ± 0.8%).

E. *Serum iron and iron binding capacity.* Serum iron and iron binding capacity were measured in 30 patients. Serum iron was found increased before the treatment (108.6 ± 51.6 μg%) and the iron binding capacity diminished (250.2 ± 45.7 μg%). After starting treatment, a reduction of serum iron (49.0 ± 15.6 μg%) was observed in all patients. The normalization of this value following treatment paralleles with the normalization of RBC survival and the increase of reticulocytes (Patanè and Musumeci, 1968).

F. *Intestinal iron absorption.* The intestinal iron absorption following a test dose of ferrous sulphate labelled with ^{59}Fe was studied in 6 children. It was

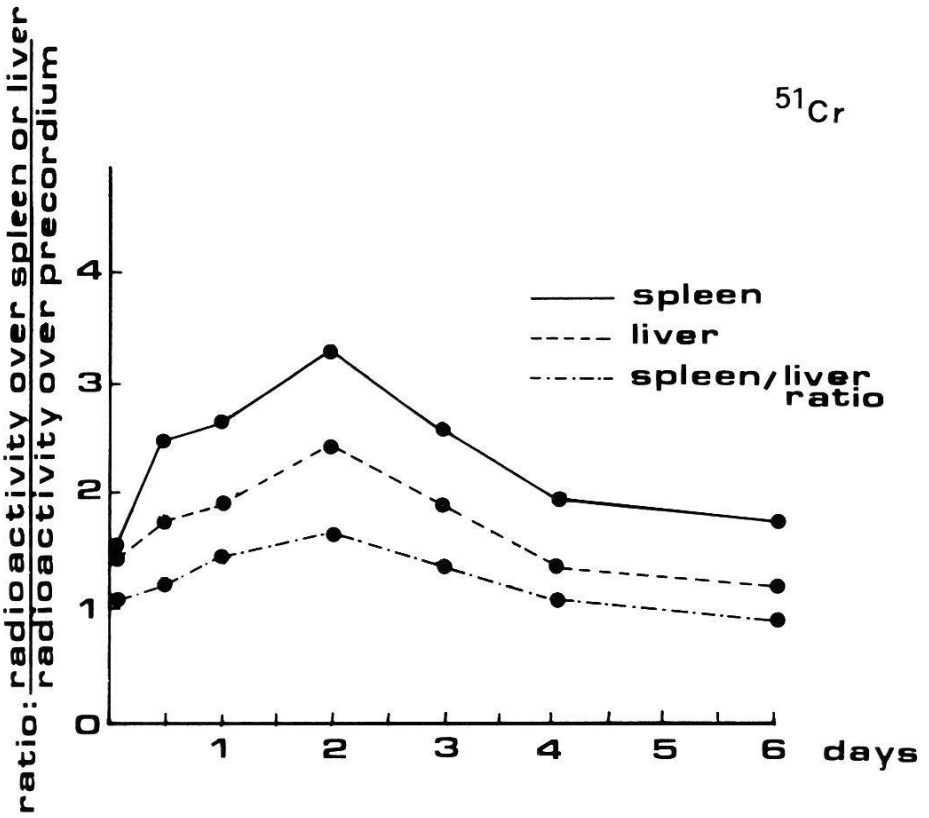

Fig. 1. Surface radioactivity curves over spleen and liver with ^{51}Cr labelled red cells.

Table 3. Iron plasmatic clearance and iron turnover

Case Nr.	^{59}Fe plasmatic clearance T½ min	^{59}Fe RBC incorporated before Glucantime treatment (%)	^{59}Fe RBC incorporated during Glucantime treatment (%)
1	20	76	100
2	22.5	47.5	89.7
3	15	50	46
4	15	70	94
5	19	74	89.1
	18.3 ± 3.3	63.5 ± 13.7	83.8 ± 21.6

found always increased with values ranging between 41 and 77.6% (normal range: 6–12%) (Musumeci et al., 1971).

G. *Iron kinetics.* The study of iron kinetics with ^{59}Fe in 5 patients showed a marked increase of serum iron clearance, while the percentage of ^{59}Fe incorporated by the red cells was low or at low limit of normal (Table 3). The surface radioactivity curves over the sacrum showed in the first hours a progressive increase followed by a decrease when new red cells were introduced in the circulation. The curve of the spleen radioactivity showed a progressive increase before treatment and a reduction soon after, confirming that the spleen is the

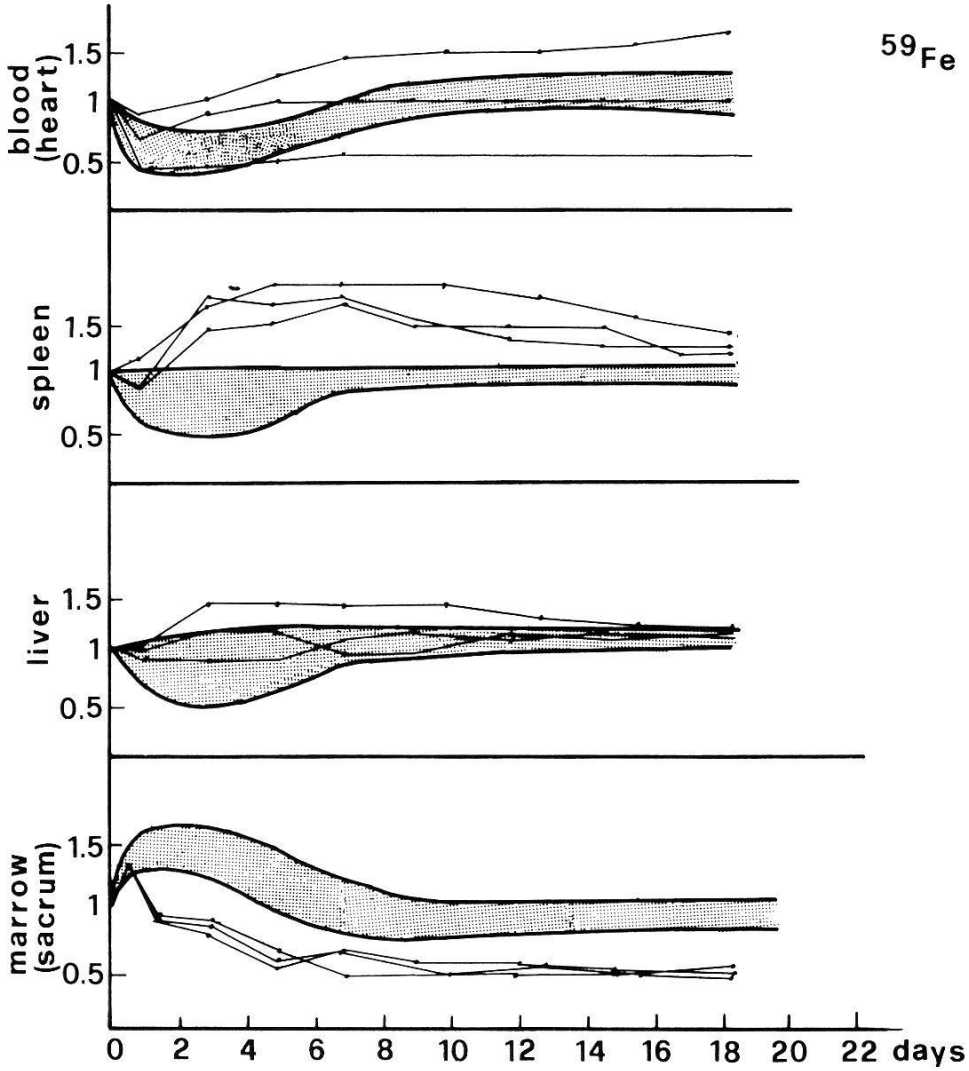

Fig. 2. Surface radioactivity curves over spleen, liver, sacrum and heart with ^{59}Fe.

district where the major RBC destruction and of reticulocytes takes place (Fig. 2). Only in 2 cases was delayed response to Glucantime treatment (Fig. 3). In the first one, RBC survival curves became normal after Glucantime treatment, while the curve of ^{59}Fe uptake remained flat. Only after an oral supplement, when iron serum decreased from 91 μg% to 26 μg%, did we obtain a rapid utilization of ^{59}Fe by reticulocytes. In the second case, both red cell survival and ^{59}Fe uptake, in spite of Glucantime treatment and oral iron supplement, remained unchanged (Musumeci et al., 1974b). Later, we found out that Glucantime was not absorbed just because carelessly it had been injected in two gluteal abscesses developed following injection of other drugs. After draining them, we obtained a complete normalization.

2. A. *Leucocyte count.* Total and differential leucocyte counts are reported in Tables 4 and 5. In all patients there was marked leukopenia without difference between the first and the second age group and between the second and the third one, while the difference was statistically significant between the first and the third ($p < 0.005$). The differential count showed a progressive decrease of lymphocytes and of monocytes according to the age of patients, while the

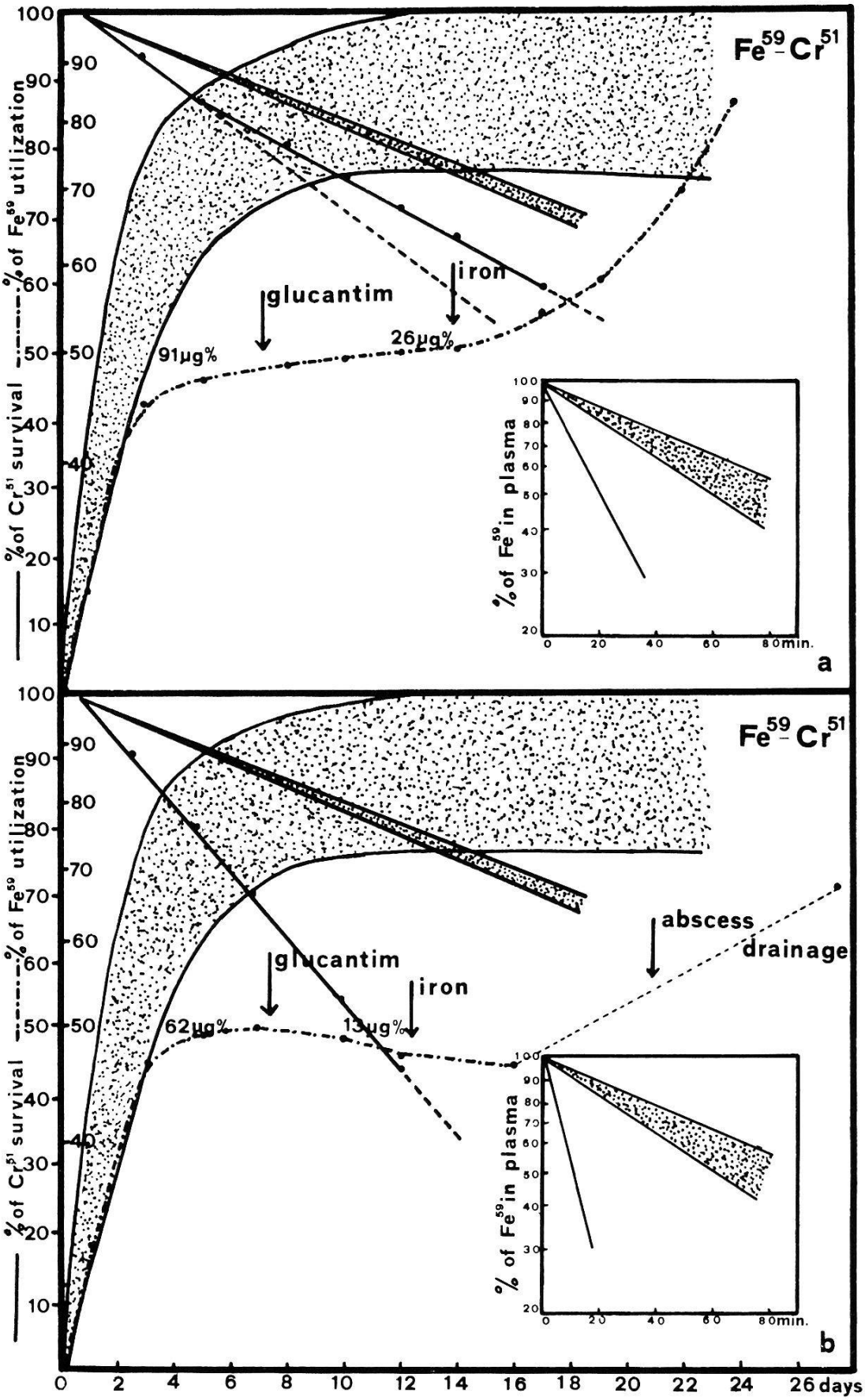

Fig. 3. Iron kinetics with ^{59}Fe and red cell survival with ^{51}Cr in 2 patients.

Table 4. Total leucocyte count

Range age (years)	Number of cases	Leucocyte count ($10^3/mm^3$)	
0–3	44	5.1 ± 2.0	$p < 0.005$*
4–7	10	4.1 ± 2.1	
8–12	2	2.9 ± 1.0	
Total	56		

* Student's t test

percentage of neutrophils remained unchanged. Lymphocytes and monocytes in absolute were significantly different between the first and the third group ($p < 0.025$) and between the second and the third group, respectively ($p < 0.005$). No immature cells were found in peripheral blood stained by the conventional method.

B. *Neutrophil survival.* The study of neutrophil survival with $DF^{32}P$ in 2 patients and with ^{51}Cr in 3 patients (Musumeci et al., 1976, 1978) showed a marked peripheral destruction of neutrophils which appeared more marked in the spleen. The study of the pools showed a marked expansion of the marginal pool at the expense of the circulating one, while the total pool remained unchanged (Table 6). In all patients the neutrophils bone marrow reserve, measured after hydrocortison hemisuccinate injection, was reduced probably owing to an increase of peripheral destruction or to neutrophil immission from bone marrow into the marginal pool.

C. *Nitroblue tetrazolium (NBT) test.* In 9 patients, where the neutrophil function was studied (Schilirò et al., 1977), NBT test and phagocytosis were found normal (Table 7). Also the bactericidal activity of neutrophils was normal.

3. *Platelets and hemostasis.* Platelet count, as well as the platelet survival, resulted lower in 5 patients with acute K. A. (Table 8). Fibrinogen and the coagulation time resulted normal while the clot retraction time, as well as prothrombin time, were found to be higher. In 2 patients, in whom thrombocytopenia was more marked, the survival of the fibrinogen, studied with ^{75}Se-methionine, decreased and fibrinogen level diminished before treatment (Musumeci et al., 1974c).

4. A. *Serum proteins.* In Table 9 we report both the values of the serum proteins, determined by the method of Kingsley-Gornall, and their fractions, evaluated by acetate cellulose electrophoresis, according the patient age. We found a significant difference in the values of serum proteins between the first and the third age group ($p < 0.0025$). Moreover, the difference was significant between the first and the second age group only for $alpha_1$, $alpha_2$, and beta-globulin ($p < 0.00125$). No abnormal gamma-globulin fraction was discovered by the conventional method. Serum lability reaction of Brahmachari (1928) and

Table 5. Leucocyte differential count

Range age (years)	Number of cases	Leucocyte differential count ($10^3/mm^3$)			
		Lymphocytes	Neutrophils	Monocytes	Eosinophils
0–3	44	3.7 ± 1.5	1.1 ± 0.7	0.3 ± 0.2	0.06 ± 0.03
4–7	10	2.9 ± 1.9 $p < 0.03$*	1.0 ± 0.4	0.2 ± 0.1 $p < 0.005$*	0.04 ± 0.02
8–12	2	1.6 ± 0.6	1.2 ± 0.5	0.1 ± 0.0	0.04 ± 0.02
Total	56				

* Student's t test

Table 6. 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)

Case Nr.	PMN ($T_{½}$ h)	TBGP	CGP (cells 10^7/kg)	MGP	GTR (cells 10^7/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 acute ill	3.53 ± 1.9	92.26 ± 48.8	11.66 ± 8.3	80.61 ± 42.2	457.51 ± 158.9
Normal*	6.60 ± 1.64	65.00 ± 22.4	31.70 ± 11.10	33.30 ± 16.00	179.00 ± 74.30

* Normal values derived from Wintrobe (1967)

Table 7. Results of NBT test and bactericidal activity in visceral leishmaniasis

Subjects	NBT score (%)	Bactericidal activity*
1	6	
2	2	
3	8	
4	6	
5	6	
6	8	90–95
7	9	90–95
8	4	90–95
9	5	90–95
Normal controls (mean)	9.1 ± 4.0	90–95

* Percentage of Staphylococci killed at 60 min

Auricchio and Chieffi (1935) resulted positive in the acute phase of the disease. In 20 patients, controlled after the clinical recovery, there were still serum protein alterations, characterized by an increase of gamma-globulins and by inverted A/G ratio, without any relation to the lenght of time from recovery (Musumeci et al., 1977).

B. *Antibodies.* The presence of autoantibodies against RBC was demonstrated in 7 cases of K. A. by an antiglobulin consumption test (Musumeci et al., 1974a (Table 10). The direct Coombs' test was negative in all patients.

C. *Isoagglutinins.* Six patients, studied before and after Glucantime, presented no significant changes, showing that these antibodies are not involved in the hyperglobulinemia of K. A. (Sorge et al., 1972).

D. *Immunoglobulins.* Immunoglobulins were estimated by single radial immunodiffusion with the method of Mancini et al. (1965), using antisera purchased by Behringwerke. IgG in 10 children with K. A. resulted increased in the acute phase of the disease (Brucchieri and Patanè, 1968). IgA increased during illness in 4 cases and IgM in 5 cases. In another group of 8 children (Pavone and Patanè, 1969) the rise of IgA, IgG and IgM was always observed in the acute phase of disease. Several years after clinical recovery, when alterations of serum protein fractions were still evident (Table 11) IgM and IgG were found constantly high (Musumeci et al., 1977).

E. *Lymphocyte subpopulations.* In 8 children with acute K. A., B and T lymphocytes were normal. The N cell counts were significantly lower ($450.66 \pm 303.68 \times 10^6/l$; normal values: $896.00 \pm 482.01 \times 10^6/l$) (Musumeci et al., 1980). The K cell activity was similar ($12.97 \pm 4.22\%$) to that observed in normal controls ($10.35 \pm 4.34\%$).

Table 8. Coagulation studies

Case Nr.	Age in months	Platelets survival		Fibrinogen survival		Plasma prothrombin activity (%)	Coagulation time (min)	Bleeding time (min)	Euglobulin lysis time (h)
		(mm^3)	(days)	(mg%)	(days)				
1	31	200,000	7	375	5.5	60	8	5	4.5
2	21	80,000	5.5	300	1.5	40	11	8	2.5
3	13	60,000	6	339	1.5	30	12	9	2
4	30	226,000	4.5	425	5	70	7	4.5	4.5
5	21	55,000	6	494	5	50	10	10	3.5
Controls									
1	74	300,000	9	350	6	100	5	4	5
2	84	295,000	9.5	400	6.5	100	5	3.5	5

Table 9. Serum proteins and fractions

	Range age (years)			
	0–3	4–7	8–12	Total
Number of cases	44	10	2	56
Proteins (g/100 ml)	6.60 ± 0.74	6.16 ± 1.10	8.13 ± 1.00	
		$p < 0.0025$*		
Protein fractions (%)				
α_1-globulin	8.09 ± 2.25	10.10 ± 4.08	8.02 ± 3.12	
		$p < 0.012$*		
α_2-globulin	13.03 ± 4.90	16.75 ± 5.56	7.80 ± 2.10	
		$p < 0.012$*		
β-globulin	11.66 ± 3.70	9.00 ± 3.16	14.00 ± 1.50	
		$p < 0.012$*		
γ-globulin	30.14 ± 10.20	34.00 ± 13.04	43.00 ± 8.10	

* Student's t test

Table 10. Antiglobulin consumption test on red cells taken from patients with Kala-Azar and from normal controls

Case Nr.	Titres before incubation	Titres after incubation	
		with RBC from normal controls	with RBC from patient with K. A.
1	1/16	1/16	1/4
2	1/256	1/128	1/32
3	1/256	1/128	1/4
4	1/256	1/128	1/32
5	1/256	1/128	1/4
6	1/32	1/32	1/8
7	1/32	1/32	1/4

* The titres of Coombs' serum were determined against Rh + sensitized red cells.

Table 11. Protein electrophoresis and immunoglobulins

	Past Kala-Azar (n = 20)	Controls (n = 30)	Student's t test
Total proteins (g/100 ml)	6.59 ± 0.59	6.59 ± 0.61	
Electrophoresis:			
Albumins (%)	45.90 ± 6.47	59.43 ± 8.72	0.0005
α_1-globulins (%)	5.70 ± 1.38	5.37 ± 2.04	
α_2-globulins (%)	12.95 ± 3.27	8.43 ± 2.06	0.0005
β-globulins (%)	14.05 ± 4.65	10.62 ± 3.20	0.0025
γ-globulins (%)	21.10 ± 5.42	15.80 ± 4.91	0.0005
A/G ratio (%)	0.85 ± 0.21	1.56 ± 0.58	0.0005
Immunodiffusion:			
IgG (mg/100 ml)	1272 ± 346	997 ± 326	0.005
IgM (mg/100 ml)	181 ± 92	101 ± 42	0.0005
IgA (mg/100 ml)	137 ± 48	140 ± 72	

Discussion

Mediterranean K. A., as in other parts of the world, is always characterized by profound hematologic changes. Bone marrow biopsies have always shown an hypercellular tissue with inverted myelo-erythroid ratio (Martelli, 1920; Barberi, 1929; Roccuzzo, 1946). The myeloid precursor and megacariocytes were normal or sometimes increased, suggesting that pancytopenia in K. A. is due to increased destruction of circulating elements in the spleen (Musumeci et al., 1974b, 1976; Bada et al., 1979).

Anemia is more evident and precocious in the youngest children and it is usually of the hypochromic type. Hyperchromic and slightly macrocytic anemia has been observed in India. That is probably due to deficiency of some dietary factors preceding the onset of the disease, which aggravates the anemia.

The poor bone marrow response to the treatment, when observed, is clearly due to iron deficiency, which can precede the disease or be related to an increased uptake of iron by the reticulo-endothelial system. This last mechanism has been recognised by Aksoy et al. (1970) as responsible for anemia, when the serum iron was found at lower level. The results obtained in 2 cases, where we studied iron kinetics, have clearly demonstrated the important role of iron treatment, in order to prevent a bone marrow block at the beginning of the treatment, when the serum iron had become lower because of the disappearance of hemolysis and the increase of circulating reticulocytes.

The study with radioactive isotopes has clearly demonstrated that in K. A. pancytopenia is due to increased peripheral destruction and not to a defect in production. Moreover, the reduced survival of RBC has been attributed to an extracorpuscular factor. In fact, RBC survival was found also reduced when

transfusions of normal labelled RBC were given to patients affected by K. A. (Musumeci, 1971; Musumeci et al., 1974b). However, we can not exclude the importance of the precocious destruction of cells in the bone marrow before entering in the circulation (inefficient erythropoiesis and thrombocytopoiesis).

In our patients the intestinal iron absorption is not reduced in the acute phase of the disease. This is at variance with the results obtained by Das Gupta et al. (1956) in Indian K. A. In that case lacking dietary factors can be involved owing to intestinal mucosa changes. In fact, the coexistence of pernicious anemia with K. A. is frequent in India. As in other infectious diseases, iron binding capacity was found reduced.

There is also the constant presence of leukopenia with decrease of neutrophils in all ages and a relative increase of lymphocytes and monocytes, more marked in the first age group. Agranulocytosis, registred in Indian K. A. (Chatterryea and Sen Gupta, 1970), has never been reported in Mediterranean K. A.

The reduced number of platelets is seldom associated with hemorrhagic manifestations, as well as with alterations of coagulation factors, due to liver dysfunction (Chakrawarty et al., 1949; De Luca and Santangelo, 1961) and to an increased fibrinolytic activity of serum (Musumeci et al., 1974c).

Moreover, the characteristic of K. A. is serum globulin increase, especially gamma-globulins; it has always been recorded, not only in the acute phase of the disease, but also many years after recovery (Most and Lavietes, 1947; Stone et al., 1952; Morris et al., 1961; Benallegue et al., 1970). That is still unclear. Sen Gupta and Bhattacharryea (1953) hold that the persistent spleen congestion, left by the sickness, constitutes a continuous stimulation of the reticulo-endothelial system responsible for the hyperglobulinemic response. An alternative hypothesis could be a reactivation of the immunological response by successive contacts with Leishmaniae. This hypothesis is supported by the frequent positivity of the leishmanin skin test (Pampiglione et al., 1975) observed among house-hold and neighbour-contacts of patients with past K. A., compared to the population in general. However, the weight of evidence concerning the immunity in K. A. is that any new challenge-infection must be dealt in the skin, so that no further endothelial stimulation would take place (Bryceson, 1976).

The incidence of K. A. in the Mediterranean area has undergone a notable decrease because of the use of DDT. In our clinic the mean incidence per year before 1948 was 143 (range: 20–320); after that year it fell to 9 (range: 2–19) (Russo et al., 1975). Although the number of patients with K. A. is variable, no increase has been observed since DDT was replaced by other insecticides. Thus, K. A., even if in decline, is far from being eradicated (Paradiso, 1966) and it still holds our interest in all characteristic aspects.

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