Full Replacement of 2-Mercaptoethanol by Cysteine Plus Selenium Compounds in Augmenting DNA Synthesis of Mitogen-Stimulated Mouse Spleen Lymphocytes

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ABSTRACT. Mouse spleen lymphocytes require 2-mercaptoethanol for maximal mitogenic activation *in vitro*. Previous studies indicate that the lymphocytes are defective in the cystine transport activity and that they require 2-mercaptoethanol to utilize cystine. 2-Mercaptoethanol catalytically carries cysteine moiety into the cells in a mixed disulfide form. Because cysteine is easily oxidized to cystine in the culture medium, it has been not easy to precisely examine the effect of near-physiological concentrations of cysteine on the activation of lymphocytes. By controlling the cysteine content in the medium, we have reviewed the effect of cysteine to see if cysteine replaces 2-mercaptoethanol in enhancing the DNA synthesis of lipopolysaccharide-stimulated lymphocytes. It was found that cysteine was less effective than 2-mercaptoethanol, and that cysteine fully replaced 2-mercaptoethanol when a selenium compound was supplemented. The effects of cysteine and selenium compounds were apparently independent and additive. Among the selenium compounds examined, sodium selenite and L-selenocystine were much more effective in stimulating DNA synthesis than sodium selenate and L-selenomethionine.

Click *et al.* (7) and Chen and Hirsch (6) showed that 2-mercaptoethanol (2ME) markedly enhanced the immune responses of mouse spleen lymphocytes *in vitro*. Then, Broom *et al.* (3) showed that many mouse neoplastic lymphoid cells also required 2ME for cell growth *in vitro*. As a result of these studies, 10–50 μ M of 2ME has been routinely added to the culture media to enhance growth of murine lymphoid cells *in vitro*. We attempted to replace 2ME by various physiological substances to understand the physiological meaning of the effect of 2ME. We previously showed that in mouse lymphoma L1210 cells, near-physiological concentrations of cysteine enhanced cellular GSH content and growth of L1210 cells to the same extent as 2ME (14, 16, 17). This was based on the facts that 2ME-dependent cells required cysteine because they had a low cystine transport activity (12, 14) and that 2ME catalytically carried cysteine into cells at a high rate in a mixed disulfide form via the system(s) shared by neutral amino acids with bulky side chains, such as leucine and phenylalanine (15). It should be noted that in normal culture media, cystine but not cysteine, is available because cysteine is easily oxidized to cystine in

Abbreviations used: 2ME, 2-mercaptoethanol; GSH, glutathione; BCS, bathocuproinedisulfonate; LPS, lipopolysaccharide; Con A, concanavalin A; GSHPx, glutathione peroxidase.

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the media, although cysteine is maintained in body fluid.

In mouse spleen lymphocytes, 2ME also improved the availability of cystine (4, 25, 26) and thereby 2ME increased cellular glutathione (GSH) (35), a major metabolite of cysteine. Compared with 2ME, cysteine may be a more important physiological immunomodulator because the cysteine transport activity increases in the lymphocytes during their stimulation with mitogens (18), and macrophages have the ability to produce cysteine (34). The question which we investigate in this paper is whether cysteine fully replaces 2ME in enhancing DNA synthesis of the mitogen-stimulated lymphocytes. Although supplementing cysteine to culture media enhanced the immune responses *in vitro* (8, 11, 25), the above question was not accurately answered because of the instability of the cysteine supplemented to the culture media. Controlling the cysteine content, we have shown that cysteine is less effective than 2ME in enhancing DNA synthesis of the mitogen-stimulated lymphocytes in contrast to the growth of L1210 cells. We have found, however, that infusing selenium compounds in addition to cysteine enhanced the DNA synthesis to be as effective as 2ME. The results herein indicate that an antioxidative activity of 2ME other than enhancing cellular GSH is also important for maximal proliferation of the mitogen-stimulated lymphocytes *in vitro*.

MATERIALS AND METHODS

Materials. The materials used in this work were as follows: [methyl-³H]-thymidine (20.0 Ci/mmol), New England Nuclear, Boston, MA; Ficoll-Paque, Pharmacia Fine Chemicals, Uppsala, Sweden; RPMI 1640 medium, Gibco Lab., Grand Island, NY; Lipopolysaccharide (LPS) (W, *S. typhosa* 0901), Difco Lab., Detroit, MI; 4,7-diphenyl-2,9-dimethyl-1, 10-phenanthroline disulfonic acid disodium salt (bathocuproinedisulfonate, BCS), Dojindo Lab., Kumamoto, Japan; L-selenomethionine, Sigma, St. Louis, MO; sodium selenite, sodium selenate, and L-cysteine (free base), Wako Pharmaceutical Company, Osaka, Japan. The majority of the fetal bovine serum used in this study was from Commonwealth Serum Lab. (lot No. 279-2), Melbourne, Australia. An additional 14 lots of fetal bovine sera used were from Commonwealth Serum Lab., Filtron (Victoria, Australia), Flow Lab. (McLean, VI), M.A. Bioproducts (Walkersville, MD), and K.C. Biological (Lenexa, KA). α -Tocopherol was provided by Eisai Tsukuba Research Lab., (Ibaraki, Japan). Enzymatically synthesized L-selenocystine (9) was provided by Dr. K. Soda (Kyoto Univ.).

Cell preparations and cultures. Both sexes of C57BL/6N mice (Doken Lab., Ibaraki, Japan), ranging in age from 8 to 12 weeks, were used. The mice were fed a commercial diet (MF, Oriental Yeast Co., Tokyo, Japan) containing 0.46 ppm selenium. Mouse spleens (usually one or two) were squeezed between frosted microslide glass in the culture medium, and the cell suspension was allowed to stand in a plastic tube for a few minutes in order to sediment large tissue aggregates. Then, the upper cell suspension was layered on Ficoll-Paque and centrifuged. After centrifugation, the lymphocyte fraction was separated and the cells were washed three times in a serum-free culture medium. The medium used for the cell preparation and culture was RPMI 1640 medium supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum, 1 mM pyruvate, penicillin (50 units/ml) and streptomycin (50 μ g/ml). The lymphocytes thus fractionated were suspended in the complete culture medium, and after the cell density was adjusted to 10⁶ cells/ml, LPS (10 μ g/ml) was added and seeded in culture plates or flasks. The cells were cultured in an atmosphere of 95% air and 5% CO₂ at 37°C. The number of cells were counted using a hemocytometer. The viability of the cells was estimated by the nigrosine (0.05%) exclusion method.

Assays. For assays of [^3H]thymidine incorporation, triplicate cultures were set up in flat-bottom, 24-well plates with a total volume of 1.0 ml (1.0×10^6 cells) per well. Supplements such as cysteine, BCS, selenium compounds and 2ME were added to each well at the initiation of the culture. The stock solutions (10 or 100 mM cysteine, 2 mM BCS, and 10 mM 2ME in distilled water) were maintained at -20°C . In some experiments, cysteine was added at 8- or 12-h intervals starting at time 0. GSHPx and other enzymes were dissolved in the culture medium and filtered for sterilization. α -Tocopherol was first solubilized with ethanol and filtered for sterilization after dilution with fetal bovine serum. At the indicated time, 0.2 or 1 μCi of [^3H]thymidine (diluted with unlabeled thymidine to 0.4 or 2 Ci/mmol) was added to each well. After 1 h, 10 μl of 50 mM thymidine was added to each well, then the culture plate was cooled on ice, and the cells were collected on glass fiber filters in order to determine the amount of ^3H incorporated into trichloroacetic acid-insoluble material. Since the amount of radioactivity incorporated into the cells differed from experiment to experiment, standardization of the results was necessary to assess the effect of the supplements. Therefore, in each assay, the percent of radioactivity of the control condition, in which 20 μM 2ME was added for each experiment, was calculated.

Glutathione peroxidase (GSHPx) activity was assayed at 25°C by the method of Paglia and Valentine (27) with hydrogen peroxide as the substrate. The assay mixture contained 61 mM phosphate buffer (pH 7.0), 2.4 mM EDTA, 1 mM GSH, 0.2 mM H_2O_2 , 0.16 mM NADPH, 1 mM NaN_3 , and 0.12 U/ml glutathione-reductase. Lymphocytes were washed twice with cold phosphate buffered saline and then frozen once at -20°C . On thawing, the samples were treated with 2.5 mg/ml digitonin (13), sonicated in an ice-cold bath, and centrifuged at 3000 rpm for 10 min. The supernatant fractions were assayed for GSHPx activity and protein content by the method of Lowry *et al.* (21). Trichloroacetic acid-soluble thiol contents in the culture media were determined by 5,5'-dithiobis(2-nitrobenzoic acid) using cysteine as the standard, as described in a previous paper (18). Selenium contents of some of the sera were determined by an atomic absorption method. α -Tocopherol contents of the sera were determined by the method of Abe and Katsui (1).

RESULTS

Effect of cysteine and BCS on DNA synthesis. It was not clear whether physiological concentrations of cysteine could fully replace 2ME in enhancing DNA synthesis of mitogen-stimulated mouse spleen lymphocytes. In order to answer this question, the effect of cysteine on the time-dependent incorporation of [^3H]thymidine into the cells was compared with that of 2ME as shown in Fig. 1. In this assay, the freshly prepared lymphocytes were stimulated with LPS, and the incorporation of [^3H]thymidine was measured by 1 h-pulse labeling at 23, 35, 47, and 59 h in culture. Adding 100 μM of cysteine to the medium every 12 h significantly enhanced the incorporation of [^3H]thymidine. However, cysteine thus added was far less effective than 20 μM 2ME added only once at the initiation of the culture. The effect of cysteine was enhanced by 20 μM BCS, a nontoxic copper-specific chelating agent which improves the stability of cysteine in the culture medium (18, 24). However, cysteine plus BCS was still far less effective than 2ME, especially after 47 h.

Table 1 compares the effects of various concentrations of cysteine and various time schedules of cysteine addition on the [^3H]thymidine incorporation. In this table, the contents of nonprotein thiols (presumably cysteine) in the culture medium in some experiments at 23 and 47 h are also shown so that approximate cysteine con-

Fig. 1. Effects of the culture supplements on the time-dependent incorporation of [^3H]thymidine. Cysteine ($100\ \mu\text{M}$) was added at 0, 12, 24, 36 and 48 h. BCS ($20\ \mu\text{M}$) and 2ME ($20\ \mu\text{M}$) were added at time 0. The cultures were exposed to $1\ \mu\text{Ci}$ [^3H]thymidine ($2\ \text{Ci}/\text{mmol}$) for 1 h, as indicated.

centrations can be estimated. To maintain near-physiological levels of cysteine in the medium, $100\ \mu\text{M}$ cysteine had to be added every 8 h. Cysteine thus added had enhancing effects comparable to that of 2ME (control) on the first day (23–24 h); however, these effects decreased to roughly 50% of that of 2ME on the second day (47–48 h). When 3 mM cysteine was added at the initiation of the culture, the incorporated [^3H]thymidine at 47 h was 50% of that of the control. Adding $100\ \mu\text{M}$ cysteine every 12 h, or 1 mM cysteine at the initiation of the culture, was less effective than the above conditions of cysteine supply. In the presence of BCS, the oxidation of the added cysteine was inhibited and the effect of cysteine was enhanced. However, the effect of cysteine plus BCS was roughly 50% of that of 2ME on the second day (47–48 h). BCS did not enhance the effect of 2ME on the [^3H]thymidine incorporation. In conclusion, cysteine with or without BCS under various concentrations could not enhance the DNA synthesis as effectively as 2ME on the second day of the culture.

Effects of selenium compounds on DNA synthesis. Examining the reason why cysteine was less effective than 2ME, we found that various selenium compounds added to the culture medium enhanced the [^3H]thymidine incorporation. Figure 2 shows the effects of inorganic selenium compounds, sodium selenite and sodium selenate, on the [^3H]thymidine incorporation at 47 h. In the presence of cysteine ($100\ \mu\text{M}$ /every 12 h) and BCS, selenite at 10^{-7} to $10^{-6}\ \text{M}$ and selenate above $5 \times 10^{-6}\ \text{M}$ showed maximal effects (nearly equal to 2ME), suggesting that selenite was more

TABLE 1. EFFECTS OF CYSTEINE AND 2-MERCAPTOETHANOL (2ME) IN THE PRESENCE OR ABSENCE OF BATHOCUPROINEDISULFONATE (BCS) ON THE DNA SYNTHESIS AND ON THE NONPROTEIN THIOL CONTENTS IN THE CULTURE MEDIUM OF THE LPS-STIMULATED LYMPHOCYTES

Supplements	[³ H]Thymidine Incorporation 1 h-pulse label		Non-protein Thiols in the Medium	
	23–24 h (% cpm of each control)	47–48 h	at 23 h	at 47 h (μ M)
(Without BCS)				
2ME (20 μ M)	100 (control) ^a	100 (control) ^b	4	7.0 $\pm$ 2.0 (n=3)
None	25.0 $\pm$ 2.2 (n=3)	6.9 $\pm$ 3.2 (n=11)	3	5.5 $\pm$ 1.3 (n=4)
Cys (100 μ M/every 8 h)	93	51	9	12
Cys (100 μ M/every 12 h)	37	21, 16		
Cys (1 mM)		21		10
Cys (3 mM)		50		20
(With 20 μ M BCS)				
2ME (20 μ M)		100		27
None		18.4 $\pm$ 8.3 (n=5)	9	7.7 $\pm$ 2.5 (n=3)
Cys (25 μ M/at 4 h and every 8 h) ^c	93	54	15	26
Cys (50 μ M/every 8 h)	123	49.6 $\pm$ 7.6 (n=5)	31	46, 35
Cys (50 μ M/every 12 h)	97	32		
Cys (100 μ M/every 12 h)	82	46.6 $\pm$ 7.3 (n=12)		36
Cys (500 μ M)		53		24

One million fresh lymphocytes were cultured in one ml medium with LPS (10 μ g/ml) and the supplements indicated. Values are the mean of triplicate assays or the mean of more than three independent experiments $\pm$ SEM.

^a The incorporated radioactivities in the controls were, on average, 26.8 $\pm$ 4.4 (n=5) percent of those in the controls labeled between 47 and 48 h.

^b The incorporated radioactivities in the controls labeled with 0.2 μ Ci [³H]thymidine (0.4 Ci/mmol) varied in each experiment between 21890 $\pm$ 1233 and 42180 $\pm$ 1987 cpms per culture.

^c Cysteine (25 μ M) was added at 0, 4, 8, 16, 24, 32, and 40 h.

than one order of magnitude effective than selenate. Without additions of cysteine and BCS, selenite above 5×10^{-8} M and selenate above 5×10^{-6} M significantly enhanced the [³H]thymidine incorporation. The effects of cysteine plus BCS and the selenium compounds appear to be additive under these assay conditions. Selenite was cytotoxic above 5×10^{-6} M if cysteine plus BCS was present.

Figure 3 shows the effects of organic selenium compounds, L-selenocystine and L-selenomethionine, on the [³H]thymidine incorporation. In the presence of cysteine and BCS, selenocystine above 10^{-6} M (as selenium) and selenomethionine above 5×10^{-6} M showed maximal effects. Without the additions of cysteine and BCS, selenocystine and selenomethionine markedly enhanced the [³H]thymidine incorporation.

Table 2 shows viable cell densities at 48 h under different culture conditions. The densities increased markedly in cultures supplemented with cysteine plus BCS plus selenite (or selenocystine), and with 2ME respectively, correlating well with the results of [³H]thymidine incorporation labeled at 47 h (Figs. 2 and 3).

Cellular GSHPx activities. Table 2 also lists cellular GSHPx activities of the lymphocytes freshly prepared and those cultured for 2 days under different culture

Fig. 2. Dose-dependent effects of sodium selenite (●) and sodium selenate (▲), in the presence (—) or absence (---) of cysteine (100 μ M/every 12 h) and BCS (20 μ M), on the [3 H]thymidine incorporation. Values are expressed by % cpm of the control (+2ME). Pulse-labeling was performed between 47 and 48 h.

conditions. The enzyme activities per mg soluble cell protein decreased to 16 and 22% of the initial activity when cultured for 2 days with cysteine plus BCS, and with 2ME, respectively. Since the GSHPx activity may indicate selenium status in the cells, this result suggests that the cells become deficient in selenium under those culture conditions. However, sodium selenite and selenocystine enhanced the enzyme activity only slightly.

Fig. 3. Dose-dependent effects of selenocystine (●) and selenomethionine (▲) in the presence (—) or absence (---) of cysteine (100 μ M/every 12 h) and BCS (20 μ M), on the [3 H]thymidine incorporation. Values are expressed by % cpm of the control (+2ME). Pulse-labeling was performed between 47 and 48 h. The molar concentration of selenocystine was doubled here since there are two Se per one molecule.

TABLE 2. EFFECTS OF CYSTEINE (100 μ M/EVERY 12 h) PLUS BCS (20 μ M), SODIUM SELENITE (5×10^{-7} M), SELENOCYSTINE ($5/10^{-7}$ M AS SE), AND 2ME (20 μ M) ON CELLULAR GSHPx ACTIVITY AND ON CELL VIABILITY AND DENSITY AT 48 h OF LPS-STIMULATED LYMPHOCYTES

Supplements	GSHPx Activity ^a		Cell Viability (%)	Viable Cell Density (cell/ml $\times 10^{-6}$)
	Unit per mg soluble cell protein	Unit per 10^7 viable cells		
(Fresh 0 h)	0.153 $\pm$ 0.008	0.020 $\pm$ 0.008	99 $\pm$ 1	1.0
(After 48 h)				
None	0.048 $\pm$ 0.008	0.006 $\pm$ 0.001	69 $\pm$ 7	0.52 $\pm$ 0.02
Cys + BCS	0.024 $\pm$ 0.008	0.008 $\pm$ 0.002	84 $\pm$ 4	0.86 $\pm$ 0.02
Na ₂ SeO ₃	0.048 $\pm$ 0.009	0.013 $\pm$ 0.002	74 $\pm$ 3	0.72 $\pm$ 0.15
Cys + BCS + Na ₂ SeO ₃	0.056 $\pm$ 0.006	0.020 $\pm$ 0.001	95 $\pm$ 1	1.46 $\pm$ 0.32
Selenocystine	0.057 $\pm$ 0.002	0.016 $\pm$ 0.003	75 $\pm$ 5	0.68 $\pm$ 0.12
Cys + BCS + Selenocystine	0.038 $\pm$ 0.006	0.014 $\pm$ 0.002	93 $\pm$ 2	1.41 $\pm$ 0.19
2ME	0.034 $\pm$ 0.009	0.010 $\pm$ 0.005	96 $\pm$ 1	1.60 $\pm$ 0.08

Values are the means of three independent experiments ($\pm$ SEM).

^a One unit = 0.5 μ mol NADPH oxidized per min at 25°C.

Effects of α -tocopherol on DNA synthesis. Since α -tocopherol, a natural antioxidant, enhanced an *in vitro* immune response (5), we compared the effects of α -tocopherol on the DNA synthesis with those of selenite and 2ME. As shown in Table 3, α -tocopherol has an enhancing effect similar to that of selenite. The enhancing effect of α -tocopherol was saturated at about 1 μ g/ml (2.3×10^{-6} M), and it was slightly less effective than selenite either in the presence or absence of cysteine plus BCS.

Variations among the serum lots. Table 4 summarizes the effects of 14 randomly selected lots of fetal bovine serum on the lymphocyte activation under different culture conditions. The second column indicates the relative incorporated radioactivity per culture in the presence of 2ME. These values varied from 27 to 117% of the No. 1 lot (used in this report), suggesting that the activation varied widely with

TABLE 3. EFFECTS OF α -TOCOPHEROL ON THE DNA SYNTHESIS OF LPS-STIMULATED LYMPHOCYTES IN THE PRESENCE OF ABSENCE OF CYSTEINE (100 μ M/12 h) AND BCS (20 μ M)

Supplements	[³ H]Thymidine Incorporation (47–48 h) (% cpm of the control)	
	without Cys and BCS	with Cys and BCS
None	9	44
α -Tocopherol, 0.1 (μ g/ml)	13	56
0.5	32	83
1.0	38	88
2.0	36	87
Na ₂ SeO ₃ (5×10^{-7} M)	45	99
2ME (20 μ M)	100 (control) ^a	
2ME + α -Tocopherol (2.0 μ g/ml)	96	

Values are calculated from the mean cpms of triplicate cultures.

^a The incorporated radioactivity in the control samples labeled with 0.2 μ Ci [³H]thymidine (0.4 Ci/mmol) was 27427 $\pm$ 864 cpm.

TABLE 4. VARIATIONS AMONG FETAL BOVINE SERUM LOTS

Serum Lots No.	[³ H]Thymidine Incorporation (47–48 h)					Selenium content ng/ml	α-Tocopherol content ng/ml	
	2ME (cpm/culture) % of No. 1 lot	Ratios × 100						
		None	Se	Cys, BCS	Cys, BCS, Se			
		2ME	2ME	2ME	2ME			
1.	100	7 ± 3	43 ± 5	47 ± 7	99 ± 4	20	58	
2.	89	8	37	39	88			
3.	88	10	42	60	95	23	57	
4.	97	11	36	53	94	19	43	
5.	67	12	33	70	96			
6.	56	13	27	68	96			
7.	46	16	25	90	101			
8.	60	18	35	80	93			
9.	84	23	39	79	96			
10.	103	25	44	73	90	15	53	
11.	62	26	39	76	85			
12.	27	26	30	81	93			
13.	117	28	43	79	97	22	51	
14.	92	28	42	71	93			
15.	112	29	44	73	90			
		19.5 ± 7.7	36.9 ± 6.2	70.9 ± 13.0	93.4 ± 4.1	19.8 ± 3.1	52.4 ± 6.0	
		Mean ± SEM (No. 2 to 15)					(mean of 5)	(mean of 5)

Randomly selected serum lots (No. 2 to 15) from 4 companies were compared with the No. 1 lot (used in this report) for the effect of $[^3\text{H}]$ thymidine incorporation by LPS-stimulated lymphocytes. Each serum was heat-treated (56°C for 30 min) and supplemented to the medium Se (Na_2SeO_3 , 5×10^{-7} M) and 2ME (20 μM).

the serum lot used. The third column indicates the enhancing effect of 2ME, and the 14 serum lots were thus numbered accordingly. Supplementing 5×10^{-7} M selenite enhanced the $[^3\text{H}]$ thymidine incorporation in all serum lots. Cysteine plus BCS increased the incorporation rate from 19.5 ± 7.7 to $70.9 \pm 13.0\%$ on average. Cysteine plus BCS plus selenite increased the incorporation rate up to $93.4 \pm 4.1\%$ of that of 2ME. Table 4 also shows the selenium and α -tocopherol content of five serum lots. Because these contents did not differ significantly among the serum lots, they were not related to the variations between lots.

DISCUSSION

The results in this report show that 2ME, which enhances proliferation of the mitogen-stimulated lymphocytes *in vitro*, can be fully replaced by two physiological substances, cysteine and a selenium compound. A single addition of 20 μM 2ME was as effective as the repeated addition of 100 μM cysteine every 12 h in the presence of 20 μM BCS and an appropriate concentration of a selenium compound. The action of 2ME as the carrier of cysteine has been established in L1210 cells (15) and confirmed in mouse spleen lymphocytes (26). As shown in Table 1, the enhancing effect of cysteine on the $[^3\text{H}]$ thymidine incorporation was saturated at physiological concentrations of cysteine, where it is evidenced that human and rat plasma contains 10–20 μM cysteine (20, 28). Because the effect of selenium com-

pounds is not apparently related to the transport of cysteine or cystine, 2ME must have another function in addition to that as a cysteine-carrier in enhancing DNA synthesis of the LPS-stimulated lymphocytes. This is a distinct difference in the 2ME requirement of lymphocytes from that of L1210 cells for cell proliferation.

It is confirmed that in the absence of 2ME, relatively high concentrations of the selenium compounds are required for maximal enhancement of DNA synthesis of the mitogen-stimulated lymphocytes in an *in vitro* culture. The lymphocytes required 10^{-7} to 10^{-6} M selenite for maximal effect, which was roughly one order of magnitude higher than that required by the human diploid fibroblast for maximal clonal cell growth (23). Usually, the selenium requirement of cultured cells is studied under a low content of serum because the serum contains selenium (23). The fetal bovine serum used in this study contained 20 ng/ml (2.5×10^{-7} M) selenium, with a value near the average of the 5 serum lots assayed (Table 4). Since the culture medium contained 10% (v/v) serum, the selenium content in the medium, *i.e.*, about 2.5×10^{-8} M, seems insufficient to support the DNA synthesis of the lymphocytes, although it is unknown what types of selenium compounds are included in the serum. It should be noted that the serum lots showed variable selenium dependence, as shown in Table 4, although their contents of selenium and α -tocopherol did not differ greatly. This may suggest that the sera contain another factor(s) which alters the effect of selenium compounds.

The dependence of immune responses on selenium has been extensively studied in experimental animals. Berenshtein (2) first reported a potentiation of humoral immunity by selenium. Rabbits, in which sodium selenite was administered, produced higher antibody titers against a typhoid vaccine than control rabbits fed a selenium-deficient diet. Spallholz *et al.* (30, 31) reported that dietary or intraperitoneally injected selenite enhanced antibody titers as well as the number of antibody producing cells against sheep red blood cells in mice. The similar effect of dietary selenite on immunity was reported in chicks (22). In the *in vitro* culture system, lymphocyte activation in response to mitogens decreased in animals fed diets deficient in selenium, as reported in the study of peripheral lymphocytes from pigs (19) and dogs (29), and spleen lymphocytes from rats (10). In those *in vitro* experiments, however, the effects of selenium compounds during activation were not examined. The results of this report indicate that in the activation of normal spleen lymphocytes with LPS, the selenium requirement appears after 24 h in culture. In the presence of cysteine and BCS, adding selenite either at 0 h or 24 h in culture shows almost the same enhancement effect when the [3 H]thymidine incorporation is measured at 47 h (data not shown). This may suggest that oxidative damage may gradually be accumulated within the lymphocytes, or that the lymphocytes may gradually be deficient in selenium, as implied by the fact that intracellular GSHPx activity decreases during the culture period (Table 2).

α -Tocopherol is also important in immune responses *in vivo* and *in vitro*. Tengerdy *et al.* showed that α -tocopherol used as a diet supplement enhanced the immune responses in chicks (32) and also in mice (33). Campbell *et al.* (5) showed that α -tocopherol was as effective as 2ME in enhancing the immune responses of normal spleen lymphocytes *in vitro*. Their result is apparently inconsistent with ours. In our assay system, α -tocopherol was far less effective than 2ME if cysteine was not supplemented (Table 3). This discrepancy may be elucidated by the differences of the assay conditions. First, Campbell *et al.* (5) cultured the cells under a high-cell density and a low-oxygen tension (8.5%). These conditions may reduce the requirement

of cysteine supply. Second, they counted the number of antibody-producing cells after 5 days in culture, but they did not monitor the early phase of the immune response.

The important finding in this report is that both the selenium compounds and α -tocopherol enhance DNA synthesis in a manner different from that of cysteine. It is noted that a selenium compound is still required by the cells containing sufficient amounts of cellular cysteine and GSH; adding 100 μ M cysteine every 12 h in the presence of BCS is sufficient to maintain high intracellular GSH levels on the second day (data not shown). In this assay system, the antioxidant activity of selenium compounds may not be elucidated by the cellular GSHPx because selenite and selenocystine were not effective in maintaining GSHPx activity (Table 2). Also, it cannot be determined at present how 2ME at 10^{-5} M functions in a manner similar to selenium compounds. Further studies on these problems should elucidate the mechanism of the action of selenium in enhancing DNA synthesis of the lymphocytes.

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