

MECHANISM OF AUGMENTATION OF THE ANTIBODY
RESPONSE IN VITRO BY 2-MERCAPTOETHANOL
IN MURINE LYMPHOCYTES

I. 2-Mercaptoethanol-induced Stimulation of the
Uptake of Cystine, an Essential Amino Acid

BY HITOSHI OHMORI AND ITARU YAMAMOTO*

*From the Department of Medicinal Biochemistry, Faculty of Pharmaceutical Sciences, Okayama University,
Tsushima-naka 1-1-1, Okayama 700, Japan*

It has been reported by many authors that a variety of thiol compounds augment various lymphocyte reactions in vitro, including antibody responses (1, 2), induction of cytotoxic T cells in the mixed lymphocyte culture (3, 4), and the DNA synthetic response to mitogens (5-7). 2-Mercaptoethanol (2-ME),¹ one of the most effective thiols, was shown to be a polyclonal activator of both T cells (8) and B cells (9). Although 2-ME is widely used as an effective additive to lymphocyte cultures to elicit in vitro antibody responses, its mechanism of action still remains to be elucidated, particularly at the molecular level. Opitz et al. (10) have proposed that 2-ME does not act directly on lymphocytes, but interacts with fetal calf serum (FCS) to convert its component(s) into activated form, which in turn is directly involved in the augmentation of the primary antibody response to sheep erythrocytes (SRBC) (11). Sidman and Unanue (12) also demonstrated that the enhancing effect of 2-ME was mediated by 2-ME-activated serum factor(s) in anti-Ig antibody-induced proliferation of murine B cells. The chemical nature of these 2-ME-activated factor(s), however, has not been investigated in detail. On the other hand, Goodman et al. (9, 13, 14) have reported that 2-ME could act directly on late-maturing, resting murine lymphocytes, including both T cells and B cells, to trigger their proliferation and differentiation. They have reported (15) that synergistic cooperation between T and B cells was involved in the activation process by 2-ME. It has been recently proposed by Hoffeld and Oppenheim (16) that a major role of 2-ME in the augmentation of antibody response in vitro is to convert oxidized glutathione (GSSG) in the serum into reduced glutathione (GSH). The former compound had inhibitory effect on antibody response, whereas 2-ME was found to reverse its inhibition. Thus, we have no final conclusion as to the mechanism of action of 2-ME. In a previous paper (7), we compared the enhancing activities of various thiol compounds and found that antibody response was markedly augmented by cysteine, which has higher reduction potential than

* To whom correspondence should be addressed.

¹ *Abbreviations used in this paper:* Con A, concanavalin A; DNP, dinitrophenyl; DTT, dithiothreitol; FCS, fetal calf serum; GSH, reduced glutathione; GSSG, oxidized glutathione; LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; MEM, Eagle's minimum essential medium; PBS, phosphate-buffered saline; PFC, plaque-forming cells; SRBC, sheep erythrocytes; [³H]TdR, tritiated thymidine; TNP, trinitrophenyl.

GSH and thus is not able to reduce GSSG into GSH. Moreover, both cysteine and cystine enhanced the response as effectively as 2-ME if they were added at higher concentrations (2.5–5 mM). When fresh cysteine, but not cystine, was fed serially every 12 h, the dose-response curve of the compound shifted distinctly to lower concentrations. If one considers that cysteine is readily oxidized to cystine in the culture conditions (17), it is suggested that lymphocytes could be activated by both cysteine and cystine, but the former compound would be used more efficiently than the latter. These observations prompted us to investigate whether cyst(e)ine is required as an essential amino acid in antibody response *in vitro* and whether 2-ME could facilitate, in some way, the utilization of cystine that is contained in RPMI 1640 medium as a cysteine source. A preliminary report dealing with these problems has been published (18).

Materials and Methods

Mice. Female BALB/c mice were purchased from Japan Charles River Breeding Laboratories (Tokyo, Japan) and were used during 10–15 wk of age.

Chemicals and Culture Media. The reagents used in the present experiments were obtained from the following sources: 2-ME and L-cysteine hydrochloride, Tokyo Kasei, Tokyo; dithiothreitol (DTT) and GSH, Sigma Chemical Co., St. Louis, MO; L-cystine dihydrochloride, Ishizu Seiyaku, Osaka, Japan. RPMI 1640 medium and Eagle's minimum essential medium (MEM) were purchased from Nissui Pharmaceutical Company (Tokyo). FCS (lot 31K1101) was obtained from Gibco Laboratories, Grand Island Biological Company (Grand Island, NY). RPMI 1640 medium, from which cystine and GSH were omitted, was prepared by using commercially available ingredients (amino acids, vitamins, and inorganic salts) according to the composition reported by Moore et al. (19). Culture medium was usually supplemented with penicillin G (50 $\mu\text{g}/\text{ml}$) and streptomycin (50 $\mu\text{g}/\text{ml}$).

Antigens and Mitogens. SRBC stored in sterile Alsever's solution was obtained from Nishinippon Sheep Farm (Fukuyama, Japan). SRBC were washed twice with sterile saline before use. Dinitrophenyl (DNP)₃₆-Ficoll was prepared by coupling ϵ -DNP-L-lysine (Sigma Chemical Co.) and Ficoll 400 (Pharmacia Fine Chemicals, Uppsala, Sweden) with cyanuric chloride (Wako Chemicals, Tokyo) as a cross-linker, according to the method by Haba and Hamaoka (20). Trinitrophenylated lipopolysaccharide (TNP-LPS) was prepared by reacting trinitrobenzene sulfonate (Tokyo Kasei, Tokyo) with LPS by the procedure as described by Jacobs and Morrison (21). LPS was the phenol-water extracts of *Escherichia coli* 055 B5 (Difco Laboratories, Detroit, MI). Concanavalin A (Con A) was the product of E. Y. Laboratories (San Mateo, CA).

Lymphocyte Suspension. Dissociated spleen cells were prepared in MEM by teasing spleens on rough stainless mesh with spatula. This preparation was passed through stainless mesh (40-mesh) to remove large debris followed by washing once with MEM and were finally suspended in RPMI 1640 medium supplemented with 10% FCS (RPMI-FCS) at an appropriate cell density. When cells were cultured in cystine-free RPMI 1640 medium, the cells were washed before culture twice with Hank's balanced salts solution to avoid the contamination of cyst(e)ine. The number of viable cells were counted by trypan blue exclusion test.

Culture of Spleen Cells for Primary Antibody Response and Assay of Antibody-forming Cells. Spleen cells (8×10^6) were cultured with SRBC (2×10^6), DNP-Ficoll (20 ng/ml), or TNP-LPS (1 $\mu\text{g}/\text{ml}$) in 2 ml of RPMI-FCS at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 4 d using 24-well multi-dish culture plates (NUNC, Kamstrup, Denmark). In the case of polyclonal antibody synthesis, the same number of cells were cultured in the presence of 50 $\mu\text{g}/\text{ml}$ LPS for 2 d. When cystine-free RPMI 1640 medium was used, 6×10^6 spleen cells were cultured with 2×10^6 SRBC in 1 ml of the medium containing 10% FCS and varying concentrations of cystine for 4 d as described above. Cystine concentrations were expressed as those of half-cystine in this paper. Antibody synthesis was assayed by enumerating the number of hemolytic plaque-forming cells (PFC) by the method of Jerne and Nordin (22). Lightly conjugated TNP-SRBC were prepared as described by Rittenberg and Pratt (23) for detecting anti-TNP PFC. Polyclonal antibody synthesis was assayed by using SRBC as the target. The

number of direct PFC was counted in all cases. All cultures were carried out in duplicate or triplicate. Typical data from several repeated experiments were presented. Standard error did not usually exceed 10–15% of the mean value.

DNA Synthetic Response. Using 96-well microculture plates (NUNC), spleen cells (1×10^5) were cultured in triplicate with Con A (2.5 $\mu\text{g}/\text{ml}$) or LPS (25 $\mu\text{g}/\text{ml}$) in 200 μl of cystine-free RPMI 1640 medium containing 5% FCS at 37°C for 48 h in the humidified atmosphere of 5% CO_2 and 95% air. Where indicated, 2-ME or varying concentrations of cystine was supplemented. Cells were then radiolabeled for 18 h with 0.5 μCi of [^3H]thymidine ([^3H]TdR) (20 Ci/mmol, Radiochemical Center Amersham, Amersham, England). The cultures were harvested with automatic cell harvester (Labo Mash 101, Labo Science, Tokyo) onto glass filter strips. Incorporated radioactivities were counted with liquid scintillation counter (Aloka Model LSC 700).

Uptake of ^{35}S -Cyst(e)ine by Murine Lymphocytes. In this experiment, RBC were removed from spleen cell suspensions according to the procedure described by Mishell (24) with slight modifications. In brief, spleen cell pellets were rapidly suspended in 9:1 mixture of 0.16 M NH_4Cl and 0.17 M Tris-HCl buffer (pH 7.65) at the density of $2\text{--}3 \times 10^7$ cells/ml and were kept for 2 min at room temperature. Cell suspensions were then underlaid with FCS and centrifuged at 500 g for 5 min. The cell pellets were washed once with MEM and twice with Hanks' balanced salts solution at 4°C. 4–6 million RBC-depleted spleen cells were incubated in 1 ml of cystine-free RPMI 1640 medium supplemented with 10% FCS and 0.1 mM ^{35}S -L-cystine (as half cystine) (0.5 μCi , RCC Amersham) at 37°C in the humidified atmosphere of 5% CO_2 and 95% air for the time indicated. When uptake reactions were performed for <1 h, the medium was buffered with 20 mM Hepes (Sigma Chemical Co.) (pH 7.2) and incubated in air. When cysteine uptake was investigated, the mixture of 1 mM cysteine and ^{35}S -cystine (5 $\mu\text{Ci}/\text{ml}$), which was kept at room temperature for 30 min in the presence of 2 mM DTT, was used as ^{35}S -cysteine preparation. The uptake reactions were terminated by adding 4 ml of cold phosphate-buffered saline (PBS) followed by centrifugation at 1,000 g for 2 min. The pellets were again washed with 4 ml of cold PBS and finally suspended in 1 ml of PBS. Incorporated radioactivities were counted in ACS II scintillation cocktail (Radiochemical Center Amersham) using liquid scintillation counter (Aloka, LSC 700).

Enrichment of T and B Cells. RBC-depleted murine spleen cells (2×10^7) were suspended in 1 ml of MEM buffered with 10 mM Hepes (pH 7.2) (MEM-Hepes) containing monoclonal anti-Thy-1,2 (1:500, Olac 1976, Blackthorn, England) or anti-Ig (1:3) and were kept below 4°C for 30 min. Anti-Ig were prepared in our laboratory by repeated immunization of rabbits with purified BALB/c IgG fraction. After centrifugation and washing once with MEM-Hepes, the cells were resuspended in 0.7 ml MEM-Hepes. Upon addition of 0.1 ml of guinea pig complement, the cells were incubated at 37°C for 30 min with occasional stirring, centrifuged, washed three times with MEM, and finally suspended in an appropriate volume of the culture medium. The treatment with anti-Thy-1,2 killed 40–50% of spleen lymphocytes and reduced Con A-induced [^3H]TdR uptake by 85–95% without significantly affecting the response to LPS. On the other hand, anti-Ig treatment resulted in 50–55% killing and decreased LPS-induced [^3H]TdR uptake by 80–85%, whereas Con A response was not significantly altered. T cell enrichment was also performed by passing spleen cells through a nylon wool column as described by Julius et al. (25).

Depletion of Adherent Cells. Spleen cells from which RBC were removed previously were depleted of adherent cells by passage over Sephadex G-10 column (Pharmacia Fine Chemicals) as reported by Ly and Mishell (26) with some modifications. Briefly, spleen cell suspension in MEM-Hepes containing 5% FCS (1×10^8 cells/ml) was applied to Sephadex G-10 column (2 ml bed vol/ 10^8 cells) which was previously equilibrated with the same medium. Adherent cell-depleted spleen cells were recovered by eluting the column with MEM-Hepes containing 5% FCS (average recovery, 40%).

Results

Augmentation of Primary Antibody Response by 2-ME and Cysteine. Table I shows that the addition of 2-ME at an optimal concentration (1×10^{-5} M) resulted in a marked

TABLE I
Effect of 2-ME on the Antibody Response to SRBC, DNP-Ficoll, and TNP-LPS

2-ME (1×10^{-5} M)*	Anti-SRBC PFC/culture‡		Anti-TNP-PFC/culture‡		
	No antigen	SRBC	No antigen	DNP-Ficoll	TNP-LPS
-	240 ± 90	660 ± 60	230 ± 30	400 ± 40	510 ± 90
+	300 ± 30	2295 ± 315	470 ± 110	1830 ± 270	2040 ± 140

* Spleen cells (8×10^6) were cultured with various antigens in the presence or absence of 1×10^{-5} M 2-ME as described in Materials and Methods.

‡ Mean ± SE.

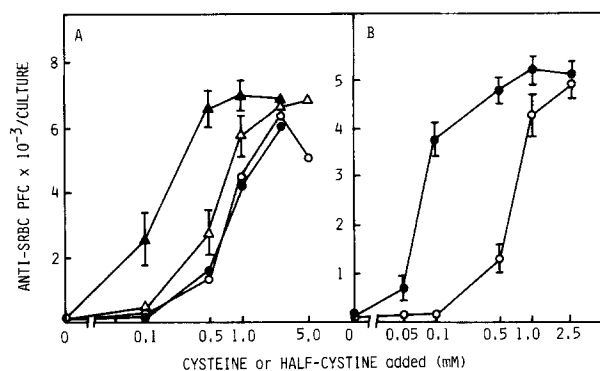


FIG. 1. Effect of cysteine or cystine concentrations on the antibody response to SRBC in vitro in the presence or absence of 2-ME. Spleen cells (6×10^6) were cultured with 2×10^6 SRBC in 1 ml of cystine-free RPMI 1640 medium containing 10% FCS in the presence of the indicated amount of cysteine or half-cystine for 4 d. Where indicated, fresh solution of cysteine or cystine was added serially every 12 h eight times. In this case, the concentrations indicate the total amount of cysteine or half-cystine after the final addition. (A) (○) cystine, (●) cystine added serially every 12 h, (△) cysteine, (▲) cysteine added serially every 12 h. (B) (○) cystine, (●) cystine plus 2-ME (1×10^{-5} M).

enhancement of the primary antibody responses in vitro to three different antigens (SRBC, DNP-Ficoll, and TNP-LPS). The magnitude of enhancement varied from two- to fivefold depending on the lots of FCS and the spleen cell pools used. We have tested various thiol compounds for their capacity to augment the antibody response in vitro, and have found that cysteine could also enhance the response (7, 18). Although data are not shown here, cysteine was as effective as 2-ME (1×10^{-5} M) when it was added at 5 mM at the start of the culture.

Requirement of Cyst(e)ine in Antibody Response. Cyst(e)ine is known to be an essential amino acid in many mammalian cell lines (27). RPMI 1640 medium contains cystine (65 mg/liter) and GSH (1 mg/liter). The medium, devoid of cystine, was prepared to examine whether the amino acid is required as a nutrient in the antibody response. In the experiment of Fig. 1A, the effect of increasing concentrations of cyst(e)ine on the antibody response to SRBC was examined by using the cystine-free culture medium. It was shown that the immune response was not elicited unless cyst(e)ine was supplemented to the medium. Omission of GSH did not significantly affect the response, and GSH (1 mg/liter) could elicit no response in the absence of cystine, as reported previously (18). Therefore, GSH was usually supplemented when the following experiments were performed in cystine-free conditions. The effect of cyst(e)ine and GSH that would contaminate from RBC and FCS appeared to be negligible,

because whole splenocyte suspensions responded to the antigen absolutely depending on exogenously added cyst(e)ine (Fig. 1 A). These observations clearly showed that cyst(e)ine is an essential nutrient in the antibody response of murine lymphocytes. The PFC response reached maximum with 2.5–5.0 mM cysteine or half-cystine. Interestingly, the dose-response curve of cysteine, but not of cystine shifted distinctly to lower concentrations when fresh solutions of these amino acids were fed serially eight times, every 12 h. We confirmed that cysteine (0.1–2.5 mM) was readily oxidized to cystine under our culture conditions as reported by Toohey (17). Thus, it is likely that lymphocytes could use cysteine more efficiently than cystine for eliciting the antibody response. On the other hand, the dose-response curve of cystine was shifted to lower concentrations by approximately one order of magnitude (Fig. 1 B). It should be noted that 2-ME did not augment the response when half cystine was absent or when an optimal concentration of half cystine (2.5 mM) was present. Namely, it only enhanced the response when suboptimal doses of cystine were present in the culture medium. It was demonstrated that 2-ME did increase significantly the viability of lymphocytes even in the absence of cystine. The enhancing effect of 2-ME on antibody response, however, could not be explained only by the nonspecific improvement of cell viability, because similar shift of dose-response curve was seen when the data were plotted as the number of PFC/ 10^6 viable cells as shown in Fig. 2. Similar results were obtained when DNP-Ficoll, a thymus-independent antigen, was used (data not shown).

Cystine Requirement in DNA Synthetic Response to Mitogens and Polyclonal Antibody Synthesis. Data in the previous section showed that spleen cells as a whole required cyst(e)ine for developing antibody-forming cells. To investigate the role of cystine in the proliferation and differentiation of T and B cells separately, cystine requirement was examined in the DNA synthetic responses to T cell-specific mitogen (Con A) and B cell-specific mitogen (LPS). As illustrated in Fig. 3 A and B, both responses to Con A and LPS were completely dependent on exogenously added cystine, and reached maximum with 1–2.5 mM half-cystine. The addition of 1×10^{-5} M 2-ME resulted in the shifting of the dose-response curves to lower concentrations, as in the case of the

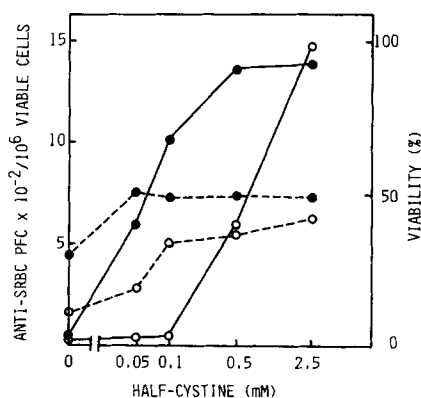


FIG. 2. Effect of cystine concentrations on the antibody response to SRBC in vitro and the viability of lymphocytes in the presence or absence of 2-ME. Spleen cells (6×10^6) were cultured with 2×10^6 SRBC in 1 ml of cystine-free RPMI 1640 medium containing 10% FCS and varying concentrations of half-cystine in the presence (●) or absence (○) of 1×10^{-5} M 2-ME for 4 d. (—) antibody response. (---) viability after culture.

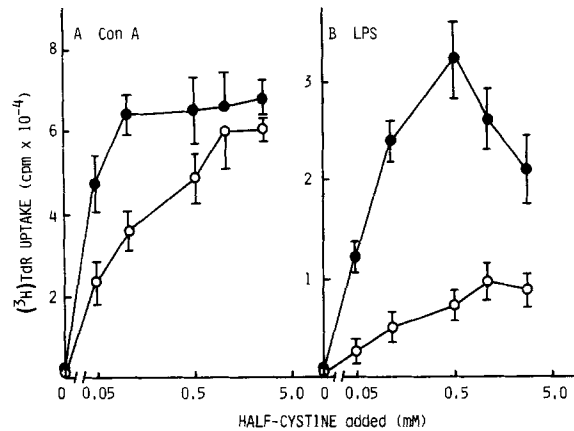


FIG. 3. Effect of cystine concentrations on the DNA synthetic response to mitogens in the presence or absence of 2-ME. Spleen cells (1×10^5) were cultured for 48 h with Con A ($2.5 \mu\text{g/ml}$) or LPS ($25 \mu\text{g/ml}$) in 0.2 ml of cystine-free RPMI 1640 medium containing 5% FCS and varying concentrations of half-cystine in the presence (●) or absence (○) of 1×10^{-5} M 2-ME. The cells were then pulsed with [^3H]TdR ($0.5 \mu\text{Ci/well}$) for 18 h.

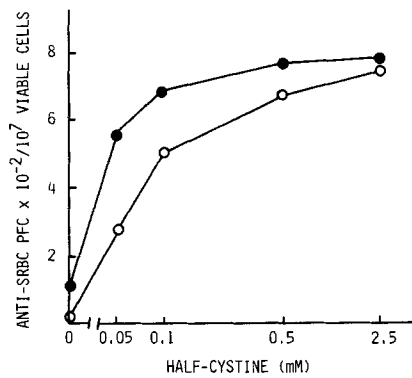


FIG. 4. Effect of cystine concentrations on the polyclonal antibody response induced by LPS in the presence or absence of 2-ME. Spleen cells (8×10^6) were cultured with $50 \mu\text{g/ml}$ LPS in 1 ml of cystine-free RPMI 1640 medium containing 10% FCS and varying concentrations of half-cystine in the presence (●) or absence (○) of 1×10^{-5} M 2-ME. Polyclonal antibody response was assayed as the number of anti-SRBC PFC.

antibody response. More pronounced stimulation by 2-ME was observed in the proliferation of B cells than in that of T cells. Polyclonal antibody synthesis induced by LPS was similarly shown to require cystine as demonstrated in Fig. 4. 2-ME augmented the response particularly when suboptimal doses of cystine were added to the culture medium. The characteristic feature of DNA synthetic response and polyclonal antibody response was that these responses were significantly elicited with 0.05–0.1 mM half cystine in contrast to antibody response, which did not occur with this level of cystine. The results described thus far clearly demonstrated that cyst(e)ine is an essential amino acid in the proliferation and differentiation of lymphocytes. Commercially available RPMI 1640 usually contains ~ 0.41 mM half-cystine, which was shown to be a suboptimal level for maximal response to antigens and mitogens by murine lymphocytes.

Stimulation of ^{35}S -Cystine Uptake by 2-ME. The above data suggested that lymphocytes would use cystine more efficiently than cystine, the use of which, however, might be stimulated by 2-ME. The following experiments using ^{35}S -cyst(e)ine were carried out to directly prove these speculations. When RBC-depleted murine spleen cells were incubated with 0.1 mM ^{35}S -half-cystine or ^{35}S -cysteine, cysteine was incorporated into the cells five to six times as rapidly as cystine (Fig. 5 A). Addition of 1×10^{-5} M 2-ME resulted in ~ 2.5 -fold acceleration of ^{35}S -cystine uptake. When the cells were incubated for 30 min in this condition, $\sim 30\%$ of the incorporated radioactivity was found in trichloroacetic acid-precipitable fraction. 2-ME stimulation was still observed when the incubations were prolonged up to 24 h (Fig. 5 B). We confirmed that 2-ME had no effect on the viability of lymphocytes during these incubation periods. Although data were not presented, omission of FCS did not affect the rate of 2-ME-stimulation of cystine uptake. Fig. 6 illustrates the correlation between cystine concentrations in the medium and the amount of incorporated cystine in the presence or absence of 2-ME. Cystine added at 2.5 mM (as half-cystine) caused

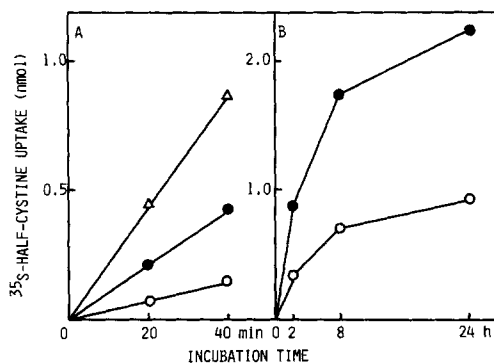


FIG. 5. Effect of 2-ME on the rate of cystine incorporation into murine lymphocytes. RBC-depleted spleen cells (6×10^6) were incubated at 37°C for the time indicated in 1 ml of cystine-free RPMI 1640 medium supplemented with 10% FCS and 0.1 mM ^{35}S -half-cystine or ^{35}S -cysteine (0.5 μCi). (A) Medium buffered with 20 mM Hepes (pH 7.2) and incubations performed in air. (B) Reaction mixture incubated without adding Hepes in the humidified atmosphere of 5% CO_2 and 95% air. (○) ^{35}S -cystine, (●) ^{35}S -cystine plus 2-ME (1×10^{-5} M), (Δ) ^{35}S -cysteine (in the presence of 0.2 mM DTT).

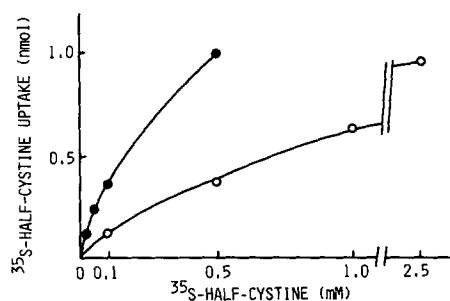


FIG. 6. Effect of cystine concentrations on its incorporation into murine lymphocytes in the presence (●) or absence (○) of 2-ME. RBC-depleted spleen cells (5×10^6) were incubated with increasing concentrations of ^{35}S -half-cystine in 1 ml of cystine-free RPMI 1640 medium containing 10% FCS and 20 mM Hepes (pH 7.2) at 37°C for 30 min. Where indicated, 1×10^{-5} M 2-ME was added to the medium.

a significant intracellular accumulation of the amino acid that was approximately the same as that when 0.5 mM half-cystine was present together with 1×10^{-5} M 2-ME. Similar stimulation of cystine uptake by 2-ME was observed both in T and B cell-enriched lymphocytes as shown in Fig. 7. The rate of enhancement was approximately the same in both lymphocyte fractions. Depletion of adherent cells did not affect the enhancement by 2-ME. Fig. 8 shows the comparison between the effects of 2-ME concentrations on antibody response and on ^{35}S -cystine uptake in RPMI-FCS, which contains 0.37 mM half-cystine. A close correlation was observed between these two dose-response profiles, thus suggesting that a major role of 2-ME in augmenting the antibody response would be to facilitate the utilization of cystine by lymphocytes.

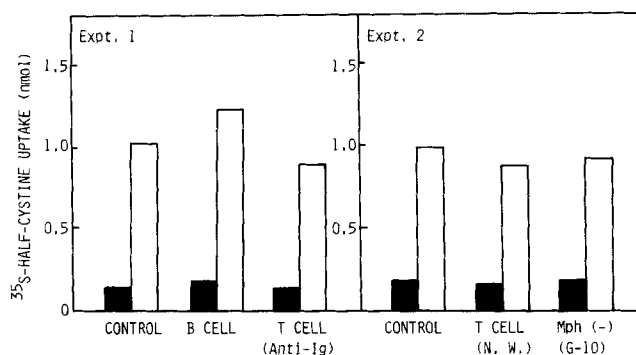


FIG. 7. Stimulation of cystine uptake in the presence (□) and absence (■) of 2-ME in T cell-enriched, B cell-enriched and adherent cell-depleted murine lymphocytes. RBC-depleted spleen cells were enriched for T cells or B cells by the treatment with anti-Ig or anti-Thy-1,2 in the presence of guinea pig complement, respectively. In experiment 2, T cell-enrichment was performed by utilizing nylon wool column (N.W.). Adherent cell-depleted lymphocytes (Mph⁻) were prepared by passing spleen cells through Sephadex G-10 column. These lymphocytes preparations (6×10^6) were incubated with 0.1 mM ^{35}S -half cystine (0.5 μCi) in 1 ml of HEPES-buffered cystine-free RPMI 1640 medium containing 10% FCS at 37°C for 30 min. Where indicated, 1×10^{-5} M 2-ME was added to the medium.

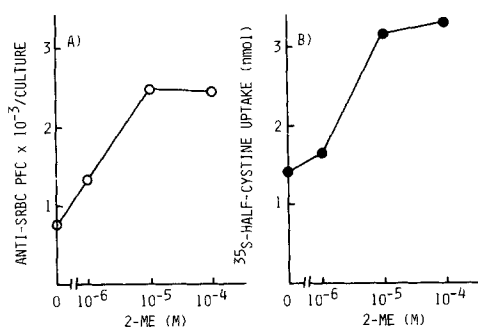


FIG. 8. Dose-response profiles of 2-ME in the enhancement of the antibody response in vitro and cystine uptake in murine lymphocytes. (A) Effect of 2-ME concentrations on the antibody response to SRBC in vitro. Spleen cells (8×10^6) were cultured with 2×10^6 SRBC in 2 ml of RPMI-FCS in the presence of varying concentrations of 2-ME for 4 d. (B) Effect of 2-ME concentrations on ^{35}S -cystine uptake by murine lymphocytes. RBC-depleted spleen cells (5×10^6) were incubated in 1 ml of RPMI-FCS containing ^{35}S -cystine (2 μCi) for 60 min at 37°C in the humidified atmosphere of 5% CO_2 and 95% air.

Discussion

2-ME could augment primary antibody responses *in vitro* not only to SRBC (a thymus-dependent antigen) but also to DNP-Ficoll and TNP-LPS (thymus-independent antigens). It has been reported that macrophages and B cells are involved in the immune response to TNP-Ficoll (28). On the other hand, the response to TNP-LPS requires neither macrophages nor T cells (29). We confirmed that depletion of either adherent cells or T cells from murine spleen cells did not affect the augmentation of the response to TNP-LPS by 2-ME. Therefore, B cells would be the target of 2-ME action at least in this case. On the other hand, Chen and Hirsch (30) and Lemke and Opitz (11) have proposed that 2-ME could substitute for the role of macrophages in the primary antibody response to SRBC. They removed macrophages from spleen cells by glass adherence (30) or carbonyl iron treatment (11). Other authors, however, have demonstrated that the response to SRBC (26) or TNP-Ficoll (28) was dramatically abrogated even in the presence of 2-ME when spleen cells were depleted of macrophages by passage through Sephadex G-10 column. We also confirmed that 2-ME could not substitute for the function of macrophages in Sephadex G-10-treated spleen cells. These findings indicated that 2-ME could not totally substitute for the role of macrophages, but rather suggested that a class of adherent cells which could not be removed by glass adherence or carbonyl iron treatment but could be depleted by Sephadex G-10 column might be involved in supporting antibody response as accessory cells in the presence of 2-ME.

Eagle et al. (27) have reported that a number of mammalian cell lines require cyst(e)ine for their growth because these cells are devoid of cystathionase, a key enzyme for the biosynthesis of cysteine from methionine. Our present experiments clearly show that cyst(e)ine is an essential amino acid in the proliferation and differentiation of both T cells and B cells because the following lymphocyte reactions were absolutely dependent on cyst(e)ine in the culture medium: (a) antibody responses to both thymus-dependent and thymus-independent antigens, (b) proliferative response to Con A or LPS, and (c) polyclonal antibody synthesis induced by LPS. Cyst(e)ine could not be replaced by 1 mM methionine, indicating that there would be some deletions on the metabolic pathway from methionine to cysteine in murine lymphocytes. Commercially available RPMI 1640 contains 0.41 mM half-cystine, which is a suboptimal dose judging from the dose-response curve of cyst(e)ine in Fig. 1-3. At least 2.5 mM half-cystine was necessary for eliciting maximal antibody response. 2-ME only shifted the dose-response curve of cystine in the antibody response (Fig. 1 B). It could not elicit the antibody response in the absence of cystine, nor did it enhance the maximal response when 2.5 mM half-cystine was present. These results suggested that 2-ME would enhance antibody response through stimulating the uptake of cystine contained in RPMI 1640 medium at a suboptimal concentration. This was clearly demonstrated by the experiments using ^{35}S -cystine (Fig. 5, 6). The augmentation by 2-ME of the antibody response was not solely due to nonspecific increase in the viability of whole spleen lymphocytes as shown in Fig. 2, but rather would be attributable to the stimulated expansion of specific clones of lymphocytes.

It appears that the stimulation of cystine uptake by 2-ME is not only due to the fact that 2-ME reduces cystine to liberate cysteine, which was shown to be incorporated more rapidly. Free thiol groups became undetectable within 2 h after the addition of 1×10^{-4} M 2-ME to the culture medium (data not shown), but 2-ME

enhancement of cystine uptake could still be observed during 8–24 h of incubation. We found that a major reaction product between 2-ME and cystine was the mixed disulfide after free thiol group had disappeared. Moreover, it was demonstrated that the mixed disulfide itself was incorporated ~80% as rapidly as cystine into murine lymphocytes and was readily metabolized to cysteine and GSH. The detailed data will be presented elsewhere (H. Ohmori and I. Yamamoto, manuscript in preparation).

Broome and Jeng (5) have reported that the growth rate of L 1210, a lymphoma cell line, was accelerated by various sulfide and disulfide compounds including 2-ME. It was also reported that the cell line could grow well in the presence of macrophages (31) or fibroblasts (32). Bannai and Ishii (33) have found that thiol compounds, including cysteine, were continuously secreted from fibroblasts. While our work was in progress, a mechanism of action of 2-ME similar to that presented above was reported concerning the growth stimulation of L 1210 by 2-ME (34, 35). It was demonstrated that a 2-ME dependent line of L 1210 incorporated cystine very slowly but the rate was markedly accelerated by 2-ME, whereas a 2-ME-independent variant showed an efficient uptake of cystine which was no longer enhanced by 2-ME (34). It was also reported that freshly isolated human leukocytes incorporated cystine only one-tenth as rapidly as cysteine (36). It is likely that such a low efficiency of cystine uptake would be common to lymphoid cells.

2-ME-dependent enhancement of cystine uptake was not mediated by FCS components, because similar stimulation was observed when FCS was either present or absent in the medium (unpublished data). Opitz et al. (10, 11) have reported that the incubation of FCS with 2-ME followed by lyophilization to remove free 2-ME resulted in the formation of activated serum factor(s) that could support antibody response as effectively as 2-ME added directly to the culture medium. We have obtained several lines of evidence indicating that the activation of FCS by 2-ME is due to the carrying over of 2-ME bound to serum proteins through disulfide bonds. It was also demonstrated that this protein-bound 2-ME could be effective for stimulating cystine uptake (H. Ohmori and I. Yamamoto, manuscript in preparation).

Hoffeld and Oppenheim (16) have reported that 2-ME-enhancement of the primary antibody response is mediated by its action on GSH in the serum. Our data indicated that cyst(e)ine at 2.5–5 mM augmented the response as effectively as 2-ME. The reduction potential of cysteine (-0.21 V) is higher than GSH (-0.24 V). Thus, cysteine is unable to reduce GSSG into GSH. On the contrary, it was found that oxidation of GSH was accelerated catalytically in the presence of cystine which is readily formed from cysteine (unpublished data). We observed that antibody response was elicited when GSH (1 mM) was supplemented to cystine-free RPMI 1640 medium, thus indicating that GSH could be a source of cysteine. As in the case of cystine, 2-ME augmented antibody response induced by suboptimal concentrations of GSH (unpublished data). Therefore, it is likely that 2-ME would increase the intracellular levels of GSH and cysteine by accelerating the uptake of these compounds, thus offering the more favourable conditions for lymphocytes to proliferate and differentiate. Our experiments using cystine- and GSH-free culture medium indicated that exogenously added GSH was not required for eliciting antibody response if cystine was present (18). However, particular roles of GSH contained in

the serum in supporting the antibody response, as proposed by Hoffeld and Oppenheim (16, 37) cannot be ruled out.

It has been reported by Shiigi and Mishell (38) that high antibody responses were elicited in the absence of 2-ME when particular lots of FCS (supportive lots) were used. They have demonstrated that such lots of FCS were often contaminated by gram-negative psychrophilic microorganisms that conditioned the culture medium to produce adjuvant and mitogenic activities (38, 39). We compared the dose-response curves of cystine in antibody responses when the culture medium was supplemented with a "good" and a "deficient" lot of FCS. We observed no significant differences between them, thus indicating that good lots of FCS would not support the response through facilitating cystine uptake (unpublished result).

Stimulation of cystine uptake would not be a sole mode of action of 2-ME in augmenting antibody responses *in vitro*. It has been observed (40, 41) that human fibroblasts fell into the deficiency of intracellular glutathione and readily started to die due to oxidative damages after the cells were transferred into cystine-free medium. These authors have reported that various antioxidants, including thiol compounds, were effective in preventing the cells from being necrotic under cystine-deficiency. Our data also showed that 2-ME could significantly improve the viability of murine lymphocytes when the cells were cultured in cystine-free conditions (Fig. 2). Therefore, such a function of 2-ME (perhaps as an antioxidant) would be responsible, in part, for the augmentation of antibody responses as recently reported by Hoffeld (42). The present paper demonstrated that cystine was a limiting nutrient when murine lymphocytes were cultured in RPMI-FCS, and suggested that every factor affecting cystine utilization would modify antibody responses *in vitro*.

Summary

The mechanism of augmentation of the primary antibody response *in vitro* by 2-mercaptoethanol (2-ME) was investigated. By using cystine-free RPMI 1640 medium, it was demonstrated that cyst(e)ine was absolutely required for eliciting the following murine lymphocyte reactions: antibody response to sheep erythrocytes, proliferative response to concanavalin A or lipopolysaccharide (LPS), and polyclonal antibody response induced by LPS. The maximal antibody response was attained with 2.5–5 mM cysteine or half-cystine. The serial feeding of fresh cysteine markedly amplified its capacity to support antibody response particularly when cysteine concentration was suboptimal. Such an effect was not observed in the serial addition of cystine. On the other hand, the dose-response curve of cystine was dramatically shifted to lower concentrations by the addition of 2-ME (1×10^{-5} M), which alone could not elicit the antibody response in the absence of cystine, nor could it augment furthermore the maximal response induced by 2.5 mM half-cystine. Commercially available RPMI 1640 medium contains 0.41 mM half-cystine, which proved to be a suboptimal concentration for eliciting the maximal response. ^{35}S -cystine was incorporated into murine lymphocytes five to six times more slowly than ^{35}S -cysteine. The rate of cystine uptake, however, was accelerated by 2.5-fold in the presence of 1×10^{-5} M 2-ME. A close correlation was observed between dose-response profiles of 2-ME in augmenting the antibody response and the stimulation of cystine uptake. These results strongly suggest that one of the roles of 2-ME in augmenting the antibody response *in vitro* is

to facilitate the use of cystine contained in RPMI 1640 medium only at a suboptimal concentration.

We thank Miss Etsuko Kasahara for her excellent secretarial assistance.

Received for publication 16 November 1981 and in revised form 19 January 1982.

References

1. Click, R. E., L. Benck, and B. J. Alter. 1972. Enhancement of antibody response in vitro by mercaptoethanol. *Cell. Immunol.* **3**:156.
2. Click, R. E., L. Benck, and B. J. Alter. 1972. Immune response in vitro. I. Culture conditions for antibody synthesis. *Cell. Immunol.* **3**:264.
3. Bevan, M. J., R. Epstein, and M. Cohn. 1974. The effect of 2-mercaptoethanol on murine mixed lymphocyte cultures. *J. Exp. Med.* **139**:1025.
4. Harris, J. W., H. R. MacDonald, H. D. Enger, F. W. Fitch, and J.-H. Cerrotini. 1976. Increased cytotoxic T lymphocyte activity induced by 2-mercaptoethanol in mixed leukocytes: kinetics and possible mechanism of action. *J. Immunol.* **116**:1071.
5. Broome, J. D., and M. W. Jeng. 1973. Promotion of replication in lymphoid cells by specific thiols and disulfides in vitro. *J. Exp. Med.* **138**:574.
6. Fanger, M. W., D. A. Hart, and J. V. Wells. 1970. Enhancement by reducing agents of the transformation of human and rabbit peripheral lymphocytes. *J. Immunol.* **105**:1043.
7. Ohmori, H., and I. Yamamoto. 1981. Activation of murine lymphocytes by 2-mercaptoethanol and related thiol compounds and its mechanism. I. Relationship between mitogenic activities and augmenting effects on antibody synthesis in vitro. *Immunopharmacology.* **3**:333.
8. Igarashi, T., M. O. Kada, T. Kishimoto, and Y. Yamamura. 1977. In vitro induction of polyclonal killer T cells with 2-mercaptoethanol and essential role of macrophages in this process. *J. Immunol.* **118**:1697.
9. Goodman, M. G., and W. O. Weigle. 1977. Nonspecific activation of murine lymphocytes. I. Proliferation and polyclonal activation induced by 2-mercaptoethanol and α -thioglycerol. *J. Exp. Med.* **145**:473.
10. Opitz, H.-G., U. Opitz, H. Lemke, G. Hewlett, W. Schreml, and H.-D. Flad. 1977. The role of fetal calf serum in the primary immune response in vitro. *J. Exp. Med.* **145**:1029.
11. Lemke, H., and H.-G. Opitz. 1976. Function of 2-mercaptoethanol as a macrophage substitute in the primary immune response in vitro. *J. Immunol.* **117**:388.
12. Sidman, C. L., and E. R. Unanue. 1978. Control of proliferation and differentiation in B lymphocytes by anti-Ig antibodies and a serum-derived cofactor. *Proc. Natl. Acad. Sci. U. S. A.* **75**:2401.
13. Goodman, M. G., J. M. Fidler, and W. O. Weigle. 1978. Nonspecific activation of murine lymphocytes. III. Cells responding mitogenically to 2-mercaptoethanol are typical unstimulated lymphocytes. *J. Immunol.* **121**:1899.
14. Goodman, M. G., J. M. Fidler, and W. O. Weigle. 1978. Nonspecific activation of murine lymphocytes. IV. Proliferation of a distinct, late maturing lymphocyte subpopulation induced by 2-mercaptoethanol. *J. Immunol.* **121**:1905.
15. Goodman, M. G., and W. O. Weigle. 1979. Nonspecific activation of murine lymphocytes. V. Role of cellular collaboration between T and B lymphocytes in the proliferative and polyclonal responses to 2-mercaptoethanol. *J. Immunol.* **122**:1433.
16. Hoffeld, J. T., and J. J. Oppenheim. 1980. Enhancement of the primary antibody response by 2-mercaptoethanol is mediated by its action on glutathione in the serum. *Eur. J. Immunol.* **10**:391.
17. Toohey, J. I. 1975. Sulfhydryl dependence in the primary explant hematopoietic cells. Inhibition of growth in vitro with vitamin B₁₂ compounds. *Proc. Natl. Acad. Sci. U. S. A.* **72**:73.

18. Ohmori, H., and I. Yamamoto. 1982. A mechanism of augmentation of antibody response in vitro by 2-mercaptoethanol: Facilitation of cystine uptake in murine lymphocytes. *Int. J. Immunopharmacol.* In press.
19. Moore, G. E., R. E. Gerner, and H. A. Franklin. 1967. Culture of normal human leukocytes. *JAMA (J. Am. Med. Assoc.)* **199**:519.
20. Haba, S., and T. Hamaoka. 1974. Method for the preparation of DNP-conjugated proteins or polysaccharides. In *Methods in Immunological Experiments B*. Edited and published by the Japanese Society for Immunology. 1129.
21. Jacobs, D. M., and D. C. Morrison. 1975. Stimulation of a T-independent primary anti-hapten response in vitro by TNP-lipopolysaccharide (TNP-LPS). *J. Immunol.* **114**:360.
22. Jerne, N. K., and A. A. Nordin. 1963. Plaque formation in agar by single antibody-producing cells. *Science (Wash. D. C.)* **140**:405.
23. Rittenberg, M. B., and K. L. Pratt. 1969. Anti-trinitrophenyl(TNP) plaque assay. Primary response of BALB/c mice to soluble and particulate antigens. *Proc. Soc. Exp. Biol. Med.* **132**:575.
24. Mishell, B. B. 1980. Preparation of mouse cell suspensions. In *Selected Methods in Cellular Immunology*. B. B. Mishell and S. M. Shiigi, editors. Freeman Publications, San Francisco. 3.
25. Julius, M., E. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* **3**:645.
26. Ly, I. A., and R. I. Mishell. 1974. Separation of mouse spleen cells by passage through columns of Sephadex G-10. *J. Immunol. Methods.* **5**:239.
27. Eagle, H., C. Washington, and S. M. Friedman. 1966. The synthesis of homocystine, cystathionine, and cystine by cultured diploid human cells. *Proc. Natl. Acad. Sci. U. S. A.* **56**:156.
28. Boswell, H. S., S. O. Sharrow, and A. Singer. 1980. Role of accessory cells in B cell activation. I. Macrophage presentation of TNP-Ficoll: evidence for macrophage-B cell interaction. *J. Immunol.* **124**:989.
29. Boswell, H. S., A. Ahmed, I. Scher, and A. Singer. 1980. Role of accessory cells in B cell activation. II. The interaction of B cells with accessory cells results in the exclusive activation of an Lyb-5⁺ B cell subpopulation. *J. Immunol.* **125**:1340.
30. Chen, C., and J. G. Hirsch. 1972. The effects of mercaptoethanol and of peritoneal macrophages on the antibody-forming capacity of nonadherent mouse spleen cells in vitro. *J. Exp. Med.* **136**:604.
31. Nathan, C. F., and W. D. Terry. 1975. Differential stimulation of murine lymphoma growth in vitro by normal and BCG-activated macrophages. *J. Exp. Med.* **142**:887.
32. Tanapat, P., E. Gaetjens, and J. D. Broome. 1978. Growth promoting and inhibitory activities of 3T3 and other cell lines for thiol dependent lymphoma cells in vitro (40088). *Proc. Soc. Exp. Biol. Med.* **157**:517.
33. Bannai, S., and T. Ishii. 1980. Formation of sulfhydryl groups in the culture medium by human diploid fibroblasts. *J. Cell. Physiol.* **104**:215.
34. Ishii, T., I. Hishinuma, S. Bannai, and Y. Sugita. 1981. Mechanism of growth promotion in mouse lymphoma L1210 cells in vitro by feeder layer or 2-mercaptoethanol. *J. Cell. Physiol.* **107**:283.
35. Ishii, T., S. Bannai, and Y. Sugita. 1981. Mechanism of growth stimulation of L1210 cells by 2-mercaptoethanol in vitro. *J. Biol. Chem.* **256**:12387.
36. Schneider, J. A., K. H. Bradley, and J. E. Seegmiller. 1968. Transport and intracellular fate of cysteine-³⁵S in leukocytes from normal subjects and patients with cystinosis. *Pediatr. Res.* **2**:441.
37. Hoffeld, J. T., and J. J. Oppenheim. 1980. The capacity of fetal calf serum to support a

- primary antibody response in vitro is determined, in part, by its reduced glutathione content. *Cell. Immunol.* **53**:325.
38. Shiigi, S. M., and R. I. Mishell. 1975. Sera and the in vitro induction of immune responses. I. Bacterial contamination and generation of good fetal bovine serum. *J. Immunol.* **115**:741.
 39. Shiigi, S. M., R. R. Capwell, K. H. Crabstein, and R. I. Mishell. 1977. Sera and the in vitro induction of immune responses. III. Adjuvant obtained from gliding bacteria with properties distinct from enteric bacterial lipopolysaccharide. *J. Immunol.* **119**:679.
 40. Bannai, S., H. Tsukeda, and H. Okumura. 1977. Effect of anti-oxidants on cultured human diploid fibroblasts exposed to cystine-free medium. *Biochem. Biophys. Res. Commun.* **74**:1582.
 41. De Brabander, M., H. Van Belle, F. Aerts, R. Van De Viere, and G. Genens. 1979. Protective effect of levamisole and its sulfhydryl metabolite OMPI against cell death induced by glutathione depletion. *Int. J. Immunopharmacol.* **1**:93.
 42. Hoffeld, J. T. 1981. Agents which block membrane lipid peroxidation enhance mouse spleen cell immune activities in vitro: relationship to the enhancing activity of 2-mercaptoethanol. *Eur. J. Immunol.* **11**:371.