PROMOTION OF REPLICATION IN LYMPHOID CELLS BY SPECIFIC THIOLS AND DISULFIDES IN VITRO*

Effects on Mouse Lymphoma Cells in Comparison with Splenic Lymphocytes

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(Received for publication 4 May 1973)

Lymphocyte division has been the subject of many studies from the point of view of cellular immunology and antibody synthesis. Blast cell transformation and mitosis occur both in vitro and in vivo under stimulus from a number of agents which act on the cell surface, particularly in experimental situations by lectins (1).

Although the sequence of events which connect the mitogenic stimulus to DNA synthesis is still incompletely known and several different cell types may be involved, a number of preliminary changes have been recognized. Thus, for example, the cyclic AMP level in lymphocyte cultures rises within 2 min of stimulation (2), membrane biosynthesis increases within ½ h of stimulation (3), nuclear template activity increases within 2 h (4). It is of considerable importance to an understanding of the neoplastic change in lymphoid cells to know the extent to which such processes and factors controlling them are retained in replicating lymphoma cells, and the extent to which they are altered.

We here report a comparison, between the effects of certain thiols and disulfides which we have found to be necessary for the growth of mouse lymphoma cells in vitro, and their effects in enhancing the action of mitogens on splenic lymphocytes.

Our observations stem from the finding that mouse lymphoma cells of the line L1210 (V) which had previously been established in culture only with great difficulty (5), proliferate in a medium formulated by Balk (6). The specific growth promoting component of this medium was found to be its high concentration of added L-cysteine (1.5 mM) (7). As will be described, a number of thiols and disulfides can substitute for L-cysteine, some at very low concentration (to < 1 μM). Precise structure activity relationships exist. Furthermore, 13 of 23 other mouse leukemic and neoplastic lymphoid cell lines were also thiol-disulfide dependent in vitro.

* Supported by grant CA-08045 of the National Institutes of Health.

The effect of four agents which promote the growth of L1210 (V) cells has been examined on mitogen-stimulated lymphocytes. All enhanced the uptake of thymidine but there was considerable variation of response in relation to the mitogen preparation used, and under some conditions thiols and disulfides were inhibitory.

We conclude that many lines of mouse lymphoma cells demonstrate with heightened sensitivity a character of normal lymphocytes, the potential for growth promotion by thiols-disulfides: with the lymphoma cells these substances have become obligatory for growth in vitro.

Materials and Methods

Reagents.—Thiols and disulfides were of reagent grade, purchased from Eastman Organic Chemicals Div., Rochester, N. Y. or K and K Laboratories, Plainview, N. Y. Other chemicals, including those used in preparing culture media were obtained from Mann Research Laboratories Inc., New York and Sigma Chemical Co., St. Louis, Mo., as their most highly purified grades. Mitogens were obtained from Difco Laboratories, Detroit, Mich., (Bacto-phytohemagglutinin—*P*), Wellcome Reagents Ltd, Beckenham, England, (phytohemagglutinin, reagent grade and purified), and Pharmacia Fine Chemicals Inc., Piscataway, N. J., (Concanavalin A, homogeneous protein, purified by affinity chromatography). In subsequent descriptions these are designated D-P, BW-R, BW-P, and Con A, respectively.

Culture media.—For initial experiments, Balk's medium was made up with calcium 1 mM, and 10% calf serum (Microbiological Associates Inc., Bethesda, Md.) heated at 56°C for 30 min. In later experiments, amino acids with the composition of Eagle's basal medium (L-cystine 0.05 mM) (8) were substituted for those in the original formulation. RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) with 10% heated calf serum was used for culture of splenic lymphocytes. Dulbecco's medium (9) was prepared from lyophilized powder (Grand Island). Glucose was added to give a concentration of 4.0 g/liter, and horse serum Baltimore Biological Laboratories, Cockeysville, Md.) to 10%. Penicillin (50 U/ml) and streptomycin (50 U/ml) were used in all media. Substances under test for growth promoting activity were added to basic media from sterile-filtered solutions and dispensed into T-flasks which were incubated for 24 h at 37°C with air—5% CO_2 atmosphere, before cell inoculation.

Cells.—Normal spleens and thymuses were obtained from pooled organs of 5–10 mice ($B6D2F_1$) in each experiment. Cells were put into suspension by pressing the organs through a fine stainless steel screen into culture medium. Heavy particles were allowed to settle in a cylinder and the remaining suspension was centrifuged at 1,000 rpm. Two to three washes removed (in the supernatants) all but a few red cells. Counts were made in a hemocytometer after dilution in 0.4% Trypan blue—normal saline.

Lymphoma cells of lines shown in Table IV were carried as ascites tumors except for EG-2 which was obtained from spleen suspension. Ascites cells were washed twice in Ringer's solution before inoculation into cultures. L1210 (V) was originally obtained through the kindness of Dr. Dorris Hutchison of the Sloan-Kettering Institute, New York, and was carried in $B6D2F_1$ mice (Jackson Memorial Laboratories, Bar Harbor, Maine). Numerous other lines were kindly provided by Dr. R. Herberman of the National Cancer Institute. The mouse hemangioendothelioma BW6473 was obtained from Jackson Memorial Laboratories as a subcutaneous tumor of 129/J mice; suspensions were made as described for splenic cells.

3T3. SV101 (an SV40-transformed subline) and other nonlymphoma lines described in the text were grown in Dulbecco's medium with 10% heat-inactivated calf serum using 25 cm^2 flasks (Falcon Plastics Div. of BioQuest, Oxnard, Calif.), counts were made after detaching the cells by treatment with 0.1% trypsin (Difco) in balanced salt solution, for 30–60 min at room temperature.

TABLE I

Tests for Growth Promotion of L1210(V) Cells by Derivatives of Thioethane and Other Sulfur-Containing Substances

R	R_1	R_2	R_3	R_4	Substances tested	Concentration range tested	Maximum and minimum concentrations promoting growth
					(*a*) Derivatives with substitution on carbons R_1 R_3 HS—C—C—H R_2 R_4	*mM*	*mM*
					ethanethiol		0.1 –1.0
	O				thioacetic acid	0.01 –1.0	
	COOH	CH_3			α-mercaptoisobutyric acid	0.01 –1.0	
	COOH		COOH		mercaptosuccinic acid	0.01 –2.0	
	SH		OH	CH_2OH	α-mercaptoglyceraldehyde	0.01 –1.0	
	CH_3	CH_3	NH_2	COOH	DL-penicillamine	0.005 –2.0	
	COOH				2-mercaptopropionic acid	0.01 –1.0	
			CH_3		1-propanethiol		0.1 –1.0
	CH_3		NH_2		2-propanethiol		0.1 –1.0
					cysteamine		0.005 –0.5
			OH		2-mercaptoethanol		0.002 –0.2
			COOH		3-mercaptopropionic acid		0.01 –1.0
			SH		dithioethane	0.01 –1.0	
			O CH_2CH_2SH		β-mercaptoethylether	0.01 –1.0	
			CH_2SH		1,3-propanedithiol	0.01 –1.0	
			$(CH_2)_5CH_3$		1-octanethiol	0.01 –1.0	
			$(CH_2)_{15}CH_3$		*n*-octadecyl mercaptan	0.01 –1.0*	
			NH_2	COOH	L-cysteine		1.5 –3.0
			NH_2	COOH	D-cysteine	1.0 –3.0	
			NH-glutamic	CO-glycine	glutathione	0.5 –3.0	
			O	OH	mercaptoacetic acid	0.001 –1.0	
			OH	CH_2OH	α-thioglycerol		0.0002–1.0
			OH	CHOH CH_2SH	dithiothreitol		2.0 –4.0
			SH	CH_2OH	2,3-dimercaptopropanol	0.002 –2.0	
			SH	CH_3	1,2-propanedithiol	0.01 –1.0	
					(*b*) Derivatives with substitution of thiol H R_1 R_3 RS—C—C—H R_2 R_4		
CH_3			NH_2	COOH	*S*-methyl-D-cysteine	1.0 –3.0	

TABLE I—*Continued*

R	R_1	R_2	R_3	R_4	Substances tested	Concentration range tested	Maximum and minimum concentrations promoting growth
						mM	*mM*
HO_2			NH_2	COOH	cysteine sulfinic acid	0.01 –1.0	
HO_3			NH_2	COOH	cysteic acid	0.01 –1.0	
HO_3			NH_2		taurine	1.0 –5.0	
CH_3			CH NH_2 COOH		L-methionine	1.5 –3.0	
$(CH_2)_2$ OH			OH		thiodiglycol	0.01 –1.0	
CH_2 COOH			O	OH	thiodiglycolic acid	0.01 –1:0	
					(*c*) Ring compounds		
					o-mercaptobenzoic acid	0.01 –1.0	
					2-mercaptopyrimidine	0.01 –1.0	
					2-mercaptopyridine	0.01 –1.0	
					thioproline	0.01 –1.0	
					thioglucose	0.01 –1.0	
					thionicotinamide	0.01 –1.0	
					thiodigalactoside	0.01 –1.0	
					(*d*) Other substances		
					thiourea	0.01 –1.0	
					thioacetamide	0.01 –1.0	
					α-lipoic acid	1×10^{-5}–0.1	
					sodium sulfide	0.0005–0.25	
					insulin	0.0004–0.08‡	

L1210(V) cells from ascites tumors were inoculated (1.5×10^5 cells) into 25 cm^2 plastic flasks containing 3 ml Balk's medium modified to have the amino acid composition of Eagle's basal medium. They were incubated at 37°C with air—5% CO_2. Without a growth promoting supplement, the cell count declined 20–50% in 72 h, and more than half the cells were then permeable to Trypan blue.

* Dilutions of medium saturated with thiol at 37°C.

‡ Soluble insulin, U/ml.

RESULTS

Structural Specificity of Thiols-Disulfides for Growth Promotion of L1210 (V) Cells.—L-Cysteine, the first substance which we found to have growth promoting activity for L1210 (V), can be depicted as a substituted ethanethiol (Table I). Tests soon showed that the simpler substance was even more effective; a variety of compounds related to it were therefore examined. The experimental method was simple; a fixed number of ascites tumor cells were inoculated into medium supplemented with the compound under test. Growth was considered to have taken place if the number of cells after inoculation at least doubled in 72 h.

It will be seen in Table I that only one substance in which there was substitution of hydrogen on the primary carbon, promoted the growth of L1210 (V), and in this case (2-propanethiol) substitution was by a single methyl group. On the other hand, four compounds in which one hydrogen on the secondary carbon was substituted were highly active, some even more so than ethanethiol. The order of increasing effectiveness was substitution with methyl, carboxylic, amino, and alcoholic groups: 2-mercaptoethanol was effective at a concentration as low as 2 μM. By contrast, substitution of ethanethiol with five other groups resulted in ineffective compounds. In particular, a second thiol group appeared inhibitory.

Substances with two substituents on the secondary carbon were sometimes active, and indeed the most powerfully growth promoting agent of all, α-thioglycerol which was effective at a concentration of 0.2 μM belonged to this category. As stated earlier L-cysteine was effective when used in a relatively high concentration (1.5–4.5 mM); the D-isomer was not only inactive, but when mixed in equal amount with the L-form, at total cysteine concentrations of 1.0–3.0 mM, it completely prevented growth. Glutathione (GSH) was also inactive, but it inhibited the growth promoting action of 2-mercaptoethanol (10 μM) only at a concentration at least 50 times greater. The substance with the largest chain length to be effective, dithiothreitol (four carbons) was unusual in that it was a dithiol, and it is the only compound of this kind which we have found to promote growth. Two points should be noted, that the thiol groups were separated by a four carbon chain, and that the agent was only effective at high concentration (2.0–4.0 mM). Because of the discrepancy with earlier observations in which dithiothreitol was thought to be more effective than its isomer dithioerythritol, a commercial preparation was purified by resublimation at 37°C in vacuo (10). The purified product (m.p. 40°C) did not differ in potency from dithioerythritol.

No substance was effective in promoting growth of L1210(V) cells in which the thiol hydrogen was substituted, for instance *S*-methyl-L-cysteine; or the sulfur oxidized, for instance the physiologically important substance, cysteine sulfinic acid. No substance containing an aromatic ring was effective, nor were a number of other sulfur containing substances including α-lipoic acid.

Growth promotion was also demonstrated using the disulfides corresponding to thiols previously shown to be active, as may be seen in Table II. Direct measurements showed that in fact added thiols were rapidly oxidized in solution under conditions of cell culture. Thus no free SH groups were detected in L-cysteine containing medium after 4 h at 37°C, and even with dithiothreitol only 7.5% remained after 24 h (Table III).

TABLE II

Growth Promoting Activity of Thiols Compared with Corresponding Disulfides

Thiol	Effective concentration for growth promotion (as meq/liter thiol)	
	−SH	−S−S
L-cysteine	1.5 −4.5	3.0 −4.5
cysteamine	0.005–0.2	0.1 −0.2
2-mercaptoethanol	0.002–0.2	0.002–0.2
dithiothreitol	2.0 −4.0	2.0 −4.0

Experimental method as in Table I.

TABLE III

Oxidation of Thiols in Tissue Culture Medium (37°C pH 7.2)

Thiol	Half-life	Initial thiol remaining at 24 h
	h	%
L-cysteine	1.6	0.5
cysteamine	2.5	0.5
2-mercaptoethanol	5.9	5.2
dithiothreitol	<8.0*	7.5

Thiols were dissolved (0.5 mM) in Balk's medium with amino acid composition of Eagle's basal medium, but lacking phenol red or serum, and incubated in flat T-flasks. Cysteine was measured by the method of Bydalek and Poldoski (11). Color development was slower with cysteamine and 2-mercaptoethanol; optical density readings were made after standing the reactants at room temperature for 20 and 100 min, respectively. Oxidation of dithiothreitol (4.0 mM) was followed by optical density readings at 283 nm, as described by Cleland (10).

* The rate of oxidation was initially linear, but became more rapid as the concentration of thiol dropped to low levels. This may be related to the catalytic effect of small quantities of L-cysteine in the medium (8, 10).

Since our standard technique was to equilibrate media with compounds under test for 24 h before lymphoma cell inoculation, it is clear that the cells were actually exposed to disulfides. Nonetheless, as can be seen from Table II, added thiol was in some cases more effective than disulfide. Possibly, mixed disulfides formed by combination of thiols with components of the medium had higher growth promoting activity than the simple disulfides.

A few tests have been performed in which cells were inoculated into medium

immediately after the addition of thiol. With 2-mercaptoethanol, growth stimulation was observed at 0.5 μM. This figure is $\frac{1}{4}$ of that required for thiol added 24 h earlier. Cysteamine was active at 5.0 μM when added at both times. By contrast L-cysteine (0.1–3.0 mM) and dithiothreitol (0.1–4.0 mM) added freshly were toxic, and no growth was observed.

Establishment of Permanent Lines of L1210(V).—The experimental system just described measured cell division only in the first 72 h of culture, using a specific basic medium (Balk's). Further tests showed, however, that permanent lines did not become established. Thus, as shown in Fig. 1, approximately five

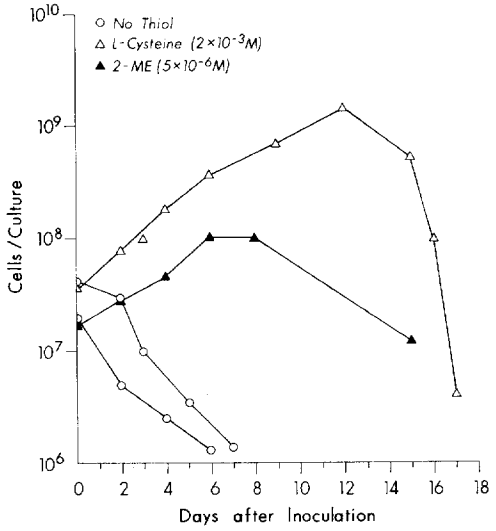


FIG. 1. Growth patterns of L1210 (V) cells in Balk's medium with supplements of L-cysteine and 2-mercaptoethanol. Glass T-flasks contained 20 ml Balk's medium modified to contain amino acids of Eagle's basal medium and 10% calf serum. Thiol-supplements were added and equilibrated for 24 h before inoculation with 1×10^7 L1210 (V) ascites cells. Cell counts were made at 48-h intervals, and where growth had occurred, the cell suspension was partially replaced with fresh medium so that the number of viable cells in each culture was returned to that of the original inoculum.

cell divisions occurred in the first 12 days in medium supplemented with L-cysteine (3 mM) but thereafter the cultures declined, and only about 2% of the cells survived at 17 days. Even fewer divisions (two to three) occurred with 2-mercaptoethanol supplementation, but 12% of cells were viable at 15 days. These were capable of producing ascites tumors after inoculation into mice (1×10^3 living cells). The recipients died after 7–10 days, which is characteristic for the original cell line. The reason for the limited growth potential in vitro is not clear, but it should be noted that the medium we used, compared with others, contained a low level of folic acid and other vitamins (6, 8).

When cells were inoculated into Dulbecco's medium with 10% horse serum and suitable supplements of 2-mercaptoethanol (5–100 μM) growth was observed continuously (Fig. 2). At lower concentrations only a limited number of divisions (3 at 1 μM, 11 at 2.5 μM) occurred, after which the cells died. Similar results were found with cysteamine; cultures grow indefinitely using 10–100 μM; at 5 μM only 12 divisions occurred. The results with L1210(V) cells prompted examination of a variety of other lymphoma cell lines.

Response of Various Mouse Leukemia Cell Lines to Medium Supplemented with Thiol.—Since 2-mercaptoethanol added to Dulbecco's medium proved to

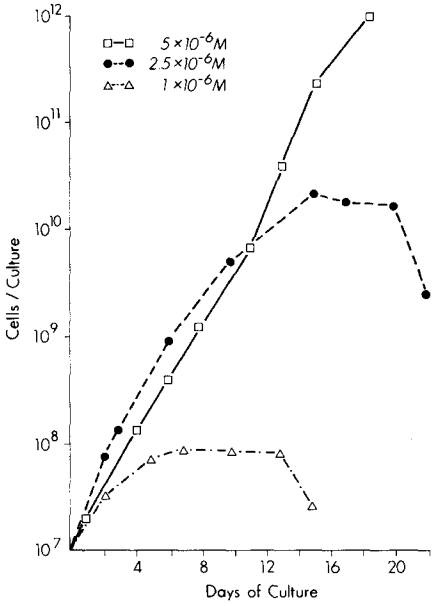


FIG. 2. Growth patterns of L1210 (V) in Dulbecco's medium with supplements of 2-mercaptoethanol. Method as in Fig. 1, but using Dulbecco's—10% horse serum medium.

be particularly effective in the previous experiments, this thiol and medium were chosen for examination of other lymphomas. Cells of different lines showed several kinds of response (Table IV). First, the majority tested (13 of 22) resembled L1210 (V) in that division only occurred and cultures only became established in medium to which thiol had been added. These cell lines were of a number of different types. Some lymphoid cell lines, for instance EL-4 and MCDV-12 possessed the θ-antigen, but others such as L1210 (V) and RBL-5 did not (13).[1] One line (C1498) is reported to have originated as a myeloid leukemia, but neutrophil granules were lost on subsequent passage. One line, LSTRA, has IgG surface receptors, which are found on monocytes, (13).

[1] These observations have been confirmed by Dr. Celso Bianco.

TABLE IV

Effects of 2-Mercaptoethanol Supplementation of Dulbecco's Medium on the Ability of Mouse Tumor Cells to Proliferate in Culture

Culture characteristic	Tumor	Type	Strain of origin	Ref.
Growth only in thiol-supplemented medium	L1210 (V)	Lymphoblastic lymphoma	DBA/2	5
	P1534	Lymphoblastic lymphoma	DBA/2	12
	EL-4	Lymphoblastic lymphoma	C57BL/6	14
	MCDV-12	Lymphoblastic lymphoma	BALB/c	14
	MBL-2	Lymphoblastic lymphoma	C57BL/6	14
	EG-2	Lymphoblastic lymphoma	C57BL/6	14
	CP	Lymphoblastic lymphoma	C3H/HE	15
	RBL/5	Lymphoblastic lymphoma	C57BL/6	14
	LSTRA	?Monocytic leukemia	BALB/c	14
	C1498	?Myeloid leukemia	C57BL	13
Initial growth promotion in thiol-supplemented medium, but cultures later become established in unmodified medium.	6C3HED-S	Lymphoblastic lymphoma	C3H	15
	6C3HED-R	Lymphoblastic lymphoma	C3H	15
	RL-2	Lymphoblastic lymphoma	BALB/c	17
	PU5-1	Lymphoblastic lymphoma	BALB/c	
No growth in thiol-supplemented or unmodified medium	RCS 5	Reticulum cell sarcoma	SJL/J	18
	RCS 19	Reticulum cell sarcoma	SJL/J	18
	5563	Plasmacytoma	C3H	14
	E Gross	Lymphoblastic lymphoma	C57BL	17
	ASL 1	Lymphoblastic lymphoma	A	17
Cultures easily established in unmodified medium	L5178Y	Lymphoblastic lymphoma	DBA/2	19
	BW5147	Lymphoblastic lymphoma	AKR	12, 13
	RADAI	Lymphoblastic lymphoma	A	17
	MOPC 41	Plasmacytoma	BALB/c	14

Duplicate cultures, inoculated with 1×10^7 viable cells, were set up in 20 ml 2-mercaptoethanol-supplemented (5 μM) or unmodified Dulbecco's medium containing 10% horse serum. Cultures were observed daily by phase contrast microscopy and periodic cell counts were also made. In cultures which failed to proliferate no increase in cell count was observed at any time, and by 14 days, viable lymphoma cells were not demonstrable by Trypan blue exclusion.

Secondly, a few lines, notably RL-2 and PU5-1, a line which is unusual in possessing characteristics of B cells, that is, by having surface γ-M globulin and under certain conditions showing C3 receptor sites,[2] grew vigorously after inoculation in medium supplemented with thiol, (Fig. 3 *a*). In its absence the number of living cells at first declined, but subsequently the cultures began to grow. These results probably indicate that the initial inoculum was heterogeneous, so that it contained a small number of thiol-disulfide independent cells, and a majority of dependent cells. Cultures of 6C3HED cells with thiol-disulfide consistently showed the pattern of growth in Fig. 3 *b*. After about three divisions, growth ceased, the population declined, but eventually increased again. At this stage subcultures no longer showed dependence on thiol-disulfide. Evidently, the population whose growth was promoted by thiol-disulfide had only limited growth potential under the existing conditions of culture.

[2] Asofsky, R. Personal communication.

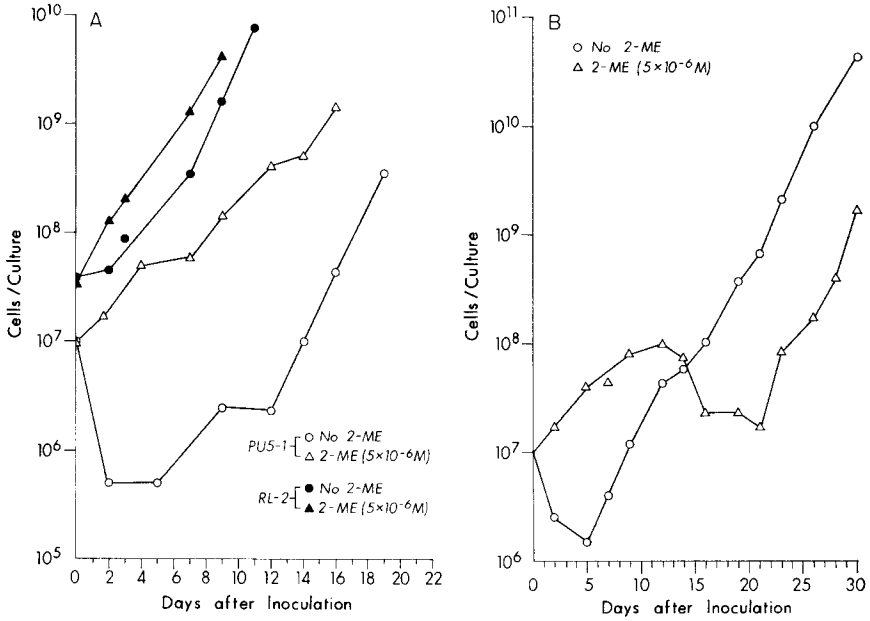


FIG. 3. Effect of 2-mercaptoethanol supplementation of Dulbecco's medium on the growth patterns of (*a*) PU5-1 and RL-2 cells and (*b*) 6C3HED-S. Method as in Fig. 1, but using Dulbecco's—10% horse serum medium.

In contrast to the two patterns of response which have been described, five lymphoma cell lines failed to become established whether thiols-disulfides were present or not. In the case of two of these, however, RCS5 and RCS19, viability, as judged by Trypan blue exclusion, was improved in 2-mercaptoethanol supplemented medium. Thus after 4 days, respectively 49 ± 2% and 45 ± 3% of the number of cells inoculated were viable compared with 21 ± 5% and 9 ± 2% in unsupplemented medium.

The growth patterns of three lymphoma cell lines which have previously been cultured in this laboratory and elsewhere in standard media were not significantly different in medium to which 2-mercaptoethanol had been added. The generation time was 30 ± 2 h for L5178Y in unmodified Dulbecco's medium, and 27 ± 3 h in medium supplemented with 2-mercaptoethanol (5 μM). The corresponding figures for BW5147 were 35 ± 2 h and 32 ± 1 h. To extend these results, it was necessary to determine whether effects of 2-mercaptoethanol at the concentrations now used could be detected on non-neoplastic lymphocytes and other cells.

Effects of Thiols-Disulfides on Lymphocytes and Other Cells.—In a number of preliminary experiments using cultures of mouse splenic lymphocytes, supplementation of media with L-cysteine (1–3 mM) and 2-mercaptoethanol (5–100 μM) failed to cause any increase in cell count or substantial uptake of thymi-

dine. On the other hand, in media to which 2-mercaptoethanol had been added there was a considerable augmentation of thymidine incorporation by mitogen-stimulated lymphocytes. Related observations have been made in lymphocyte cultures of other species by Fanger et al. (20).

In our experiments, lymphocytes failed to take up more than traces of thymidine after the first 24 h of culture unless mitogen was present. The maximum thymidine uptake of cells stimulated by mitogen alone (column 1 of Fig. 4) occurred in the 48–72 h period, and subsequently declined. Compared with these results, an eightfold increase in thymidine incorporation was brought about in the 24–48 h period when thiol had been added at 100 μM (column 3)

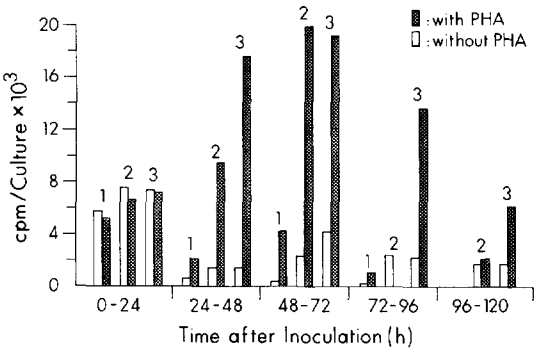


FIG. 4. Effect of 2-mercaptoethanol on the uptake of [2-^{14}C]thymidine by spleen cells in vitro. A suspension of pooled spleen cells from 8 B6D2F$_1$ mice, washed twice in culture medium, was inoculated into T-flasks (3×10^6 cells in 3 ml). RPMI 1640 medium was used with 10% calf serum, supplemented for some cultures with phytohemagglutinin-*P* (D-P 3 μl/culture). 1 μCi [2-^{14}C]thymidine (13.5 mCi/mmol, New England Nuclear, Boston, Mass.) was added to replicate cultures at either 0, 24, 48, 72, or 96 h. After 24 h exposure to thymidine the cell suspension was removed and homogenized ultrasonically. 50 μl aliquots were placed on Whatman 3 mm disks, washed as described by Fowler et al. (21) and counted in toluene-liquifluor. Concentrations of 2-mercaptoethanol: (*a*) 0; (*b*) 5 μM; (*c*) 100 μM.

and a 4.5-fold increase was seen in the 48–72 period. A 13-fold increase occurred at 72–96 h, but by this time the actual number of counts taken up was past the peak value. With 2-mercaptoethanol added at 5 μM (column 2), the maximum demonstrated increase, 4.8 times the control, occurred in the 48–72 h period. No cell line was established in any culture.

To explain these results, our first question was whether the thiol-disulfide might act by increasing cell viability. Splenic lymphocytes survive rather poorly in vitro, thus, in a typical experiment after 24 h 28 $\pm$ 3% survived, as judged by ability to exclude Trypan blue, at 48 h 11 $\pm$ 2% and at 72 h 5 $\pm$ 0%. In medium supplemented with 2-mercaptoethanol (5 μM), however, cell survival was 44 $\pm$ 2% at 24 h, 26 $\pm$ 2% at 48 h and 17 $\pm$ 1% at 72 h. Similarly, mitogen-stimulated and agglutinated lymphocytes showed a larger proportion

of unstained cells in the presence of the thiol-disulfide than in its absence, for instance, at 48 h 20 ± 2% and 45 ± 1% of the cells, respectively. Thus, improved viability may be a significant factor in the increased effect of mitogens in the presence of thiols-disulfides but it is probably not the whole explanation as further experiments show.

Our next question was whether thiols-disulfides would promote the growth of other kinds of cells. Thymocytes were therefore set up in cultures with a similar method to that used for splenic cells (Fig. 4). The [2-^{14}C]thymidine uptake (5.5×10^6 cpm/culture) was high in the first 24 h, but afterwards fell off (to 4.2×10^6) between 24 and 48 h. There was no significant change in this pattern in media supplemented with either 2-mercaptoethanol (5 and 100 μM) of L-cysteine (3.0 mM). Thymic cells of the hybrid mouse we used ($B6D2F_1$) showed only a minimal response to phytohemagglutinin (BW-R), which was not increased by these thiols-disulfides. Similarly the substances had no demonstrable effect in increasing viability of thymic cells; for instance, 67 ± 2% survived at 24 h and 8 ± 2% at 72 h in controls, while in medium supplemented with 5 μM 2-mercaptoethanol, corresponding figures were 66 ± 5% and 8 ± 1%.

Cells of a number of nonlymphoid lines have been examined in culture. 3T3 cells grew to a constant cell density ($5.1 \pm 1.3 \times 10^4$ cells/cm^2) in Dulbecco's medium with 10% calf serum whether or not it was supplemented with 2-mercaptoethanol (5–100 μM) or L-cysteine (1–3 mM). Generation times (30 ± 2 h) of nonconfluent cultures were not affected. Similarly, generation times were unchanged in HeLa, SV101, and primary cultures of the mouse hemangioendothelioma 129/J. No change was observed in the growth rate of a primary rabbit kidney cell culture, when it was tested on its third passage. After trypsinization, cells of the fourth passage failed to grow to confluence whether the medium had added thiol or not.

In the systems we have used therefore, splenic lymphocytes and certain mouse lymphoma cell lines appeared closely related in their response to thiols-disulfides. A more detailed comparison of these cell types was therefore undertaken.

Comparison of Growth Promoting Activity of Thiols-Disulfides on Splenic Lymphocytes and Different Lymphoma Cell Lines.—Experiments were now performed to determine whether the same rules of structural specificity for growth promoting agents would hold for splenic lymphocytes and thiol-disulfides-dependent lymphoma cell lines in general. Four compounds active on L1210 (V) were used for both categories of cells, which were cultured in the same medium, RPMI 1640 with 10% calf serum. Splenic lymphocytes were stimulated by a number of different mitogen preparations. Three were derived from *Phaseolus*, one was a relatively crude extract with high mitogenic activity containing several different mitogens, BW-R, the second was a partially purified and less strongly mitogenic extract from which polysaccharide had been removed, D-P

(22) and the third was a more highly purified extract which had little hemagglutinating activity, BW-P. The fourth mitogen was highly purified Con A. For the present experiments concentrations of mitogens giving maximal stimulation were used, as determined by preliminary experiment. In each case their effect was measured by uptake of [*methyl*-^{3}H]thymidine in 48–72 h cultures. Compared with controls containing no mitogen, thymidine uptake was increased by factors of 13.1 ±2.1, 2.7 ± 1.0, 4.5 ± 1.1, and 5.4 ± 1.1, respectively by these preparations.

The results are shown in Fig. 5 *a–d*; taken together they demonstrate a number of general points. First, lymphoma cells of three lines responded in a generally uniform way to the different thiols-disulfides used. In further experiments dithiothreitol (1–3 mM) promoted the growth of all three lines, while substances (GSH, DL-penicillamine and 2-mercaptopropionic acid) which were ineffective for L1210 (V) in the concentrations shown in Table I, did not promote growth of EL-4 or MBL-2.

Secondly, under appropriate conditions the mitogenic response of lymphocytes was increased by the four thiols-disulfides active on lymphoma cells. In order of increasing effectiveness the agents may be ranked: cystamine, 3-mercaptopropionic acid, 2-mercaptoethanol, and α-thioglycerol. In other experiments, cysteamine and dithiodiglycol had similar effects to the corresponding disulfide (cystamine), or thiol (2-mercaptoethanol). The concentrations of thiols-disulfides which promoted growth of lymphoma cells were usually the same as those which augmented the effects of mitogens on lymphocytes. In some instances however, notably with α-thioglycerol, the lymphoma cells showed a greater sensitivity, and growth promotion was optimal at 1/10 the level required for lymphocytes. The substances described in the previous paragraph which failed to promote growth of lymphoma cells, had no activity on the lymphocyte preparations.

Thirdly, whenever growth promotion by thiols-disulfides occurred, it was greater for the purified mitogenic proteins Con A and BW-P than for the more complex preparations BW-R and D-P. The mitogen preparations in themselves vary considerably in effectiveness, but this cannot explain such differences. It might be thought that BW-R, the most effective mitogen, was acting so strongly that all susceptible cells were replicating, and therefore very little promotion of its effect was possible. However, other experiments showed no greater promotion by thiols-disulfides when the mitogen was at suboptimal concentration. Furthermore D-P when used alone was the least effective mitogen but thiols-disulfides behaved with it in a generally similar manner to BW-R. Nor can the findings be explained by a temporal difference in the promoting effect of thiols-disulfides on the various mitogens. Further detailed experiments showed that in each case maximal effects were characteristic of the 48–72 h period. It appears therefore that the mitogenic stimuli given by these agents have qualitative differences which are reflected in the degree to which their action can be promoted by thiols-disulfides.

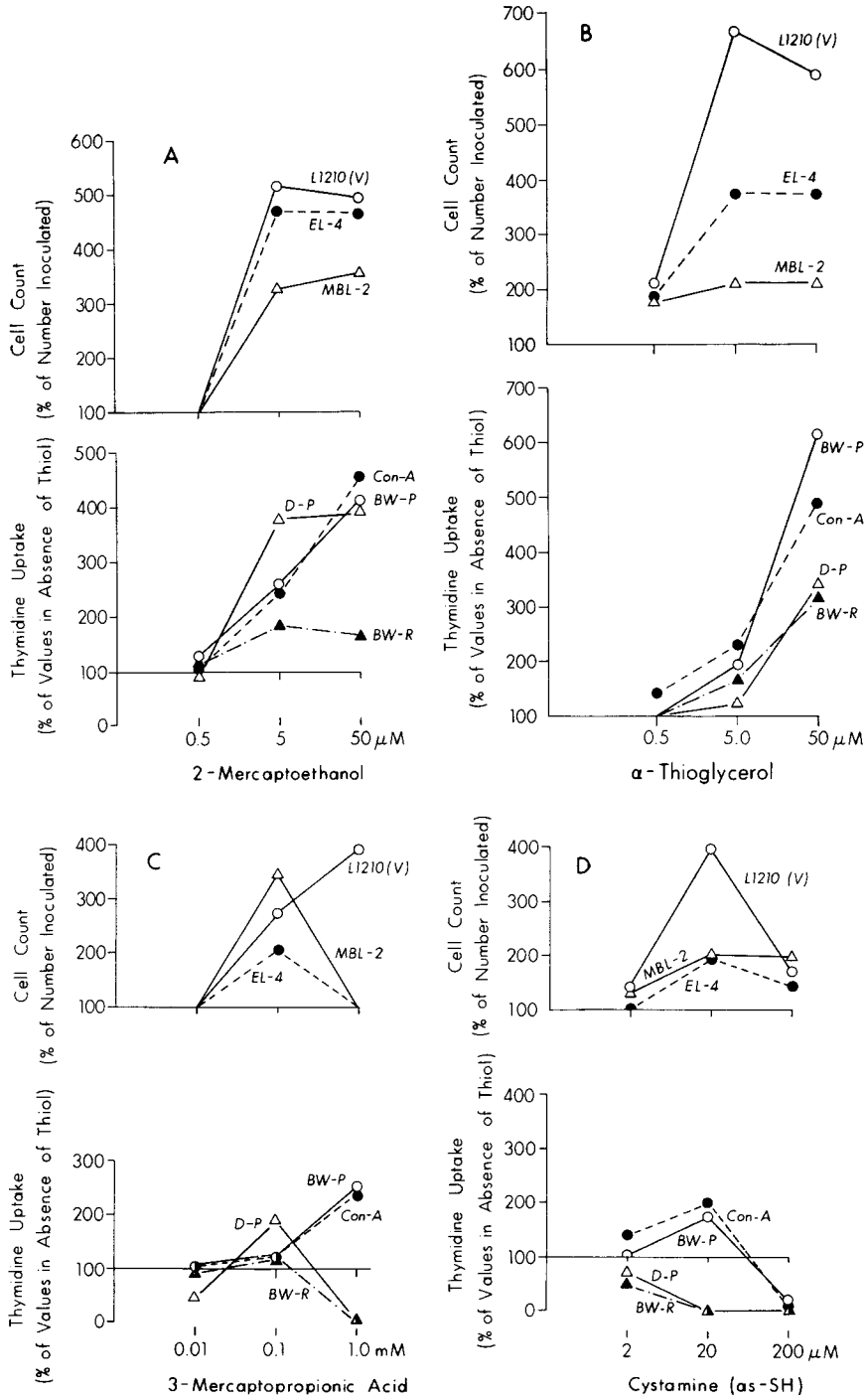


FIG. 5. Growth promoting effects of thiols-disulfides on three lymphoma cell lines in comparison to effects on the mitogenic responses of splenic lymphocytes. (*a*) 2-mercaptoethanol; (*b*) 3-mercaptopropionic acid; (*c*) α-thioglycerol; (*d*) cystamine. Lymphoma cells were cultured using a similar technique to that described in Table I. The figures on the vertical axis show the percentage of cells present at 72 h compared with the number inoculated. Splenic lymphocytes (3×10^6 cells in 3 ml) were cultured as described in Fig. 4, but were exposed to 10 μCi [*methyl*-^{3}H]thymidine, 6.7 Ci/mmol, (New England Nuclear) from 48–72 h. Mitogens were used in the following amount per culture: D-P 3 μl, BW-R 30 μl, BW-P 12 μg, Con A 30 μg. The figures on the vertical axis indicate the uptake of thymidine as percent of that occurring with mitogen stimulation in the absence of thiols-disulfides.

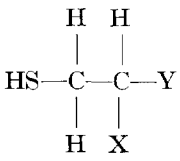
The fourth point demonstrated by the experiments is that under some circumstances thiols and disulfides can inhibit mitogenic effects. This was seen with 3-mercaptopropionic acid which at 1 mM promoted the mitogenic action of BW-P and Con A, but inhibited that of BW-R and D-P. Even more strikingly, cystamine had an inhibitory effect on BW-R and D-P at all the concentrations used, though it caused promotion of the action of the other two mitogens. These differential effects provide evidence that the thiols-disulfides have a direct effect on the mitogenic process; they cannot readily be explained on the basis of a growth permissive action of thiols on the cells in culture, such as has been demonstrated in studies of the viability of splenic cells in vitro. They can have two general explanations, first, that thiols-disulfides may be acting to modify the interaction of mitogen with receptor sites so that a stronger mitogenic stimulus results. One possibility is that new receptor sites are exposed. The inhibitory effect of some compounds might then be similar to that obtained by using a high concentration of mitogen. Alternatively, thiols-disulfides may act intracellularly to modulate one or more rate controlling steps leading from the mitogenic stimulus on the surface of the lymphocyte to DNA synthesis. These possibilities are under further investigation.

DISCUSSION

The results described show that the potential for growth promotion in vitro by specific thiols and disulfides is a characteristic shared by non-neoplastic and many lines of neoplastic lymphoid cells in the mouse. In the present experiments, these effects have not been observed with thymic cells or cells of non-lymphoid origin.

A number of recent studies have described effects of thiols on lymphocytes in vitro. Fanger et al. showed an increased effect of mitogens on human and rabbit lymphocytes in the presence of L-cysteine and glutathione (20). The latter substance has not shown activity in our systems. Click et al. described increased plaque-forming ability of lymphocytes and a potentiation of mixed lymphocyte reactions when media were supplemented by 2-mercaptoethanol or other thiols (23–26). All these results have been attributed to the reducing action of thiols. Our findings show that this is unnecessary, and indeed, under conditions of cell culture added thiols are present for only short periods before atmospheric oxidation occurs.

From experiments on growth promotion of L1210 (V), a provisional set of rules of structure-activity relationships for thiols can be formulated, on the basis of the structure of thioethane:



(*a*) Active compounds are aliphatic. (*b*) The sulfhydryl is primary and unsubstituted. (*c*) One hydrogen on the primary carbon may be substituted with a CH_3, but not with a polar group, the other should be unsubstituted. (*d*) The secondary carbon may have OH or NH_2 (not SH) as one substituent (X) and/or another substituent (Y) which may be COOH or the continuation of the carbon chain to a total length of <8 carbons. (*e*) There is steric specificity. Disulfides of thiols with these characteristics are also active.

The relationship of structural characteristics to the mechanism of growth promotion is not known; possibly they determine combining ability with specific substances or receptor sites. It is also unclear whether the thiol or its disulfide is the physiologically active form. If disulfides enter the cell they can readily be reduced (10). The redox potention of DPN^+ is -0.330 V at pH 7.0, considerably less than that of cysteine (-0.21 V). Dithiothreitol, the growth promoting substance with the lowest redox potential($-$ 0.332 V) will be reduced from the cyclical oxidized form less readily, but flavoproteins have even lower potentials (-0.67 V, ref. 27). Although the reactivity of thiols is more familiar in cellular systems, disulfides can have distinct biological effects. For instance, Skrede has shown that cystamine increases mitochondrial permeability; cysteamine does not have this property (28). Furthermore, the changing ratio of free and bound sulfhydryl groups (the glutathione cycle) has long been known to have particular importance in mitosis (29).

In contrast to L1210 (V) various other lymphoma cell lines show different responses to thiols-disulfides in vitro, some indeed are capable of growing independently and some do not grow even if thiols-disulfides are present. But amongst the numerous lines which do depend on these substances there appear to be similar structure-activity relationships. With lymphocytes, however, whether a particular concentration of thiol-disulfide will promote replication or be inhibitory depends on the mitogen preparation used. Nonetheless, under appropriate conditions, normal lymphocytes can be stimulated by all the agents examined which are active on L1210 (V) cells.

A significant question posed by the experiments is how the effects of thiols-disulfides on lymphoma cells are related to those on lymphocytes. Their effect on the growth of lymphoma cells may be permissive, and have its counterpart in the observed effect of thiols-disulfides in increasing the viability of splenic lymphocytes in vitro. On the other hand, we have presented evidence that thiols-disulfides act directly on replicative processes of mitogen-treated lymphocytes. A similar action may occur on lymphoma cells, which may be considered in this respect, to differ from lymphocytes only by the lack of a defined mitogenic stimulus.

A further question concerns the conditions or substances in vivo which are equivalent to the thiols-disulfides used in the present systems. Chen and Hirsch have shown that 2-mercaptoethanol restores the primary antibody-forming ability of lymphocyte preparations depleted of surface-adherent cells (30). There is also evidence that similar depletion inhibits the mitogenic response of

lymphocyte preparations to phytohemagglutinin (31). Since the macrophage is the principal cell type removed during these experiments it is possible that thiols-disulfides substitute for a factor or factors provided in the whole animal by this cell. In this connection, it has been observed that mouse lymphoma cells under some conditions depend for survival and growth in culture on the presence of macrophages introduced at the time of primary inoculation. This is particularly so during nutritional deprivation (32). Further studies are required to clarify these questions; they may significantly increase our knowledge of cellular relationships and growth factors for lymphoid tumors in general.

SUMMARY

Numerous lines of mouse lymphoid tumors (13 of 22 tested) showed, with increased sensitivity, a property of normal mouse splenic lymphocytes, the potential for growth promotion in vitro by specific thiols added to standard culture media. For lymphoma L1210 (V), structure activity relationships were examined; 9 of 30 thiols promoted growth; the most active was α-thioglycerol, effective at 0.2 μM. Thiols became oxidized under conditions of tissue culture and had half-lives of less than 8 h. Disulfides of active thiols promoted growth of lymphoma cells.

The mitogenic response of splenic lymphocytes to lectins was increased by thiols-disulfides which promoted the growth of lymphoma cells, but the response varied with the mitogen preparation used and under some conditions thiols-disulfides were inhibitory.

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