SOME BIOCHEMICAL ASPECTS OF LEUKAEMIAS: THE APPEARANCE OF A SOLUBLE DISULPHIDE IN THE BLOOD IN CHRONIC GRANULOCYTIC LEUKAEMIA

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THERE have been many references in the literature implicating an altered metabolism of protein-free sulphydryl compounds in the leukaemias: Contopoulas and Anderson (1950) claimed that the "leukaemic" leucocyte contains more glutathione than the corresponding cell of healthy people, though this observation was not confirmed by the later work of Hardin *et al.* (1954). White, Mider and Hoston (1943) demonstrated a dependence on dietary cystine for the development of a mouse leukaemia, while Weisberger and Levine (1954) have shown that sulphur amino-acids are metabolised at a faster rate by the leucocytes of patients suffering from granulocytic leukaemia than by the leucocytes of healthy donors (both *in vivo* and *in vitro*). Bearing in mind this increased avidity of "leukaemic" leucocytes for sulphur amino-acids, one of us (K. R. H.) has been studying a model enzyme system designed to lower the level of cysteine in the circulating free amino-acid pool, with a view to exploiting the elevated amino-acid requirement of these cells in chemotherapy (Bergel *et al.*, 1958, 1961, 1962).

In the present study we have been concerned with biochemical factors which may be associated with the treatment-resistant phase in chronic granulocytic leukaemia (CGL). Many patients in the later stages of the disease develop the well-known blast-cell crisis, but it is not certain how far treatment resistance is associated with this transformation. Most of the leukaemic patients which form the subject-matter of this report were treated with the alkylating agent busulphan (1·4 dimethanesulphonyloxy butane); at some stage during treatment a resistance to therapy develops, and this drug is no longer capable of controlling the proliferation of immature leucocytes. It is known that busulphan reacts with sulphydryl compounds both *in vivo and in vitro* (Roberts and Warwick, 1961a, 1961b). Further, the response of certain transplantable tumours in the mouse to treatment with an alkylating agent may be related to the ratio of protein-free thiol to protein-bound thiol (Calcutt and Connors, 1963). We wondered, therefore, whether there might be detectable abnormalities in the levels of protein-free sulphydryl (SH) compounds in the blood of those patients undergoing treatment with busulphan; furthermore, the developing resistance to busulphan might be reflected in changes in the soluble SH levels. We were interested also in the level of protein-free disulphide compounds (SS) in blood: hitherto there has been little emphasis on the ratio of protein-free sulphydryl to protein-free disulphide, yet this ratio is probably a more sensitive indication of metabolic abnormality than gross changes in SH levels. (Tomita (1961) measured soluble sulphydryl-disul-

phide ratios in the blood in three cases of CGL, but did not observe any appreciable deviation from the ratios found in blood samples from healthy volunteer donors.)

MATERIALS AND METHODS

Chemicals were obtained from Hopkin and Williams (Analar or GPR grades), or B.D.H. (Analar or Laboratory Reagent grades). Reduced glutathione was supplied by Sigma Chemical Company.

The amperomatic procedure of Thompson and Martin (1958) was adopted, with slight modification to the composition of the titration mixture, for estimation of sulphydryl concentration. Blood (20 ml.) was taken by cubital venepuncture and transferred to a sealed, carbon monoxide (CO)-filled heparinised bottle at 0° C. (In the case of controls, 50–100 ml. of blood was taken in order to obtain sufficient leucocytes for analysis.) Alternatively, heparinised blood was gassed immediately with carbon monoxide for 5 minutes. For whole blood estimations, 1 ml. of the CO-saturated blood was homogenised with 9 ml. of 3 % sulphosalicylic acid at 0° C. and the resultant mixture allowed to stand at 0° C. for 20 minutes, before removal of the protein precipitate by centrifugation or filtration. The concentration of protein-free sulphydryl compounds (" free-SH ") in the supernatant solution was determined by pipetting aliquots of the latter (usually 0·5 ml.) into a titration mixture (pH 7·3) consisting of : 2·7 ml. of $M/1$ tris hydroxymethyl-amino methane, 2·2 ml. of $N/1$ HNO_3, 0·6 ml. of 0·1 % gelatin, 0·2 ml. of $M/1$ KCl, 1 ml. of 0·1 M ethylene diamine tetracetic acid, 10 ml. of deionised water, and titrating with 10^{-3} M silver nitrate, using the electrode assembly recommended by Thomson and Martin (1958). For the determination of protein-free disulphides, a second aliquot of the deproteinised whole blood was reduced in an Aimer electrolytic desalter, and titrated with 10^{-3} M silver nitrate as described above, yielding the " total–SH " titration. The difference between the " total–SH " and " free SH " titrations was a measure of the amount of disulphide present.

Blood fractionation was carried out by dextran sedimentation at 0° C. according to Skoog and Beck (1956), or by the polybrene method of Lalezari (1962). Erythrocyte contamination in leucocyte preparations obtained by the dextran procedure was reduced by adding saponin to a final concentration of 0·2 % (Kuper, Bignall and Luckock, 1961), standing at room temperature for exactly five minutes, rapidly returning to 0° C. and removing the cells by centrifugation (350 g) at the same temperature. All cell preparations were washed twice with normal saline, resuspended in saline and counted. (In the case of leucocyte-rich plasma prepared by polybrene sedimentation, cells were counted pricr to saline washing.) A suitable aliquot of the suspension, thus obtained, containing approximately 5×10^9 erythrocytes or 5×10^7 leucocytes, was homogenised with 9 volumes of ice-cold 3 % sulphosalicylic acid. Erythrocyte SH levels were determined by adding aliquots (0·2 ml.) of the protein-free supernatant to 16·8 ml. of the titration mixture, and titrating with $0·5 \times 10^{-3}$ M silver nitrate; for leucocytes, 0·5 ml. aliquots were added to 5·0 ml. titration mixture, readjusting the pH to 7·3 with $N/1$ NaOH, and the titration completed with $0·25 \times 10^{-3}$ M silver nitrate.

Any slight contamination of leucocyte preparations with erythrocytes and *vice versa*, was corrected for by the solution of pairs of simultaneous equations. Leucocyte preparations obtained via the dextran-saponin procedure were seldom

contaminated with erythrocytes, while the polybrene technique yielded leucocyte preparations containing an erythrocyte : leucocyte ratio of less than 1.

Reduction of these fractionated cell preparations was by the electrolytic method of Dohan and Woodward (1939) (5 ml. protein-free supernatant ; 25mA for 20 min.).

Two-dimensional paper chromatography was carried out using : tertiary butanol (70 vol.), formic acid (15 vol.), water (15 vol.) as the first phase ; iso-propanol (46·7 vol.), ethanol (23·2 vol.), formic acid (2·5 vol.), and water (27·5 vol.) for the second phase (Gutcho and Lauter, 1954). Cell or plasma prepara-tions were deproteinised with 2 vol. of 3 % sulphosalicylic acid or 8 % trichlor-acetic acid. In the latter case the bulk of the precipitant was removed from the protein-free supernatant by extraction with ether. All samples were concen-trated by evaporation *in vacuo* at 40° C. and desalted electrolytically before mixing with an equal volume of 0·5 M N-ethyl maleimide (Gutcho and Lauter, 1954) (in isopropanol) and applying to the papers.

RESULTS

Whole blood analysis

In Table I are listed the free and total SH levels in the whole blood of eight healthy volunteers, and in Table II the levels in the whole blood of eleven patients together with their haematologic data.

TABLE I.—*Free and Total SH Levels (μmoles/ml.) in Healthy Human Whole Blood*

Subject	Sex	Free SH	Total SH (after reduction)	% SS
R. C. .	. F. .	0·64	. 0·64	. 0
L. W. .	. M. .	1·12	. 1·12	. 0
J. Re .	. M. .	0·56	. 0·56	. 0
J. Ro .	. F. .	0·68	. 0·68	. 0
D. M. .	. M. .	1·24	. 1·20	. 0
J. V. .	. F. .	0·88	. 0·88	. 0
J. P. .	. M. .	1·66	. 1·72	. 4
K. H. .	. M. .	1·08	. 1·14	. 5

Fractionated blood analysis

In an attempt to discover the location of disulphide found in the whole blood of patients (CGL) such as those listed in Table II, soluble sulphydryl and disul-phide determinations were carried out on fractionated blood samples. As a result of these estimations it became apparent that patients (CGL) could be classified into three groups according to the location and concentration of soluble disulphide in the blood fractions. These groups were as follows :

(1) Untreated patients (CGL) on presentation showed the presence of soluble disulphide in the leucocyte fraction (Table IV). The total SH content of the leucocytes was not appreciably different from that of the controls (Table III).

(2) Patients (CGL) undergoing treatment with busulphan or radio-therapy exhibited a considerably lowered total SH level in the leucocytes,

compared with untreated patients in group (1) above. Soluble disulphide was still present however (Table V).

(3) A few patients undergoing treatment with busulphan showed the presence of soluble disulphide in the erythrocytes (Table VI).

In contrast, erythrocyte and leucocyte preparations from eleven healthy volunteer donors were free of soluble disulphide (Table III). In an effort to discover whether this disulphide appeared generally in cases of leucocytic or lymphocytic proliferation, we performed a similar analysis on blood samples of patients with leukocytosis of infective origin, with chronic lymphocytic leukaemia (CLL), and acute granulocytic leukaemia (AGL). Only in the case of AGL was soluble disulphide detected in a cellular fraction (Table VII).

TABLE II.—*Free and Total SH Levels in Whole Blood of Patients (μmoles/ml.)*

Subject	Sex	Free SH	Total SH (after reduction)	% SS	Diagnosis	Total W.B.C. (cells/mm.^{3})	Immature granulocyte count (cells/mm.^{3})	Blast cell count (cells/mm.^{3})	Lymphocyte count (cells/mm.^{3})
W. H.	. M.	0·98	1·08	9	CLL*	8,400	—	—	6,000
I. F.	. M.	1·02	1·12	9	CLL*	10,100	—	—	2,300
L. R.	. F.	1·00	1·04	4	CLL*	11,300	—	—	11,000
C. A.	. M.	1·24	1·32	6	CLL*	200,000	—	—	170,000
J. T.	. M.	0·96	0·96	0	PV*	5,300	—	—	—
W. O.	. M.	0·28	0·52	54	CGL (1)*	27,000	3,000	6,000	—
W. O.	. M.	0·56	0·80	30	CGL (1)*	50,000	3,800	42,000	—
J. B.	. M.	1·20	1·36	12	CGL*	29,600	5,350	—	—
G. J.	. M.	1·40	1·40	0	CGL, R*	6,500	—	—	—
B. C.	. M.	1·70	1·70	0	CGL, R*	12,000	120	—	—
A. C.	. M.	0·56	1·32	58	CGL, (2)*	80,000	20,100	300	—
A. C.	. M.	1·04	1·04	0	CGL (3)*	20,800	3,000	150	—
J. P.	. M.	0·48	2·60	80	CGLT*	5,800	450	1,100	—
J. C.	. F.	0·96	1·16	17	CGL*	38,700	11,600	387	—

* Abbreviations : CLL—chronic lymphocytic leukaemia ; PV—polycythaemia rubra vera ; CGL—chronic granulocytic leukaemia ; R—in remission ; T—terminal ;
(1) Treatment-resistant CGL with high blast-cell count. Observations separated by 2 weeks.
(2) 1 week after commencement of radiotherapy.
(3) at conclusion of radiotherapy.

TABLE III.—*Free and Total SH Levels in Blood Fractions of Healthy Human Donors*

Subject	Sex	Erythrocytes (μMoles SH/10^9 cells)		Leucocytes (μMoles SH/10^9 cells)		Plasma Total SH (μMoles SH/ml.)
		Free SH	Total SH	Free SH	Total SH	
B. C.	. M. .	0·17	0·17	. —	— .	0·30
K. H.	. M. .	0·17	0·17	. —	— .	0·21
R. B.	. M. .	0·18	0·18	. —	— .	0·22
R. C.	. F. .	0·19	0·19	. —	— .	0·18
B. T.	. F. .	0·20	0·20	. —	— .	0·16
R. Y.	. F. .	0·20	0·20	. 1·50	1·50 .	—
D. M.	. M. .	0·18	0·18	. 1·05	1·05 .	—
K. H.	. M. .	0·17	0·17	. 0·73	0·73 .	—
F. B.	. M. .	0·14	0·14	. 1·40	1·40 .	—
R. C.	. F. .	0·22	0·22	. 0·70	0·70 .	—
J. V.	. F. .	0·19	0·19	. 1·20	1·20 .	—
Means		0·18		1·1		0·21

TABLE IV.—*Free and Total SH Levels in Blood Fractions of Untreated (CGL) Patients : SS in Leucocytes (Group 1)*

Patient	Sex	Erythrocytes (μMoles SH/10⁹ cells)		Leucocytes (μMoles SH/10⁹ cells)		% SS	Plasma. Total SH (μMoles SH/ml.)	Total W.B.C. cells/mm³	Immature granulocyte count cells/mm³	Blast cell count cells/mm³
		Free SH	Total SH	Free SH	Total SH					
A. S.	M.	0·32	0·32	0·73	0·90	19	0·32	502,000	203,000	1,000
P. W.	M.	0·31	0·31	1·42	1·63	13	0·39	53,000	14,800	1,590
D. J.	M.	0·22	0·22	0·86	1·25	31	0·26	130,000	44,000	640
M. T.	F.	0·22	0·22	0·28	0·38	26	0·25	320,000	140,000	1,800
J. G.	F.	0·21	0·21	0·07	0·24	71	0·28	180,000	62,000	1,800
Means		0·25		0·67	0·88	32	0·30	—	—	—

TABLE V.—*Free and Total SH Levels in Blood Fractions of Treated (CGL) Patients : SS in Leucocytes (Group 2)*

Patient	Sex	Treat-ment	Erythrocytes (μMoles SH/10⁹ cells)		Leucocytes (μMoles SH/10⁹ cells)		% SS	Plasma Total SH (μMoles SH/ml.)	Total W.B.C. cells/mm³	Immature granulocyte count cells/mm³	Blast cell count cells/mm³
			Free SH	Total SH	Free SH	Total SH					
H. O.	M.	B*	0·17	0·17	0·04	0·10	60	0·21	35,000	6,800	20
D. H.	F.	,,	0·14	0·14	Nil	0·14	100	0·10	8,000	530	—
E. J.	M.	,,	0·25	0·25	0·05	0·16	69	0·19	62,000	12,000	900
A. J.	M.	,,	0·19	0·19	0·03	0·12	75	0·16	14,000	980	60
F. S.	F.	,,	0·33	0·33	0·05	0·14	64	0·26	70,000	20,500	1,400
E. I.	M.	,,	0·17	0·17	Nil	0·05	100	0·21	11,000	330	—
A. S.	M.	,,	0·25	0·25	0·05	0·10	50	0·25	13,000	—	—
A. C. (a)	M.	XRT*	0·27	0·27	0·03	0·06	50	0·21	16,000	—	—
A. C. (b)	M.	,,	0·27	0·27	0·17	0·31	45	0·19	35,000	1,200	210
E. B. (c)	F.	,,	0·27	0·27	Nil	0·29	100	0·21	7,000	—	—
E. B. (d)	F.	,,	0·26	0·26	Nil	0·22	100	0·20	11,000	300	—
Means			0·22		0·04	0·12	71	0·20			

* Abbreviations : B = busulphan ; XRT = X-radiation therapy
(a) In remission ; (b) Measurements taken 10 weeks after (a)—regressing.
(c), (d) Measurements separated by 11 weeks : remission maintained.

TABLE VI.—*Free and Total SH Levels in Blood Fractions of Busulphan-treated (CGL) Patients : SS in Erythrocytes* (Group 3)

| Patient | Sex | Erythrocytes (µMoles SH/10^9 cells) | | | Leucocytes (µMoles SH/10^9 cells) | | | Plasma. Total SH (µMoles SH/ml.) | Total W.B.C. cells/mm.^{3} | Immature granulocyte count cells/mm.^{3} | Blast cell count cells/mm.^{3} |
		Free SH	Total SH	% SS	Free SH	Total SH	% SS				
E. I.	M.	0·19	0·23	18	1·23	1·64	25	0·14	20,000	4,300	1,700
A. J.	M.	0·23	0·59	61	6·50	6·50	0	0·04	13,500	1,100	—
J. C.	F.	0·28	0·42	33	1·30	1·30	0	0·24	38,700	11,600	387

TABLE VII.—*Free and Total SH Levels in Blood Fractions of Patients with Leukocytosis and Leukaemia*

| Patient | Sex | Diagnosis | Erythrocytes (µMoles SH/10^9 cells) | | | Leucocytes (µMoles SH/10^9 cells) | | | Plasma. Total SH (µMoles SH/ml.) | Total W.B.C. (cells/mm.^{3}) | Poly-morphs (cells/mm.^{3}) | Lympho-cytes (cells/mm.^{3}) | Blast cell count cells/mm.^{3} |
			Free SH	Total SH	% SS	Free SH	Total SH	% SS					
J. K.	M.	L	0·17	0·17	0	0·17	0·17	0	0·32	17,300	12,800	—	—
S. M.	M.	„	0·15	0·15	0	1·43	1·43	0	0·27	10,600	5,620	—	—
M. K.	M.	„	0·21	0·21	0	1·32	1·32	0	0·25	14,600	12,400	—	—
R. P.	M.	CLL	0·18	0·18	0	0·20	0·20	0	0·12	7,400	—	3,400	—
I. F.	M.	„	0·31	0·31	0	0·05	0·05	0	0·23	20,000	—	12,000	—
J. P.	M.	AGL	0·20	0·20	48	1·57	3·02	48	0·25	37,000	—	—	32,000

L—leucocytosis ; CLL—chronic lymphocytic leukaemia ; AGL—acute granulocytic leukaemia.

Paper chromatography

By means of two-dimensional paper chromatography, it was impossible to differentiate qualitatively between the sulphur-amino acid pattern of acid-soluble fractions from untreated leukaemic patients and those from healthy donors as controls. Glutathione was the chief sulphur-containing constituent present.

<div align="center">DISCUSSION</div>

We have not discovered any marked difference between the total protein-free sulphydryl levels in the leucocytes of untreated leukaemic patients and those of normal donors. This is in agreement with the observations of Hardin *et al.* (1954), though at variance with those of Contopoulas and Anderson (1950).

A study of the data in Tables I and II indicates that in the peripheral blood of untreated patients suffering from chronic granulocytic leukaemia and the acute blast-cell transformation, an appreciable concentration of soluble disulphide was found ; in contrast, no soluble disulphide was detected in the blood of healthy donors, of patients with CGL in remission, of patients with polycythemia rubra vera, or chronic lymphocytic leukaemia. The occurrence of this disulphide in the blood from a patient with CGL and its disappearance following a course of radiotherapy is strikingly demonstrated in the data for patient A. C. (Table II). The disulphide disappeared when the leukocytic proliferation was controlled.

In the analysis of fractionated blood preparations, no attempt was made to prevent autoxidation of thiols in the plasma fraction (Rony *et al.*, 1964), and in consequence only the total thiol level is reported here.

Patients with CGL could be classified into three groups according to the location and concentration of soluble disulphide in the blood cells. The first group (Table IV) contained untreated patients with high total leucocyte counts (in the region of 50,000 to 500,000 cells per c. mm.) : protein-free disulphide was found in the leucocytes of all these patients (13–71 %), though the total sulphydryl titration was comparable to that of the controls. Thus it would appear that some of the thiol of the leucocyte fraction had become oxidised. Both erythrocyte and plasma titrations were comparable to those of the controls. Where SS determinations were carried out on the whole blood in addition to the isolated fractions, it was found that the disulphide in the leucocytes accounted for no more than approximately 20 % of the total found in the whole blood. Hence, soluble disulphide occurred also in the plasma of these patients.

The second group (Table V) contained those patients undergoing treatment with busulphan (or radiotherapy). It was apparent that the free and total SH titrations on the leucocyte fractions were very much lower (1/7th) than those found in untreated patients. Furthermore, the disulphide was not eliminated as a result of the treatment, there being a general tendency for the per cent of SS present to increase ; disulphide was not detectable in the erythrocytes, and the total SH titration in this fraction, and in the plasma seemed unaffected by the alkylating agent. It appeared, therefore, that one biochemical effect of treatment by means of busulphan or radiotherapy in patients with CGL was to lower selectively the soluble thiol concentration within the leucocytes. In addition, the presence of soluble disulphide in the leucocytes of treated patients (whose immature granulocyte and blast-cell counts were low or zero) may indicate that the leucocyte population could not be considered normal.

The third group was represented by those CGL patients having disulphide in the erythrocytes, accompanied in two cases by an elevated total SH titration (Table VI). Patients A. J., J. C. both died following an acute blast-cell transformation 8 months and 6 weeks respectively, subsequent to the date of the measurements listed in Table VI. E. I. (Table VI) is alive, and clinically very different from the other patients in this group, or in other groups : he has been maintained in remission for 5 years on continuous busulphan therapy. A further unusual feature in this patient has been the loss of a small acrocentric chromosome in all the bone marrow cells. All the marrow cells contain the Philadelphia chromosome, but the skin fibroblasts show a normal male karyotype (Speed and Lawler, 1964). However, shortly after the appearance of disulphide in the erythrocytes of this patient it became necessary to increase the daily dose of busulphan in order to reduce the rising leucocyte count. E. I. was the only patient to exhibit soluble disulphide in both red and white cells at the same time.

It is most unlikely that the appearance of disulphide in the erythrocytes is due to chemotherapy. In a control experiment, a group of rats received a single, maximum-tolerated dose (15 mg./kg.) of busulphan by force-feeding. The animals were examined at regular intervals until the peripheral blood count had returned to normal (approximately 3 weeks) : at no time was there any suggestion of disulphide accumulation in the erythrocytes.

Since there appears to be no unknown sulphydryl or disulphide compound present on chromatograms of blood preparations from leukaemic (CGL) patients, it seems possible that the lesion responsible for the abnormalities reported here may reside with the cellular glutathione reductase. We are examining this possibility, and intend to extend the work reported herein to include acute granulocytic leukaemia and other myeloproliferative disorders.

SUMMARY AND CONCLUSIONS

Studies on the distribution and concentration of non-protein sulphydryl and disulphide compounds in the blood of patients with chronic granulocytic leukaemia has revealed the following points :

1. There is no appreciable difference in the total content of soluble sulphydryl compounds in the blood cells of untreated patients with chronic granulocytic leukaemia compared with the levels in cells from healthy volunteer donors.

2. A soluble disulphide occurs in the whole blood of patients (CGL) undergoing treatment but is absent in remission.

3. Patients (CGL) can be classified into three groups according to the concentration and location of disulphide in the blood cells :

(a) GP. 1—Untreated patients (CGL), having disulphide in the leucocytes.

(b) GP. 2—busulphan or X-ray treated patients : total protein-free sulphydryl content of the leucocytes was approximately 1/7th that of GP. 1. Thus treatment reduced the total soluble sulphydryl content of the leucocytes. Disulphide was still present (50–100 % of total SH).

(c) GP. 3—Disulphide occurred in the erythrocytes before an acute blast-cell transformation in two cases.

4. Paper chromatography of fractions from untreated CGL blood indicated glutathione as the main sulphur-containing constituent present.

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