Journal of Supramolecular Structure
and Cellular Biochemistry 17:11-25 (1981)

Macrophages and Methylthio Groups in Lymphocyte Proliferation

John I. Toohey

Division of Hematology and Oncology, Department of Medicine, University of California, Los Angeles, California 90024

Macrophages are shown to replace methylthio disulfides in supporting in vitro proliferation of three cell lines previously characterized as methylthio-dependent. Macrophages have the capacity to generate methylthio groups from methylthioadenosine. It is hypothesized that macrophages stimulate cell proliferation both in normal immune systems and in certain cancers by providing an abundance of methylthio groups. Fetal calf serum is shown to contain methylthio groups. It appears that, in cell cultures containing fetal calf serum, sulfhydryl compounds stimulate cell proliferation by making the methylthio groups in the serum available to the cells.

Key words: sulfhydryl compounds, methylthio groups, macrophages, serum growth factors, methylthioadenosine

Macrophages, sulfhydryl compounds, or methylthio disulfides have been reported to be essential factors for in vitro proliferation of many lymphocytic cells and for in vitro function of several immune systems. This report summarizes the previous data on each of these growth factors and presents new findings. The combined data are consistent with the theory that the methylthio group is the essential factor made available to the cells in each case.

METHODS

Cell Culture

Culture medium was Eagle's Minimum Essential Medium (MEM) with Earle's salts purchased from Grand Island Biological Co. (GIBCO) either as the 10× concentrated solution or as the powder and supplemented with 5 mg% asparagine, 30 mg% glutamine, and 10 mg% sodium pyruvate. Serum and other additives were added as indicated in the text. For maintenance cultures, fetal calf serum (FCS) was used during the early period of this work; since mid-1979 new-born calf serum (NBCS) has been used for this purpose. No antibiotics were used. Cultures in Falcon plastic containers were incubated in 5% CO_2 at 37°C and were subcultured or counted 3 days after inoculation.

Received January 16, 1981; accepted June 8, 1981.

0275-3723/81/1701-0011$04.50

Cells

Malignant murine lymphocyte cell lines, L1210 and P388, were obtained as ascites cells in DBA/2 mice in January, 1977, through the National Cancer Institute (Arthur D. Little, supplier). Both cell lines proliferated in vitro only if provided with a sulfhydryl compound and fetal calf serum or with a methylthio disulfide and serum. They were explanted to in vitro culture in 1977 and have been maintained since then in vitro in medium containing 1×10^{-4} M S-methylthio-L-cysteine and 10% serum (fetal calf or new-born calf). MOPC 21aSH is a methylthio-dependent strain of murine plasmacytoma MOPC 21 [2]. The parent strain, which has been in use in the Department of Microbiology and Immunology at UCLA for many years, is normally methylthio independent in vitro. The methylthio-dependent strain arose in an explant made in 1976 and has been maintained in vitro since then in medium containing 2×10^{-3} M glutathione and 10% FCS or 1×10^{-4} M S-methylthio-L-cysteine and 10% serum (FCS or NBCS). Methylthio-independent cell lines, plasmacytoma LPC-1 and Friend cell line, and their maintenance were previously described [1]. Fibroblasts were from a cell line recently explanted from human skin in the Department of Pediatrics at UCLA.

Sera

Fetal calf serum (FCS) was purchased from GIBCO, horse serum from GIBCO and from Flow Laboratories, new-born calf serum (NBCS) and bovine calf serum from KC Biologicals (Lenexa, KA). Samples of sera were dialyzed for 2 days against balanced salt solution or heated at 60°C for 45 min.

Chemicals

Glutathione (GSH) and other chemicals were purchased from Sigma Chemical Co. Escherichia coli lipopolysaccharide (LPS), Corynebacterium parvum cells, and Freund's adjuvant were purchased from DIFCO. Phosphate-buffered saline without Ca or Mg (PBS) was purchased from GIBCO as the 10× concentrated solution. S-methylthio-L-cysteine (C–S–S–CH_3) [3], [^{35}S]methylthioadenosine [1], and [methyl-^{14}C]methylthioadenosine [1] were prepared as previously described.

Gas Chromatography–Mass Spectroscopy

The volatiles from 200 ml of fetal calf serum were analyzed in the apparatus and by the method described by Robinson et al [4]. Briefly, the volatiles released at 80°C were flushed with helium gas into a liquid nitrogen trap and then flashed into a gas chromatograph. The effluent from the chromatograph was analyzed continuously by a computerized mass spectrometer which recorded 12 mass spectra per minute [5]. To determine the characteristics of authentic methyl mercaptan in this system, the material was generated by adding C–S–S–CH_3 (5 μmol) and cysteine (33 mmol) to 200 ml of fetal calf serum just prior to the run.

Dialysis of FCS Against Sulfhydryl Compounds

For removal of sulfhydryl-reactive growth factor from FCS, the following procedure was found optimal. A sample of FCS was diluted in 9 vol of a solu-

tion containing 0.05 M NaCl and 0.1 M GSH, pH 7.2. It was then dialyzed in order against the following at 4°C: two changes of the above diluent during 24 hr, eight changes of PBS over 4 days, and two changes of MEM over 24 hr. The last dialysis converted the dialysate into MEM containing 10% treated serum; it was sterilized by filtration and used as culture medium. It should be noted that GSH is the best sulfhydryl compound for this purpose, since compounds such as cysteine, cysteamine, or thioglycollate autoxidize too rapidly to be effective and compounds such as mercaptoethanol or thioglycerol are extremely difficult to remove by subsequent dialysis and they have been shown to mimic to some extent the effect of the methylthio group even in the absence of serum [3].

Preparation of Adhered Macrophages

Peritoneal cells were obtained from female ex-breeder BALB/c mice by flushing the peritoneal space with sterile PBS. The cells from several mice were pooled, centrifuged, suspended in culture medium containing 10% FCS, and plated at varied dilutions in 35-mm plastic dishes. The dishes were incubated for 24 hr at 37°C in 5% CO_2 to allow the macrophages to adhere and spread, then rinsed repeatedly with PBS to remove nonadherent cells. The number of adhered cells in each group of dishes was determined by counting on an inverted microscope. The number and identity of the adhered cells was confirmed by selecting representative dishes, pouring off the PBS, fixing with methanol, staining with Wright's stain, and examining microscopically. Adhered macrophage preparations were used in subsequent experiments only if examination showed that the macrophages were flattened and had elongated cytoplasmic extensions. Rabbit alveolar macrophages were obtained by pulmonary lavage 3 weeks after intravenous injection of Freund's adjuvant. Human peripheral blood monocytes were obtained from the mononuclear fraction after separation on ficoll–hypaque [6].

Cell Culture on Macrophage Feeder Layers

Direct Contact. Methylthio-dependent cells were taken from late log phase culture, washed with fresh medium, and suspended in medium containing 10% FCS at a density of 1×10^5 cells ml. The dishes containing adhered macrophages were drained of PBS and rinsed with culture medium. Aliquots (1.5 ml) of the above cell suspensions were placed in the dishes in direct contact with the adhered macrophages. After incubation for 3 days, the free-floating cells were suspended and counted in a hemacytometer in the presence of Trypan blue.

Diffusion chamber cultures. Marbrook vessels [7] having outer chambers of 40-mm diameter and inner chambers of 12-mm diameter were used. The inner chambers were closed with dialysis membrane (Sigma, prewashed, Cat. No. 250-11) secured with boiled elastic bands. The assembled vessels were sterilized by autoclaving. Dishes containing adhered macrophages were placed in the bottoms of the Marbrook outer chambers and 3 ml of medium containing 5% FCS were placed in each dish. Other additives, such as endotoxin, GSH, or C–S–S–CH_3, were placed in the dishes. The inner chambers were immersed to a depth of 3 mm in the medium contained in the dishes. Cell suspensions of

methylthio-dependent cells, prepared as described previously, were placed in the inner chambers (1 ml per chamber). After incubation for 3 days, the cells in the inner chambers were suspended and counted in Trypan blue.

Incubation of Methylthioadenosine With Macrophages

Dishes containing 3×10^5 adhered mouse peritoneal macrophages were prepared as described previously. Then 0.3 μmol of [^{35}S]methylthioadenosine (800,000 cpm per μmol) or [methyl-^{14}C]methylthioadenosine (300,000 cpm per μmol) was added to the dishes in 1 ml of medium containing 3% FCS. Control dishes lacked macrophages. After 24 hr. of incubation at 37°C under 5% CO_2, the medium was withdrawn, placed in glass-stoppered centrifuge tubes, acidified with 0.1 ml of 12 N HCl, and extracted two times with 1.5-ml vol of diethyl ether using centrifugation to separate the phases. The ether extract was mixed with 10 ml of scintillation fluid and radioactivity was measured to calculate the amount of ether-extractable product. The medium with labeled substrates was replaced in the dishes and the procedure repeated with the same macrophages on 3 consecutive days.

Enzyme Assays on Extracts of Various Cells

Methylthioadenosine nucleoside phosphorylase and methylthiolase activities were measured in crude cell extracts by the methods previously described [8]. Blood cells for enzyme assays were obtained as follows: Normal human venous blood, 400 ml, anticoagulated with 0.1% Na_2EDTA, was centrifuged at 600*g*. Platelets were obtained from the plasma by centrifugation at 5000*g*. The blood cells were washed twice with PBS containing 0.1% Na_2EDTA to remove more platelets, then suspended in 600 ml of PBS and centrifuged on a two-step gradient of ficoll–hypaque [9]. Red blood cells were recovered from the pellet, washed with PBS, and passed through cellulose to remove residual white cells [10]. Granulocytes were recovered from the band of cells overlying 6% ficoll–16% hypaque. Mononuclear cells, overlying 6% ficoll–10% hypaque, were washed with PBS, suspended in 100 ml of MEM containing 10% FCS, and adherent cells were depleted by two consecutive incubations at 37°C for 90 min, first on plastic and then on glass. Mouse peritoneal macrophages were obtained as described above from mice injected 2 weeks previously with C parvum cells. Rabbit alveolar macrophages were obtained as described previously. Fibroblasts were grown in vitro in MEM containing 10% FCS and detached by trypsinization. L1210 cells were grown in MEM 10% FCS either in the presence of 1×10^{-4} M C–S–S–CH_3 or in flasks containing 7×10^5 adhered macrophages per 75 cm^2 of surface. Differential counts were determined on each cell preparation after spreading a sample in a cytocentrifuge and staining with Wright's stain. Each cell preparation was washed with PBS, suspended in Hepes-buffered saline [8], disrupted by sonication [1], and centrifuged at 10,000*g* for 20 min to obtain crude cell extract.

RESULTS AND DISCUSSION

Sulfhydryl-Dependent Cell Proliferation

For continuous proliferation of many cells lines in vitro, it has been found necessary to add a sulfhydryl compound to medium containing fetal calf serum.

Most of these sulfhydryl-dependent cells are malignant murine lymphocytes or lymphocyte-derived plasma cells [3, 11–18], but other cells reported to have this requirement include normal murine lymphocytes [19, 20], malignant human plasma cells [21], malignant human lymphocytes [22], and murine teratocarcinoma cells [23].

Sulfhydryl compounds are also known to stimulate normal immune systems in vitro. Immune systems stimulated in this way include antibody production by B lymphocytes [24], cytotoxicity of T lymphocytes to allogenic cells [25], and [^{3}H]thymidine uptake by lymphocytes exposed to allogeneic cells [25] or mitogens [26]. In these systems, the sulfhydryl compound in the presence of FCS appears to replace the macrophage component of the cell systems (loc. cit.).

A variety of sulfhydryl compounds have been tested for ability to support proliferation of dependent cells in the presence of fetal calf serum [11, 16]. Many sulfhydryl compounds have been found to be active. It appears that the ability to pass through the cell membrane is the criterion determining activity since molecules with charged groups and no relationship to known physiological transport mechanisms have been found to be inactive [16]. Two compounds, mercaptoethanol and thioglycerol, are exceptional in that they support some proliferation of dependent cells in the absence of serum [3]. The optimal concentration for different sulfhydryl compounds varies greatly, from 10^{-6} to 10^{-3} M [11, 16], and most compounds are toxic at supraoptimal concentrations [11]. The compound preferred by the author is glutathione (2×10^{-3} M) since it is not rapidly autoxidized [11] and does not show any toxicity [11].

Several sera have been tested for ability to support proliferation of dependent cells in the presence of GSH. The results are shown in Table I. Horse, bovine calf, and new-born calf sera failed to support significant growth of de-

TABLE I. Effect of Various Sera in Cell Cultures*

Serum	MOPC 21aSH			LPC-1	Friend cell
	No add'n	GSH	C–S–S–CH_3		
Fetal calf	1[a]	41	41	35	38
Fetal calf, dialyzed	1[a]	40	40	35	36
Fetal calf, GSH treated	1[a]	2	30	35	35
Horse (GIBCO)	1[a]	1[a]	39	32	35
Horse (Flow)	1[a]	1[a]	38	35	36
Horse, dialysed	1[a]	1[a]	40	32	35
Horse, heated	1[a]	1[a]	40	33	34
New-born calf	1[a]	3	39	30	35
New-born calf, dialyzed	1[a]	1[a]	38	30	35
Bovine calf	1[a]	1[a]	38	32	36
Bovine calf, heated	1[a]	1[a]	38	30	35
None	1[a]	1[a]	1[a]	1[a]	1[a]

*Inoculum cells were washed and suspended in culture medium at a density of 1×10^5 cells/ml. The sera were added at a concentration of 10%. Other additions were GSH, 2×10^{-3} M, or C–S–S–CH_3, 1×10^{-4} M. Cultures of 1.5 ml were contained in 35-mm dishes. Other conditions were as described under Methods.

[a]Inoculum cells dead.

pendent cells in the presence of 2×10^{-3} M GSH but all of these sera supported growth of the sulfhydryl-independent cells, LPC-1, and Friend cell. Dialysing these sera against balanced salt solution or heating them at 60°C for 45 min did not alter their effect in these cultures. All of the sera supported growth of the dependent cells in medium supplemented with 1×10^{-4} M C–S–S–CH_3 (see further discussion below).

The results of this limited sampling indicate that fetal calf serum may be unique among commercially available sera in its ability to support cell proliferation in systems of this type. This conclusion is corroborated by the fact that fetal calf serum was used in all of the previously reported sulfhydryl-dependent systems with the exception of systems using mercaptoethanol which is effective with other sera [16] or, as noted above, even with no serum [3].

Methylthio Disulfides Replace Sulfhydryl Compounds

In a previous report, it was shown that sulfhydryl compounds can be replaced by methylthio disulfides in supporting proliferation of dependent cells [3]. It has also been found that methylthio disulfides give an effect in mixed leukocyte cultures [27] identical with the effect of mercaptoethanol (J. W. Harris, personal communication). The disulfides are provided as compounds of the type R–S–S–CH_3 where the R group can be any of several groups tested. The most convenient source of RS is cysteine [3] or glucose-1-SH (unpublished). The CH_3–S group is shown to be the essential component of the disulfides since neither cystine (C–S–S–C) nor cysteine (C–SH), added in the amounts provided by the optimal concentration of C–S–S–CH_3 (1×10^{-4} M), are able to support proliferation of the dependent cells [3, 11]. The fact that compounds of the type R–S–S–CH_3 will replace sulfhydryl compounds shows that it is not the reducing effect of the sulfhydryl compounds which is ultimately required by the cells. It was shown that C–S–S–CH_3 can support continuous proliferation of dependent cells for several months in vitro in the absence of serum [3]. These findings indicate that the methylthio disulfide replaces a factor previously provided by the combination of a sulfhydryl compound and FCS.

Methylthio Groups in Fetal Calf Serum

It was previously reported that treating FCS with a reducing agent in a diffusion system causes the release of a volatile compound capable of reacting with dithionitrobenzoic acid (a reagent for measuring sulfhydryl compounds) [3]. The volatiles from FCS have been analyzed by gas chromatography–mass spectroscopy as described under Methods. The results are shown in Figure 1. The elution pattern of particles of mass 47 shows a peak emitting at 22.7 min which displays unequivocally the mass spectrum of methyl mercaptan [28]. When authentic methyl mercaptan is generated in the same system, a much larger peak with mass 47 elutes at 22.5 min and displays the mass spectrum of methyl mercaptan. These analyses show that fetal calf serum contains methylthio groups but quantitative data are not yet available. Unfortunately, sera other than FCS have not been subjected to this analysis.

When FCS is dialyzed against GSH as described in Methods, it loses its ability to support growth of methylthio-dependent cells in the presence of sulfhydryl compounds (Table I). The treatment with GSH appears to remove spec-

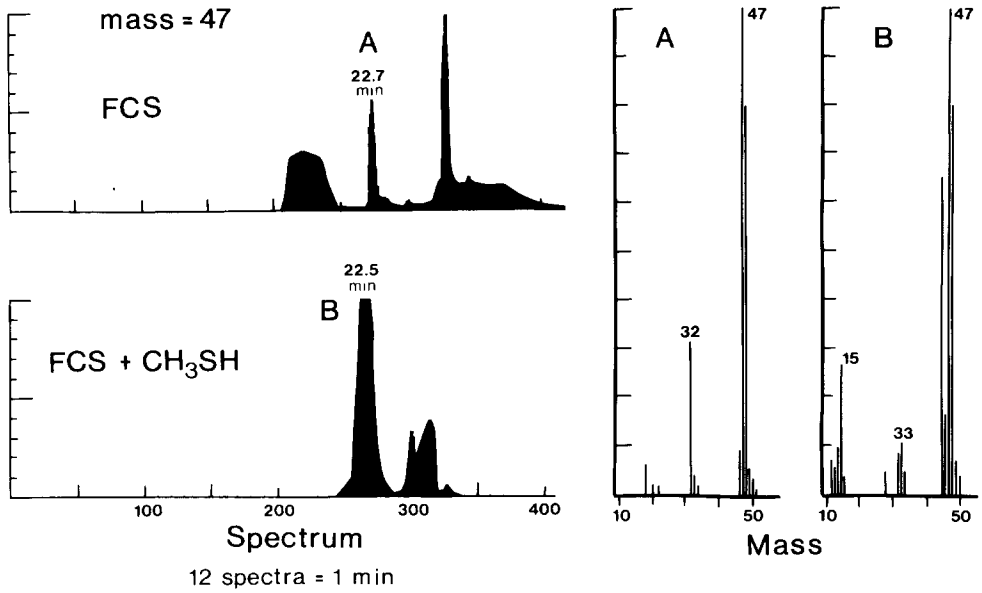

Fig. 1. GC-MS Analysis of the volatiles from fetal calf serum. Analyses were carried out as described under Methods. The elution profiles of particles of mass 47 from the gas chromatograph are shown on the left: (A) elution profile for FCS alone, full scale deflection on the vertical axis is 4600 units; (B) elution profile when methyl mercaptan was generated in the system with FCS, full scale deflection on the vertical axis is 26,200 units. Mass spectra are shown on the right: (A) spectrum of the peak eluting at 22.7 min in elution profile A; (B) spectrum of the peak eluting at 22.5 min in elution profile B.

ifically the methylthio growth factor since the serum is still capable of supporting growth of dependent cells if C–S–S–CH_3 is added and since it supports growth of methylthio-independent cells (Table I).

The above results suggest that CH_3–S groups are present in FCS as disulfides of serum proteins. Disulfides of proteins and low molecular weight thiols have been shown to occur in many natural sources (see [29], p 123, for a concise review). Of particular relevance is the report by Ferdinand et al describing a disulfide between a purified bacterial protease and an unidentified volatile mercaptan; the latter was released on treating the protein with excess reducing agent [30].

If the CH_3–S is present in FCS as a disulfide of this type, then treating with excess sulfhydryl compound (RSH) as under Methods, above, or in Ref. [3] would result in the liberation of methyl mercaptan according to the following equation:

protein-S–S–CH_3 + 2 R–SH → protein-SH + CH_3–SH + R–S–S–R.

Addition of appropriate amounts of sulfhydryl compounds would result in an equilibrium with the formation of disulfides of the type R–S–S–CH_3:

protein-S–S–CH_3 + R-SH ⇋ protein-SH + R–S–S–CH_3.

R–S–S–CH_3 could be formed also by double disulfide exchange if a catalytic amount of free SH were present ([29],p 126). The results of Broome, in which small disulfides were active in medium containing cysteine [16], can be interpreted in this way:

$$\text{protein-S-S-CH}_3 + \text{R-S-S-R} \xrightarrow{\text{X-SH}} \text{protein-S-S-R} + \text{R-S-S-CH}_3.$$

Disulfides of the type R–S–S–CH_3 are stable under cell culture conditions and, by analogy with the behavior of other small disulfides [32], would be expected to pass readily through the cell membrane if R–S contains the essential features (see above). The above hypothetical mechanism makes the CH_3–S present in FCS available to cells and provides an explanation for the effect of sulfhydryl compounds in supporting proliferation of methylthio-dependent cells.

Macrophages Replace Methylthio Groups

Macrophages and their precursor cells, monocytes, are the phagocytic cells of the reticuloendothelial system. They are essential in immune systems, not only for their role in processing antigens, but also as the source of growth factor(s) which cause lymphocytes to proliferate [31]. As noted previously, sulfhydryl compounds appear to replace the macrophage growth factor in the latter function. Also, it has been shown that macrophages and sulfhydryl compounds are interchangeable in supporting continuous in vitro proliferation of several sulfhydryl-dependent lymphocytic cell lines [13–15]. Therefore, the effect of macrophages was determined in cultures of the methylthio-dependent cells described above.

Adhered mouse peritoneal macrophages support good growth of L1210, P388, and MOPC 21aSH cells in the absence of methylthio additives (Table II). By varying the number of adhered macrophages, it is shown that the growth response in 3 days varies with the number of macrophages. At low macrophage numbers, the ratio of dependent cells to macrophages is 50 to 100. As the number of macrophages per dish is increased, the ratio decreases and macrophages in excess of 1×10^5 per dish are inhibitory. An inhibitory effect of high numbers of macrophages has been observed in other systems in which lower numbers are stimulatory [14, 24, 33, 34].

Human peripheral blood monocytes or rabbit alveolar macrophages can be used in place of mouse peritoneal macrophages in the system described in Table II, indicating that production of the growth factor is not specific to mouse cells.

TABLE II. Growth of Methylthio-Dependent Cells in Direct Contact With Macrophages*

Macrophages per dish	Cells per ml ($\times 10^5$) on Day 3		
	L1210	P388	MOPC 21aSH
None	1	1	1
1×10^4	1	3	1
2×10^4	6	9	11
5×10^4	27	21	42
1×10^5	36	33	47
2×10^5	14	21	20
5×10^5	10	15	14
None, C–S–S–CH_3 1×10^{-4} M	31	38	48

*Mouse peritoneal macrophages were used as described in Methods.

Adhered macrophages continue to support proliferation of dependent cells for long periods of time under appropriate conditions. Thus, in the system described in Table II, if 95% of the culture of free-floating cells is removed every third day and replaced by fresh medium, rapid proliferation can be maintained for at least 4 weeks with the same underlay of macrophages. If the proliferating cells are returned to standard culture conditions after that time, they are still methylthio-dependent (no data shown). This experiment suggests an analogy to the explantation of methylthio-dependent cells from in vivo to in vitro culture. Macrophages present in the initial explant are lost by adherence or dilution during subculturing and the proliferating cells then become dependent on an artificial source of methylthio groups.

In many of the studies cited in this report, medium conditioned by exposure to macrophages was used rather than macrophages themselves for supporting proliferation of dependent cells. I have not been able to find conditions for producing macrophage-conditioned medium capable of supporting growth of methylthio-dependent cells. However, it can be demonstrated in a diffusion system that macrophages release a diffusable factor which supports proliferation of these cells (Table III). In this system, the macrophages are separated from the responding cells by several millimeters of medium and a dialysis membrane. The growth response in 3 days varies with the number of macrophages, the optimal number being 3 x 10^5 macrophages per 35-mm dish. Tanapat et al [15] have also demonstrated that macrophages release a diffusable factor which supports growth of sulfhydryl-dependent L1210 cells.

In a series of 12 macrophage preparations, 50% of them released the growth factor only when endotoxin was added while the other 50% did so in the absence of added endotoxin (Table III). In view of the difficulty in avoiding endotoxin contamination, it is likely that endotoxin (or some macrophage activator) is required for the release of the growth factor by macrophages. In this context, it should be noted that sera from mice pretreated with endotoxin [12] or Freund's adjuvant [34] were shown to contain a growth factor capable of supporting in vitro proliferation of murine plasmacytoma cells. Since the sera

TABLE III. Growth of Methylthio-Dependent Cells in Diffusion Chambers With Macrophages*

Expt. No.	Additions to outer chamber		MOPC 21aSH (cells/ml (× 10^5))
	Macrophages	Other additions	
1	None	LPS	1
	5×10^4	LPS	6
	1×10^5	LPS	18
	3×10^5	LPS	37
	3×10^5	None	1
	None	C–S–S–CH_3	35
	None	GSH	38
2	None	None	1
	3×10^5	None	30

*MOPC 21aSH cells were used under the experimental conditions described under Methods. Other additions were: endotoxin (LPS), 5 μg; C–S–S–CH_3, 0.4 μmol; GSH, 6 μmol.

could be replaced by macrophages, it was concluded that macrophages in the treated mice were the source of the factors in the sera [12, 34].

The data described or referenced previously relate to the effect of macrophages on lymphocytic cells which appear, in general, to have a requirement for sulfhydryl or methylthio compounds. It should be noted that macrophages have been reported to stimulate proliferation of a variety of other cells which have not been characterized with respect to methylthio requirement. Such cells include endothelial cells [35, 36], smooth muscle cells [36], fibroblasts [37], and various malignant cells [38, 39]. Although more difficult to interpret than in vitro results, evidence for in vivo stimulation of cell proliferation by macrophages has also been presented. Macrophages or macrophage-containing cell suspensions, when admixed with tumor cells prior to injection into animals, appeared to enhance the in vivo growth of murine fibrosarcomas [40–42], carcinomas [41], and sarcomas [42, 43]. Implantation of activated macrophages in normally avascular corneas caused vascular proliferation [44, 45]. Based on these and other observations, macrophages have been attributed a stimulatory role in vivo in wound healing and fibroplasia [37, 45], neovascularization [44, 45], tumor angiogenesis [46], and cancer growth [46, 47] (see further discussion below).

Methylthio Group Generation From Methylthioadenosine

Many cell lines grow well in vitro in the absence of macrophages, sulfhydryl compounds, or methylthio disulfides. A study of extracts of cells of this type revealed the presence of an enzyme system which uses methylthioadenosine as substrate and catalyzes the formation of an ether-extractable product which contains the CH_3 group and the S atom but no ribose and no adenine [1]. This system was later shown to involve two enzymes, methylthioadenosine nucleosidase which catalyzes the phosphorolysis of methylthioadenosine to yield methylthioribose-1-phosphate and adenine and a new enzyme, given the name methylthiolase, which uses methylthioribose-1-phosphate as substrate and catalyzes the cleavage of the methylthio group from ribose [8]. These cells are, therefore, capable of generating methylthio groups according to the following reaction sequence:

(1) methionine + ATP → S-adenosylmethionine (SAM)
(2) SAM → CO_2 + descarboxy-SAM
(3) descarboxy-SAM + (amine) → polyamine + methylthioadenosine
(4) methylthioadenosine + PO_4 → methylthioribose-1-PO_4 + adenine
(5) methylthioribose-1-PO_4 → "CH_3–S" + ribose + PO_4.

Methylthio-dependent cells lack detectable methylthioadenosine nucleoside phosphorylase activity and this defect is thought to account for the requirement for methylthio groups in these cells [8]. A corollary of this theory is that methylthio (or related) groups are required for division of all cells. The exact nature of the "CH_3–S" product and its function in cell division are not known.

The generation of methylthioadenosine via reactions (1) to (3) above occurs ubiquitously in dividing cells and the rate of this process is known to be

correlated with the rate of cell division [48, 49]. Methylthioadenosine is also formed by direct cleavage of homoserine lactone from S-adenosylmethionine in pig liver [50], but this is not considered to be a major pathway in most animal cells.

Methylthio Group Generation From Methylthioadenosine by Macrophages and Other Cells

Results described previously show that macrophages produce a factor capable of supporting proliferation of methylthio-dependent cells. Assuming that the methylthio group is the factor which is ultimately required by the cells, the macrophages could produce their stimulatory effect either by secreting methylthio groups per se or by producing a factor which activates the methylthio group generating system in the responding cells. The latter mechanism does not appear to operate since L1210 cells grown in macrophages, like those grown with C–S–C–CH_3, do not contain any detectable methylthioadenosine phosphorylase activity (Table V).

However, it appears that intact macrophages can release methylthio groups from methylthioadenosine into the medium, as demonstrated in Table IV. Adhered macrophages exposed to [methyl-^{14}C] or [^{35}S]-labeled methylthioadenosine caused the release of both labels into the medium in an ether-extractable form. The product has not been conclusively identified but the facts that it is ether extractable and that the two labels are released in roughly equivalent amounts indicate that the product contains the methylthio group. The cumulative yield of labeled product in 3 days is equivalent to a molarity of about 4×10^{-5}. The optimal concentration of C–S–S–CH_3 is 1×10^{-4} M in cultures of dependent cells [3]. Therefore, under conditions of equilibrium, with both macrophages and dependent cells in the same medium, it is likely that macrophages are capable of providing methylthio groups at a rate sufficient to sustain maximal growth of dependent cells.

The activities of the enzymes, methylthioadenosine nucleoside phosphorylase and methylthiolase, have been determined in extracts of macrophages and several other normal cells (Table V). Macrophages have relatively high activities of both enzymes. Red blood cells contain nucleosidase activity but no demonstrable methylthiolase activity. Platelets contain nucleosidase activity but questionable methylthiolase activity. Granulocytes and fibroblasts contain nucleosidase activity and low but definite methylthiolase activity. It is difficult to obtain an accurate estimate of the enzyme activities in lymphocytes.

TABLE IV. Incubation of Methylthioadenosine With Adhered Macrophages

Substrate	Macrophages	Ether-extractable product (nmol/dish)		
		Day 1	Day 2	Day 3
[^{35}S]MTA	3×10^5	11	19	15
	None	1	1	1
[methyl-^{14}C]MTA	3×10^5	10	17	11
	None	1	2	1

TABLE V. Enzyme Assays on Extracts of Various Cells

Cell type	Specific enzyme activity (nmol product/mg protein)	
	Nucleosidase	Methylthiolase
Macrophages (mouse peritoneal)[a]	23	9
Macrophages (rabbit alveolar)[b]	18	11
Red blood cells	3[c]	0
Platelets	27	0.5 (?)
Granulocytes	24	3
Lymphocytes[d]	7	1
Fibroblasts	7	1
L1210 grown with C–S–S–CH_3	0	15[e]
L1210 grown on macrophages	0	13[e]

[a]Macrophages, 70%; granulocytes, 5%; lymphocytes, 25%.
[b]Macrophages, 85%: granulocytes, 10%; lymphocytes, 5%.
[c]This specific activity is artificially low because of the large amount of hemoglobin in the extract.
[d]Lymphocytes, 98%; monocytes, 2%.
[e]Partially purified methylthioadenosine nucleosidase from MOPC 21 cells [8] was added.

Because of the low cytoplasmic to nuclear ratio of lymphocytes, it is necessary to extract large numbers of cells (5×10^8 cells) in order to obtain suitable amounts of extract protein. Because, on the scale required, it is impossible to obtain lymphocytes free of monocytes and platelets, both of which have high cytoplasmic ratios and high specific enzyme activities, the extracts from lymphocytes preparations are significantly contaminated with enzymes from these other cells. Therefore, the specific enzyme activities shown for lymphocytes in Table V, although low relative to the values for some other cells, are probably erroneously high. Also, it is possible that some subpopulations of lymphocytes contain the enzymes for methylthio group generation while other subpopulations do not.

Methylthioadenosine nucleoside phosphorylase has been shown to be present in rat prostate tissues [51] and other mammalian tissues [52] and in the experimental tumors, sarcoma 180 and lymphoma L5178Y [53], but methylthiolase activity was not measured in these tissues. In any case, such measurements would not yield information on tissue-specific activities unless the tissue was first freed of blood and other cells such as Kupffer cells in liver. Moreover, in order to demonstrate maximal activities of these enzymes, it may be necessary to use tissues which are in an active state of growth or regeneration.

There are many examples of "feeder cells" which are used in cell culture to support proliferation of difficulty cultured cells (for a review of feeder cells used in human cell culture, see [22]). Specific analyses have not been carried out but it is possible that these feeder cells, at least in some cases, may be serving as a source of methylthio groups for the dependent cells. Evidence for this prediction is exemplified by the system in which a murine embryonal carcinoma cell line, normally cultured on a feeder layer of epithelioid cells, will

grow well in the absence of the feeder cells if provided with mercaptoethanol in the presence of 10% FCS [54].

Hypothesis

The fact that either macrophages or methylthio groups are required for proliferation of certain lymphocytic cells in vitro together with the finding that macrophages are capable of generating and secreting methylthio groups lead to the hypothesis that macrophages exert their stimulatory effect on lymphocyte proliferation by providing methylthio groups. This hypothesis not only provides an explanation for the stimulatory effect of macrophages in normal immune systems but may also provide a basis for implicating macrophages in promoting the proliferation of certain malignant cells in vivo. A stimulatory role of macrophages in malignant cell proliferation in vivo has been previously suspected. In 1971, Prehn and Lappe advanced the theory that cells of the immune system stimulate growth of cancers [55] but this "immunostimulation theory" does not identify the macrophage as the effective cell [56]. Cancro and Potter [57] and Metcalf [12] have speculated that adherent peritoneal cells facilitate the growth of mineral oil-induced plasmacytomas in ascites culture. In 1977, Evans discussed the possible involvement of infiltrating macrophages in promoting growth of solid tumors and speculated that some solid tumors are dependent on macrophages for a growth factor while other tumors produce the factor themselves [46]. In 1978, Salmon and Hamburger hypothesized that macrophages stimulate proliferation of both normal lymphocytes and certain malignant cells [47]. The evidence summarized in this report supports that hypothesis and extends it by defining a possible chemical mechanism. Circumstantial evidence indicates that the chemical mediator in this relationship is the methylthio group or a derivative of it.

In applying this hypothesis to cancers, it is interesting to consider the physical relationship between macrophages and malignant cells in cancer tissue. It appears that macrophages infiltrate most, if not all, solid tumors [58–60], sometimes in very large numbers. It has been reported that up to 50% of the cells in solid cancers are macrophages both in experimental animal tumors [58] and in human cancer tissues [59]. Migration in the opposite direction also occurs with high frequency as seen in the metastasis of cancers to reticuloendothelial sites (including nerve tissue, where the macrophages are represented by the glial cells). Thus, it appears that, in all cancer tissue, the malignant cells are closely associated with macrophages.

In this relationship, it is also necessary to consider the requirement of the malignant cells for exogenous methylthio groups. Only cells which are deficient in the ability to generate these groups would be expected to benefit from an external source. For example, the murine plasmacytomas, LPC-1 and MOPC 21, which have been shown to contain highly active methylthio group generating systems [1, 8], would not be expected to benefit from an association with macrophages in vivo according to the hypothesis. No malignant human cells have been characterized with respect to this enzyme system. However, the results of Epstein and Kaplan show that the in vitro growth of many malignant human lymphocytic cells is markedly stimulated by sulfhydryl compounds in the presence of 20% fetal calf serum [22]. By analogy with the data described

previously for murine lymphocytic cells, these results suggest that these malignant human cells are also methylthio dependent. Similarly, the demonstration by Hamburger and Salmon of macrophage-dependent growth in vitro of human plasmacytoma cells [21] and other cancer cells [39] suggests that these cells may be methylthio dependent.

In summary, the available evidence supports the hypothesis that macrophages promote proliferation of certain cells, particularly lymphocytes in the normal immune system and malignant cells in methylthio dependent cancers. In the immune system, macrophages may achieve amplification of the immune response by promoting lymphocyte proliferation (aside from the other functions of macrophages in this system). In cancers, the macrophage is functionally designed to destroy and remove cancer cells (a well-documented function not discussed in this report) but, by an accident of nature, they may actually promote growth of the cancer. In both systems, macrophages are thought to promote cell proliferation by providing methylthio groups which appear to have an essential, but presently unknown, role in cell division.

ACKNOWLEDGMENTS

The author is grateful to Dr. A. B. Robinson and Mark Weiss of the Linus Pauling Institute of Science and Medicine, Menlo Park, CA, for carrying out gas chromatography–mass spectrocopy analyses.

REFERENCES

1 Toohey JI Biochem Biophys Res Commun 78 1273, 1977
2 Potter M Physiol Rev 52 631, 1972
3 Toohey JI, Cline MJ Biochem Biophys Res Commun 70 1275, 1976
4 Robinson AB, Partridge D, Turner M, Teranishi R, Pauling L J Chromat 85 19, 1973
5 Matsumoto KE, Partridge DH, Robinson AB, Pauling L, Flath RA, Mon TR, Teranishi R J Chromat 85 31, 1973
6 Boyum A Scand J Clin Lab Invest Suppl 21 77, 1968
7 Marbrook J Lancet 2 1279, 1967
8 Toohey JI Biochem Biophys Res Commun 83 27, 1978
9 English D, Andersen BR J Immunol Methods 5 249, 1974
10 Beutler E "Red Cell Metabolism A Manual of Biochemical Methods New York Grune & Stratton, 1975, p 10
11 Toohey JI Proc Natl Acad Sci USA 72 73, 1975
12 Metcalf D J Immunol 113 235, 1974
13 Nathan CF, Terry WD J Exp Med 142 887, 1975
14 Hewlett G, Opitz HG, Schlumberger HD, Lemke H Eur J Immunol 7 781, 1977
15 Tanapat P, Gaetjens E, Broome JD Pros Soc Exp Biol Med 157 517, 1978
16 Broome JD, Jeng MW J Exp Med 138 574, 1973
17 Albrecht AM, Biedler JL, Hutchison DJ, Spengler BA, Stockert E Cancer Res 36 3784, 1976
18 Pourreau-Scheider N J Natl Cancer Inst 55 1467, 1975
19 Metcalf D J Immunol 116 635, 1976
20 Melchers F, Coutinho A, Heinrich G, Andersson J Scand J Immunol 4 853, 1975
21 Hamburger A, Salmon SE J Clin Invest 60 846, 1977
22 Epstein AL, Kaplan HS Cancer Res 39 1748, 1979
23 Rizzino A, Sato G Proc Natl Acad Sci USA 75 1844, 1978
24 Chen C, Hirsch JG J Exp Med 136 604, 1972
25 Bevan MJ, Epstein R, Cohn M J Exp Med 139 1025, 1974

26. Fanger MW, Hart DA, Wells JV, Nisonoff A: J Immunol 105:1043, 1970.
27. Harris JW, MacDonald HR, Engers HD, Fitch FW, Cerottini J: J Immunol 116:1071, 1976.
28. Selected Mass Spectral Data vol E-54 no. 2, serial 78 Thermodynamics Research Center and the American Petroleum Inst, Texas A & M University, 1977.
29. Jocelyn PC: "Biochemistry of the SH Group." London: Academic Press, 1972, p 122.
30. Ferdinand W, Stein WH, Moore S: J Biol Chem 240:1150, 1965.
31. Unanue ER: Amer J Pathol 83:396, 1976.
32. Eldjarn L, Bremer J, Borresen HC: Biochem J 82:192, 1962.
33. Kurland JI, Kincade PW, Moore MAS: J Exp Med 146:1420, 1977.
34. Namba Y, Hanaoka M: J Immunol 109:1193, 1972.
35. Wall RT, Harker LA, Quadracci LJ, Striker GE: J Cell Physiol 96:203, 1978.
36. Greenburg GB, Hunt TK: J Cell Physiol 97:353, 1978.
37. Leibovich SJ, Ross R: Amer J Pathol 84:501, 1976.
38. Evans R: In Fink MA (ed): "The Macrophage in Neoplasia." New York: Academic Press, 1976, p 27.
39. Hamburger AW, Salmon SE: Science 197:461, 1977.
40. Evans R: Br J Cancer 37:1086, 1978.
41. Small M, Trainin N: J Immunol 117:292, 1976.
42. Deckers PJ, Ramming KP, Pilch YH: Cancer 27:897, 1971.
43. Prehn RT: Science 176:170, 1972.
44. Clark RA, Stone RD, Leung DYK, Silver I, Hohn DC, Hunt TK: Surg Forum 27:16, 1976.
45. Polverini PJ, Cotran RS, Gimbrone MA, Unanue ER: Nature (London) 269:804, 1977.
46. Evans R: In James K, McBride B, Stuart A (eds): "The Macrophage and Cancer." Edinburg: European Reticuloendothelial Society, 1977, p 321.
47. Salmon SE, Hamburger AW: Lancet 1:1289, 1978.
48. Raina A, Janne J: Med Biol 53:121, 1975.
49. Tabor CW, Tabor H: Ann Rev Biochem 45:285, 1976.
50. Swiatek KR, Simon LN, Chao K: Biochemistry 12:4670, 1973.
51. Pegg AE, Williams-Ashman HG: Biochem J 115:241, 1969.
52. Garbers DL: Biochim Biophys Acta 523:82, 1978.
53. Savarese TM, Crabtree GW, Parks RE: Biochem Pharmacol 28:2227, 1979.
54. Oshima R: Differentiation 11:149, 1978.
55. Prehn RT, Lappe MA: Transplant Rev 7:26, 1971.
56. Prehn RT: Transplant Rev 28:34, 1976; J Nat Cancer Inst 59:1043, 1977.
57. Cancro M, Potter M: J Exp Med 144:1554, 1976.
58. Evans R: Transplantation 14:468, 1972.
59. Wood GW, Gollahon KA: J Nat Cancer Inst 59:1981, 1977.
60. Gauci CL, Alexander P: Cancer Lett 1:29, 1975.