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Sulfhydryl Dependence in Primary Explant Hematopoietic Cells. Inhibition of Growth *In Vitro* with Vitamin B_{12} Compounds

(murine leukemia/bone marrow culture/sulfhydryl oxidation/disulfide toxicity)

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ABSTRACT **Primary explants of P388, EL-4, and L1210 murine leukemia cells and of normal mouse bone marrow are shown to require sulfhydryl compounds for proliferation *in vitro*. Nine established cell lines show no stimulation by these compounds. Leukemia cells can lose the sulfhydryl dependence after various periods of adaptation to *in vitro* culture. Various sulfhydryl compounds have widely differing potencies in promoting *in vitro* proliferation of dependent cells. The effect appears to be specific for sulfhydryl groups in the reduced form. Vitamin B_{12} compounds inhibit the growth of sulfhydryl-requiring cells, apparently by catalyzing the oxidation of the sulfhydryl groups.**

Sulfhydryl compounds have received relatively little attention as essential nutrients for cells *in vitro*. In 1971, Park *et al.* (1) briefly described stimulation by cysteine and glutathione of plasmacytoma growth *in vitro*. Broome and Jeng, in 1972, reported a requirement for sulfhydryl compounds in certain murine leukemia cells *in vitro* (2, 3). Other reports describe enhancement by sulfhydryl compounds of blast transformation (4, 5), of antibody formation (6–9), and of viability (2, 8) in lymphocyte cultures. This report describes a study of the sulfhydryl dependence of some hematopoietic cells and the inhibitory effect of vitamin B_{12} compounds on these cells.

MATERIALS AND METHODS

Cell Culture. Eagle's minimum essential medium (MEM, ref. 10) was supplemented with 5–20% fetal calf serum (as indicated), 30 mg of glutamine/100 ml, 5 mg of asparagine/100 ml, 10 mg of serine/100 ml, and 11 mg of sodium pyruvate/100 ml. No antibiotics were added. In experiments involving a series of variable additives, a single homogeneous culture was used to pour all plates in the series. Variable additives were sterilized by filtration and placed in the plastic culture dishes before the culture was added.

P388 murine leukemia cells (11) and L1210 murine leukemia cells (12) were obtained from peritoneal aspirates of DBA/2 mice which were inoculated 4–6 days previously with 10^6 cells. EL-4 murine leukemia cells (13) were obtained in a similar manner from C57 mice. Leukemia cells were cultured in 1.5-ml cultures in liquid medium in 35 × 10-mm Falcon plastic plates with an initial cell density of 1×10^5 cells per ml. After incubating at 37° in 5% CO_2 for the indicated number of days (usually 3 days), the cells were suspended by swirling and diluted either 1 to 2 in trypan blue for counting in a hemocytometer or 1 to 800 in Isoton for counting in a Coulter counter.

The following cells, obtained from established *in vitro* cell lines, were grown in plastic plates or flasks as suspension or monolayer cultures: mouse fibroblasts (14), rat XC sarcoma (15), rat Walker-256 carcinosarcoma (16), human monocytic leukemia—J111 (17), human lymphoblastoid cells of T type—Molt 4 (18) and of B type—LA 96 (unpublished cell line obtained from M. Jobin and Dr. J. Fahey, UCLA), Gross-virus-infected mouse lymphoblastoid cells (unpublished cell line obtained from Dr. E. Hayes, UCLA), mouse lymphoblastic leukemia—L1210 cell line (ref. 19, strain obtained from Southern Research Institute, Birmingham, Ala.), and Friend-virus-infected mouse leukemia cell line (20). The cells which grow as attached monolayers (the first four listed), were detached with 0.25% trypsin in MEM for counting.

For bone marrow culture, the medium was made at two times the normal concentration and mixed with an equal volume of 0.6% agar at 37° before adding fresh bone marrow cells obtained from the femurs of white Swiss Webster mice. Colony-stimulating factor was provided as an extract of gravid C3H or DBA/2 mouse uterus prepared by the method of Bradley *et al.* (21). Cultures of 1.5 ml were plated as a single layer in 35 × 10-mm Falcon plastic plates. Clones of more than 20 cells were counted as colonies on the seventh day.

Vitamin B_{12} Compounds. Vitamin B_{12}, coenzyme B_{12}, and hydroxy B_{12} were purchased from Sigma Chemical Co. Factor B was synthesized by hydrolyzing vitamin B_{12} in 12 N hydrochloric acid at 65° for 5 min (22) followed by phenol extraction and chromatography on CM-cellulose using 0.05 M acetic acid as eluent. Methyl B_{12} was synthesized from vitamin B_{12} using chemical reduction by the method of Dolphin (23). The concentration of all vitamin B_{12} compounds was measured using the extinction at 367 nm after complete reaction with alkaline cyanide ($E = 30.8 \times 10^3\ M^{-1}\ cm^{-1}$).

Sulfhydryl Compounds. Dithiothreitol, mercaptoethanol, thioglycerol, thioethanolamine, cysteine, reduced glutathione, oxidized glutathione, coenzyme A, and sodium thioglycolate were purchased from Calbiochem in the highest degree of purity available. Homocysteine was purchased as the thiolactone and converted to the free thiol by dissolving in 0.5 N sodium carbonate and incubating at 55° for 30 min followed by neutralization with an equal volume of 0.5 N HCl with phenol red as an indicator. Solutions of SH compounds were prepared in double-distilled water shortly before use. Concentration of sulfhydryl (SH) groups was measured using dithionitrobenzoic acid (DTNB) by the method of Beutler (24).

Abbreviations: MEM, minimum essential medium; SH, sulfhydryl; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

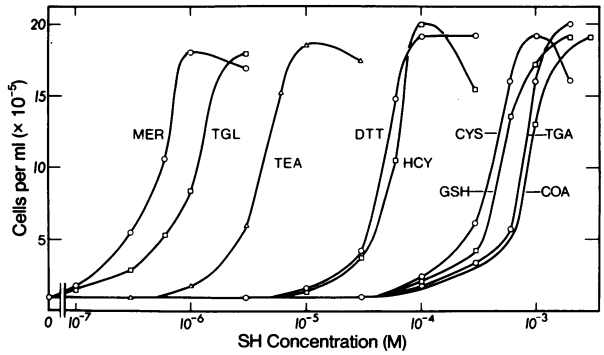

FIG. 1. Growth response of P388 leukemia cells *in vitro* to various sulfhydryl compounds. The ordinate represents cells per ml 72 hr after inoculating with 1×10^5 cells per ml in MEM containing 5% fetal calf serum and various concentrations of the SH compounds: MER, mercaptoethanol; TGL, thioglycerol; TEA, thioethanolamine; DTT, dithiothreitol; HCY, homocysteine; CYS, cysteine; GSH, glutathione; TGA, sodium thioglycolate; COA, Coenzyme A.

RESULTS

Effect of Sulfhydryl Compounds on P388 Leukemia Cells In Vitro. P388 leukemia cells taken from ascitic culture in DBA/2 mice do not proliferate *in vitro* in MEM and 100% of the inoculum cells are dead after 3 days by the criterion of trypan blue uptake. However, these cells grow luxuriantly in this medium if an SH compound is added at an appropriate concentration. Fig. 1 shows the growth response of P388 cells in the presence of varied concentrations of several SH compounds. All of the SH compounds tested stimulate proliferation at some concentration. The SH concentration producing maximum proliferation varies from about 1 μM for mercaptoethanol and thioglycerol to about 1 mM for cysteine, glutathione, thioglycolate, and coenzyme A. The stimulatory concentration range for each compound is quite narrow; the stimulatory effect rises from zero to maximum within a 10-fold increase in concentration, and there is a rapid fall-off in growth response when the concentration exceeds the optimal level (not shown for all compounds in Fig. 1).

Methionine, hydrosulfite, sulfite, ascorbic acid, cystine, and oxidized glutathione do not promote proliferation of P388 cells *in vitro*. Cystine is inhibitory at concentrations above 0.1 mM when added in the presence of stimulatory concentrations of SH compounds, causing complete inhibition of growth at about 1 mM. MEM contains 0.1 mM cystine but no reduced SH compounds. In a special preparation of MEM, in which the cystine is replaced by cysteine, the growth response to various SH compounds is essentially the same as that shown in Fig. 1 for the cystine-containing medium. Cysteine, in the absence of cystine, produces maximum growth at 1 mM. Increasing the serum content of the medium from 5% (as in Fig. 1) to 20% does not alter the requirement for SH compounds.

P388 leukemia cells can be subcultured indefinitely in SH-supplemented medium. During the first two to three subcultures, the dependence on SH compounds persists; the cells die if transferred to a non-SH-supplemented medium. However, when cells from the second or subsequent subcultures are transferred to a non-SH-supplemented medium, they proliferate at a low rate. This cell line can then be subcultured indefinitely without SH compounds, attaining the usual growth rate after two to three subcultures (20-fold increase in cell density in 3 days). The SH-independent cell line is highly malignant when transferred to DBA/2 mice. After two consecutive passages through ascitic culture in mice, these cells still retain the ability to proliferate immediately without SH supplementation when transferred back to *in vitro* culture.

Effect of Sulfhydryl Compounds on EL-4 Leukemia Cells In Vitro. EL-4 leukemia cells from ascitic culture in C57 mice show the same growth response to SH compounds as P388 during the first 3 days *in vitro*. There is no cell proliferation in the absence of SH compounds and varied concentrations of the SH compounds produce the same pattern of growth response curves as shown in Fig. 1 for P388 cells. Optimally stimulated cultures of EL-4 cells reach cell densities of 30×10^5 cells per ml in 3 days. However, EL-4 cells do not die in the absence of SH compounds and, if the primary explant cultures are incubated beyond 5 days, the cells begin to proliferate in the absence of SH compounds. These adapted cells proliferate without a lag period when transferred to non-SH-supplemented medium and the cell line can be maintained indefinitely without addition of SH compounds.

Effect of Sulfhydryl Compounds on L1210 Leukemia Cells In Vitro. L1210 leukemia cells taken from ascitic culture do not proliferate in MEM in the absence of SH compounds and 100% of the inoculum cells are dead by the third day. However, they proliferate very well in this system when SH compounds are added, showing a pattern of growth response curves which is essentially identical with that shown in Fig. 1 for P388 cells. Like P388 cells, L1210 cells can be subcultured in the presence of SH compounds. During the first seven subcultures *in vitro* these cells retain an absolute dependence on SH compounds; cells transferred to SH-deficient medium die. After seven subcultures it has not been possible to obtain a cell line of L1210 adapted to growth without SH compounds.

Effect of Sulfhydryl Compounds on Mouse Bone Marrow Culture In Vitro. As originally described by Bradley and Metcalf (25), bone marrow cells cultured in semi-solid medium give rise to colonies of granulocytes and/or macrophages when appropriately stimulated with "colony-stimulating factor." In the system described above, using MEM supplemented with 15% fetal calf serum and 1% of a high quality extract of gravid mouse uterus (but no added SH compounds), an inoculum of 15,000 nucleated mouse bone marrow cells per plate gives rise to an average of 75 colonies in 7 days. The effect of adding SH compounds to mouse bone marrow cultures is shown in column 1 of Table 1. Addition of dithiothreitol, thioglycerol, homocysteine, or glutathione causes approximately a 5-fold increase in the number of colonies and a marked increase in the size of the colonies. For each of these compounds, the stimulatory effect is maximal at concentrations between 0.3 and 1 mM. On repeated trials, cysteine, thioethanolamine, thioglycolate, and mercaptoethanol fail to produce any stimulation of colony formation. SH compounds do not replace the colony-stimulating factor which is required for bone marrow colony-formation *in vitro*.

Effect of Sulfhydryl Compounds on Established Cell Lines In Vitro. The nine established cell lines tested are listed under *Methods*. All of these cells proliferate in MEM without the addition of SH compounds and none of them show stimulation of growth when SH compounds are added. Since the MEM system contains methionine and serum, it was considered possible that these sources might fulfill an SH requirement in

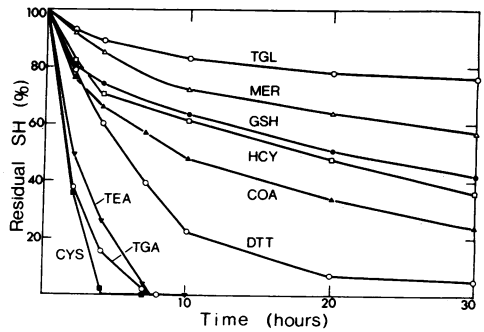

FIG. 2. Autoxidation of sulfhydryl compounds in MEM. The SH compounds were added at 2.5 mM to 1.5-ml cultures in the MEM system containing 5% fetal calf serum and 1 × 10^5 cells per ml. The cultures were incubated at 37° in 5% CO_2. Aliquots of 0.1 ml were withdrawn at various time intervals for measuring residual SH by the DTNB method. The symbols are identified in Fig. 1.

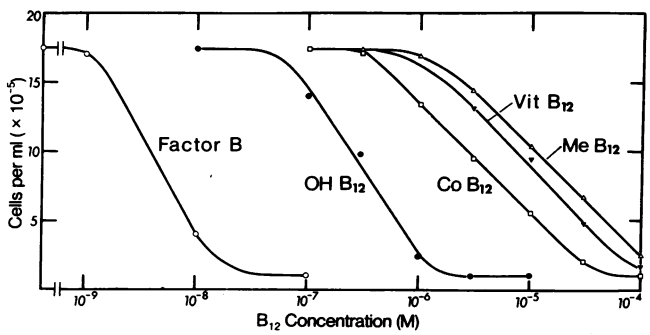

FIG. 3. Effect of vitamin B_{12} compounds on the growth of SH-stimulated P388 cells *in vitro*. The ordinate shows cells per ml 72 hr after inoculating with 1 × 10^5 cells per ml in the presence of 80 μM SH (40 μM dithiothreitol) and varied concentrations of the vitamin B_{12} compounds: Factor B; OH B_{12}, hydroxy B_{12}; Co B_{12}, coenzyme B_{12}; Vit B_{12}, vitamin B_{12}; Me B_{12}, methyl B_{12}.

these cells. However, addition of hydroxy B_{12} or Factor B to the basal medium to destroy endogenous SH groups (as described below), does not cause any inhibition of growth of any of these cells, indicating that they do not have a requirement for SH groups in the reduced form. Addition of SH compounds along with Factor B or hydroxy B_{12} causes marked inhibition when the SH concentration exceeds 0.1 mM.

Effect of Sulfhydryl Compounds on Primary Explants of Mouse Fibroblasts and Mouse Mammary Carcinoma. Primary explants of mouse fibroblasts from skin and muscle of DBA/2 mice were made in MEM containing 10% fetal calf serum and varied concentrations of SH compounds. Fibroblasts formed discs of typical cells around the tissue fragments in 5–7 days, with no subjective difference discernible between the cultures with and without SH compounds. Primary explants of spontaneous mammary carcinoma from four C3H mice, cultured in MEM containing 10% fetal calf serum and varied SH compounds, developed numerous clones of epithelioid cells on the plastic surface in 3–5 days. Addition of SH compounds had no discernible stimulatory effect and addition of Factor B (to destroy endogenous SH groups) was not inhibitory.

Autoxidation of Sulfhydryl Compounds in MEM. The rates of autoxidation of the above-named SH compounds in the MEM tissue culture system were determined by sequential measurement of SH groups using the DTNB method. Fig. 2 shows the rate of SH disappearance when the compounds are placed at 2.5 mM in the MEM system containing 5% fetal calf serum and 1 × 10^5 P388 cells per ml and incubated at 37° in 5% CO_2. An identical pattern of oxidation curves is obtained when the cells are omitted. There is a wide range of variability in the rate of SH disappearance. Cysteine, thioethanolamine, and thioglycolate oxidize rapidly, leaving no measurable SH groups within 4–8 hr. Dithiothreitol undergoes an initial rapid oxidation but a small fraction of the SH groups remains measurable for at least 48 hr. Mercaptoethanol, thioglycerol, glutathione, homocysteine, and coenzyme A are relatively resistant to oxidation in this system. The presence of fetal calf serum in the system has a marked effect in retarding the rate of SH disappearance. When serum is omitted (MEM alone), there is a rapid linear disappearance of SH groups, with concentrations reaching zero within 2–12 hr for all of the SH compounds.

Inhibition of SH-Stimulated Leukemia Cells by Vitamin B_{12} Compounds. Since it is known that vitamin B_{12} compounds catalyze the nonenzymatic oxidation of SH groups (26, 27), several vitamin B_{12} analogs were added at varied concentrations to P388 cultures containing dithiothreitol. The resulting suppression of growth is shown in Fig. 3. Factor B is the most active analog, causing 50% inhibition at 4 nM. Hydroxy B_{12} causes 50% inhibition at 0.3 μM, coenzyme B_{12} at 3 μM, and vitamin B_{12} and methyl B_{12} at about 10 μM.

Inhibition of Bone Marrow Colony Formation by Vitamin B_{12} Compounds. Table 1 shows the effect of Factor B and hydroxy B_{12} in mouse bone marrow culture with and without addition of SH compounds. The vitamin B_{12} compounds not only reverse the stimulatory effect of the added SH compounds but also inhibit colony formation in the basal medium.

Relationship of B_{12} Toxicity to the Oxidation of Sulfhydryl Groups. The above data are consistent with the hypothesis that the growth-inhibitory effect of vitamin B_{12} compounds on certain cells is due to their ability to catalyze the oxidation of SH groups. This hypothesis can be further tested by comparing the relative activities of the vitamin B_{12} compounds in the two effects. The kinetics of B_{12}-catalyzed SH oxidation were studied in the complete MEM system containing fetal calf serum but lacking cells. Activity was followed by measuring residual SH at varied intervals during incubation at 37° in

TABLE 1. *Effect of sulfhydryl compounds and vitamin B_{12} compounds on mouse bone marrow colony formation in vitro*

SH addition	B_{12} addition: None	Factor B (1 μM)	Hydroxy B_{12} (10 μM)
	(colonies per 15,000 nucleated cells)		
None	75	0	7
Dithiothreitol, 0.3 mM	~350*	0	6
Homocysteine, 1 mM	~350*	6	0
Glutathione, 1 mM	~350*	4	15
Thioglycerol, 1 mM	~350*	0	10

Cultures (1.5 ml) in MEM containing 15% fetal calf serum were inoculated with 15,000 nucleated cells per plate. Colonies were counted on the seventh day.

* Accurate counts are not possible at high colony densities.

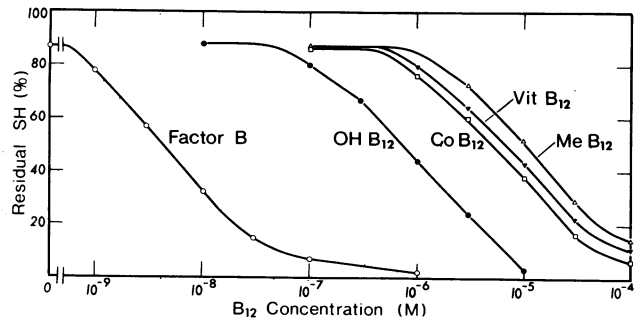

FIG. 4. Effect of vitamin B_{12} compounds on the oxidation of SH groups in MEM. MEM containing 2.5 mM SH (1.25 mM dithiothreitol) was placed in 35 × 10-mm plastic plates containing varied amounts of the vitamin B_{12} compounds. After 60 min at 37° in 5% CO_2, residual SH was measured by the DTNB method. The symbols are identified in Fig. 3.

5% CO_2. The B_{12}-catalyzed oxidation of SH groups in this system follows first order kinetics at low SH concentration and zero order kinetics at saturating SH concentration. For Factor B-catalyzed oxidation of dithiothreitol, the SH concentration producing one-half maximum velocity is 2.5 mM. This concentration of dithiothreitol was used to measure the initial velocity at varied concentrations of several B_{12} compounds. Fig. 4 shows the effect of the various B_{12} compounds in this system. The concentrations of the B_{12} compounds producing 50% destruction of SH groups in 60 min are listed in Table 2 along with the concentrations that cause 50% inhibition of P388 growth in the same system. It is seen that the different vitamin B_{12} compounds have widely differing activities in both effects, but that the relative activities for the two effects are quite similar.

DISCUSSION

Primary explant cells of three murine leukemias and of mouse bone marrow are shown to require sulfhydryl compounds for proliferation or colony formation *in vitro*. The four cell types show various degrees of SH-dependence on primary explant. P388 and L1210 leukemia cells show an initial absolute dependence on SH compounds; the cells die within 3 days in the absence of SH supplementation. EL-4 leukemia cells, although stimulated to undergo immediate proliferation by addition of SH compounds, can proliferate *in vitro* in the absence of SH compounds after a lag period of several days. Mouse bone marrow colony formation occurs in the basal medium but is markedly stimulated by SH supplementation and inhibited by SH depletion.

The SH dependence of the primary explant cells is to be contrasted with the independence of the nine established cell lines that were tested. The comparison is especially pertinent in the case of L1210 leukemia cells. The L1210 animal cell line, first described in 1949 by Law *et al.* (12) and maintained since then by animal passage, demonstrates an absolute SH dependence when transferred to *in vitro* culture, whereas the L1210 *in vitro* cell line, derived from the animal cell line in 1966 by Dixon *et al.* (19) and maintained since then *in vitro*, shows no requirement for SH compounds. It is interesting to note that, in the original establishment of the *in vitro* cell line, Dixon *et al.* used a medium containing 1 mM cysteine.

The contrast between the SH dependence of the primary explant cells and the independence of the established cell lines is corroborated by the observation that P388 and EL-4 leukemia cells lose their SH dependence after various periods of culture *in vitro*. L1210 leukemia cells appear to be more resistant to this change. The SH independence of the adapted P388 cells persists after passage through mice, indicating that a stable transformation has occurred. Similarly, when the L1210 *in vitro* cell line is passed through mice and returned to *in vitro* culture, it is found that the cells still have no requirement for SH compounds.

TABLE 2. *Relative activities of B_{12} compounds in inhibiting growth of P388 leukemia cells and in catalyzing the oxidation of SH groups*

Vitamin B_{12} compound	Concentration (μM) causing 50% inhibition of P388 growth (0.1 mM SH)	Concentration (μM) causing 50% destruction of SH groups in 60 min (2.5 mM SH)
Vitamin B_{12}	10	7
Coenzyme B_{12}	3	5
Methyl B_{12}	10	10
Hydroxy B_{12}	0.3	0.7
Factor B	0.004	0.004

From the limited series of cell types tested, some tentative conclusions can be drawn concerning the cell types that require SH compounds. As noted above, the requirement appears to occur only in primary explant cells. Since primary explants of mouse fibroblasts and mouse mammary carcinoma cells show no effect of SH supplementation, it appears that the SH requirement may be specific to certain hematopoietic cells. Since normal bone marrow cells are SH-dependent, the requirement is not specific to malignant cells. Therefore, it is tentatively concluded that the SH requirement is specific to primary explants of certain hematopoietic cells.

Several lines of evidence indicate that the SH requirement in these cells is specific for SH groups in the reduced form. The disulfides tested, cystine and oxidized glutathione, do not support growth of the leukemia cells. Both the ability to promote leukemia cell proliferation and the tendency to autoxidize in the MEM system vary over a wide range for the various SH compounds. For most compounds there is a rough correlation between the relative activities in promoting P388 growth and the abilities of the compounds to withstand autoxidation, although this correlation does not appear to apply for glutathione, thioethanolamine, and coenzyme A. The partial correlation suggests that the ability of the SH compounds to provide a sustained availability of reduced SH groups may be one factor in determining their effectiveness in promoting the growth of leukemia cells. It is interesting that the rapidly autoxidized compounds cysteine, thioethanolamine, and thioglycolate do not stimulate bone marrow colony formation in that system which involves incubation for 7 days.

The inhibitory effect of vitamin B_{12} compounds provides further evidence that the SH requirement is specific for SH groups in the reduced form. The activities of different B_{12} compounds in inhibiting P388 growth and in catalyzing SH oxidation vary over a wide range, with Factor B being 2500 times as active as vitamin B_{12}. The close correlation between the relative activities in the two actions supports the theory that the inhibition of cell proliferation by vitamin B_{12} compounds is due to destruction of SH groups which are required

by the cells in the reduced form. As stated above, the vitamin B_{12} compounds do not inhibit the growth of established cell lines in the absence of SH compounds, indicating that vitamin B_{12} compounds, at the concentrations tested, do not have an intrinsic toxicity to cells.

Several practical considerations may be derived from the above observations: Many commonly-used tissue culture media contain vitamin B_{12} at relatively high concentrations. For example, commercial McCoy's 5a medium contains 1.5 μM vitamin B_{12} (28) and many of the NCTC media contain 7.4 μM vitamin B_{12} (29). In the MEM system, these concentrations are in the toxic range for SH-dependent cells. Since vitamin B_{12} is converted to hydroxy B_{12} on exposure to light, these media probably contain significant quantities of hydroxy B_{12}, which is 30 times more toxic than vitamin B_{12}. Therefore, in research involving cell culture, it may be advantageous to determine if the cells to be cultured are SH-dependent and, if so, to select a medium that does not contain excessive amounts of vitamin B_{12}.

Secondly, most tissue culture media contain inadequate concentrations of reduced SH groups to support growth of SH-dependent cells. Many media contain no reduced SH compounds and, in those media containing cysteine, the concentration is usually much lower than that found to be optimal in the system used in this study. Therefore, the SH requirement of cells should be determined, especially in primary explant cultures, and the medium appropriately supplemented. On the other hand, excessive concentrations of SH compounds are toxic to cells and a fine balance of concentration must be achieved to obtain optimal growth of SH-dependent cells. Combining vitamin B_{12} and SH compounds in the same medium causes pronounced inhibition of all cells tested, both SH-dependent and SH-independent, presumably due to the formation of toxic disulfides. Therefore, special precaution should be taken to avoid combining these classes of compounds at reactive concentrations.

Finally, the data presented above indicate that certain malignant cells have a requirement for SH compounds when first explanted from the animal. It seems likely that the requirement exists *in vivo* and is fulfilled by natural SH compounds in the body. The ability of vitamin B_{12} compounds to prevent proliferation of the freshly explanted malignant cells leads to the speculation that the more active B_{12} compounds, such as Factor B, might be capable of inhibiting proliferation of SH-dependent malignant cells *in vivo*.

Note Added in Proof. Three additional cell types have been found to require sulfhydryl compounds on primary explant and to yield *in vitro* cell lines readily in SH-supplemented MEM. They are: lymphoma cells obtained from the thymus of Gross-virus-infected AKR mice, plasmacytoma cells-MOPC 21a obtained from ascitic culture in BALB/c mice, and cells of a transplantable reticulum cell sarcoma obtained from the spleens of SJL/J mice.

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