THE EFFECT OF 2-MERCAPTOETHANOL ON MURINE MIXED LYMPHOCYTE CULTURES*

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A number of studies have shown a requirement for adherent cells in lymphocyte suspensions to obtain a good proliferative response to allogeneic cells in vitro (1-4). In accord with this, peritoneal exudate cells treated with anti-8 serum restore the response of adherent cell-depleted cultures (5). Recently, MacDonald et al. (6) demonstrated that a nonadherent, nonantigen specific, actinomycin-D-sensitive accessory cell is also required in mixed lymphocyte cultures (MLC's) to develop cytotoxic activity. Simpson (personal communication) has stated that the cytotoxic response of nonadherent spleen cells can be restored by 2-mercaptoethanol (2-ME).

In similar studies on the development of a humoral antisheep red blood cell response in vitro, Chen and Hirsch (7) showed that cultures depleted of adherent cells could be restored by 2-ME. Click et al. (8) first reported that 2-ME could stimulate the humoral response in vitro. Many other thiols potentiate the blastogenic response of lymphoid cells (9, 10).

We have investigated the effect of 2-ME on MLC's containing as the responding population either normal or nonadherent mouse spleen cells. The effect on the proliferative (TdR uptake), and cytotoxic effector functions was measured. Heber-Katz and Click (11) have already shown that 2-ME stimulates the proliferative phase of MLC's.

Materials and Methods

Mice.—C57BL/6 females were obtained from the Jackson Laboratories, Bar Harbor, Maine, and BALB/c female mice from the Salk Institute animal colony. All mice used in this study were 8-12 wk of age.

Cell Suspensions.—Spleens or lymph nodes (inguinal, axillary, and cervical) were aseptically removed and teased with forceps in Hanks' Balanced Salt Solution (BSS). Clumps were allowed to settle for 5 min on ice and the supernate transferred and centrifuged at 1,300 rpm in a Model CL International Centrifuge (International Equipment Co., Needham Hts., Mass.) for 5 min. The pellet was resuspended in medium and viable cell counts done in PBS-trypan blue. Stimulating cells were treated with 30 μg/ml mitomycin-C for 30 min in medium and viable cell counts done in PBS-trypan blue. Stimulating cells were treated with 30 μg/ml mitomycin-C for 30 min in medium at 37°C and washed three times afterwards. Adherent cells were removed on a column of Sephadex G-10 beads as detailed by Mishell (12).

Mixed Lymphocyte Cultures.—The medium used for cultures was as described by Dutton and Mishell (13), Eagle's minimum essential medium (no. 12-126, Microbiological Associates, Bethesda, Md.) supplemented with nonessential amino acids, antibiotics, pyruvate, glutamine, and 5% fetal calf serum (FCS). 2-ME was always added at the initiation of cultures at a final concentration of 60 μM.

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35-mm tissue culture dishes (no. 3001, Falcon Plastics Div. of BioQuest, Oxnard, Calif.) were used in all experiments. They were placed in an air-tight box gassed with 83% N₂, 10% CO₂, 7% O₂ and rocked at 7 cycles/min in a 37°C room. Cultures were fed daily with a nutritional cocktail described in reference 13. FCS was omitted from the cocktail when cultures did not originally contain FCS.

Assay of TdR Uptake.—Quadruplicate cultures were labeled with 1 μCi [³H]TdR, sp act 1 Ci/mmol. They were harvested on filters (Whatman GF/c), washed successively with BSS, 10% TCA, 5% TCA, and ethanol and counted in a toluene terphenyl scintillation fluid.

Assay of Cytotoxicity.—Tissue culture-maintained P815 mastocytoma cells were labeled with [⁵¹Cr]sodium chromate (Amersham/Searle Corp., Arlington Heights, Ill.) and used as the indicator cell. The assay in Fig. 1 was performed as described by Wunderlich (14). Later experiments were assayed in plastic tubes (no. 2052, Falcon Plastics) in a final volume of 0.6 ml. Four replicate MLC’s were set up, pooled in pairs at the end, and washed once before the cytotoxicity assay was done in Dulbecco-modified Eagle’s medium supplemented with 5% FCS for 4 h.

RESULTS AND DISCUSSION

The effect of 60 μM 2-ME on the in vitro cytotoxic response of unfraccionated, and nonadherent C57BL/6 spleen cells is shown in Fig. 1. Nonadherent cells responded very poorly if at all to allogeneic cells in the absence of 2-ME. Inclusion of 2-ME not only restored the nonadherent population but

![Fig. 1](image-url)

Fig. 1. Arithmetic plot of the development of cytotoxic activity in normal and nonadherent C57BL/6 spleen cells in the presence or absence of 2-ME. Cultures contained 2 × 10⁷ responding cells and 1 × 10⁶ BALB/c mitomycin-C-treated stimulating lymph node cells. Assay performed on day 5 of culture. (A) Results expressed on a killer to target cell ratio basis. (B) The same results expressed on a per culture basis. A background release of 6% of target cells cultured alone had been subtracted. Normal spleen cells — 2-ME (□—□); nonadherent spleen cells — 2-ME (○—○); normal spleen cells + 2-ME (■—■); nonadherent spleen cells + 2-ME (●—●).
also stimulated the response of normal spleen cells. The stimulation is evident whether the results are plotted on a per culture (Fig. 1 B) or on a cell recovery basis (Fig. 1 A). The viable cell recovery at day 5 was, for normal spleen cells, 19%; normal spleen cells plus 2-ME, 28%; nonadherent spleen cells, 2.5%; nonadherent spleen cells plus 2-ME, 22%. Poor cell survival in these culture conditions without 2-ME is characteristic of nonadherent lymphoid populations.

A dissociation between proliferation and the generation of cytotoxicity can be demonstrated in MLC's (15-17). Thus, in order to compare the effect of 2-ME on both these responses we set up MLC's at higher ratios of stimulating to responding cells. 5 × 10⁶ viable normal spleen cells were cultured with 5 × 10⁶ viable mitomycin-C-treated syngeneic (C57BL/6) or allogeneic (BALB/c) normal spleen cells. Proliferation and cytotoxicity were measured on days 4, 5, and 6. Proliferation was maximal at day 4, and cytotoxicity at day 5 in cultures with and without 2-ME. Cytotoxicity was measured against ⁵¹Cr-labeled F815 cells, by making serial twofold dilutions of pairs of duplicate cultures (Fig. 2). A summary of the results is presented in Table I. At the peak of proliferation, 2-ME enhanced the antigen-stimulated DNA synthesis by a factor of 4.7, the cell recovery at day 5 in the allogeneic mixtures was increased.
TABLE I

Effect of 2-ME on the DNA Synthetic and Cytotoxic Response of Normal C57BL/6 Spleen Cells

| Cultures* | TdR incorporation (c.p.m.) | Cell recovery (day 5) | Cytotoxic activity (day 5)* |
|-----------|---------------------------|----------------------|---------------------------|
|           |                           |                      |                           |
| -2-ME, C57BL/6 + C57BL/6M | 1,600                     | 38                   | 0                         |
| -2-ME, C57BL/6 + BALB/CM | 4,644                     | 49                   | 24.4                      |
| +2-ME, C57BL/6 + C57BL/6M | 2,526                     | 47                   | 0.51                      |
| +2-ME, C57BL/6 + BALB/CM | 16,730                    | 77                   | 100                       |

* Cultures contained in 1 ml of medium 5 × 10^6 viable-responding and mitomycin-C-treated stimulating cells.

† Expressed on a per culture basis. Calculated from the data in Fig. 2 by comparing the fraction of one culture required to cause the same degree of target cell lysis.

by 2-ME less than twofold, while cytotoxic activity on a per culture basis was stimulated 4.2 times.

In six similar experiments using from 2–5 × 10^6 responding and stimulating spleen cells the antigen-induced increase of proliferation and cytotoxicity by 2-ME has varied from 3.5–15-fold. The fewer cells in the cultures the greater is the stimulation by 2-ME.

A surprising feature of using 2-ME in MLC’s was the finding that it stimulated background cytotoxicity in cultures without added allogeneic cells. This is illustrated in Fig. 2 where the syngeneic cultures with 2-ME develop cytotoxic activity at 0.51% of the level in allogeneic cultures with 2-ME. This is a very consistent finding which is never seen in cultures without 2-ME.

Considering the possibility that FCS might contain antigens cross-reactive with antigens on the target cell, we set up MLC’s in medium with 0.8% C57BL/6 serum (heat inactivated at 56°C for 5 min, stored frozen at −20°C for 24 h before use) or in medium with no serum supplement. Table II shows that in the presence of 2-ME one can detect a mixed lymphocyte stimulation of TdR uptake with both these media (18, 19). In the absence of 2-ME, serum-free cultures did not incorporate TdR at all while in 0.8% normal mouse serum (NMS) without 2-ME, the syngeneic mixture incorporated 197 cpm, and the allogeneic mixture 925 cpm. In the cultures with 2-ME cytotoxicity was detectable in both the syngeneic and allogeneic cultures containing 5% FCS, but only in the allogeneic mixture when the serum source was mouse serum. However, since the response in mouse serum is only a fraction of that obtained in FCS, this experiment does not argue very strongly for some source of serum antigenic stimulation in the development of background cytotoxicity. Furthermore, the background cytotoxicity of C57BL/6 spleen cells cultured alone in the presence of 2-ME and FCS is expressed to about the same degree on a syngeneic target cell (EL4) as on the allogeneic P815 target, and inclusion of phyto-
TABLE II

| Cultures* | TdR incorporation (h 76-92) | Cell recovery (day 5) | Cytotoxic activity (day 5)† |
|-----------|-----------------------------|----------------------|---------------------------|
| 5% FCS; C57BL/6 + C57BL/6M | 16,951 | 58 | 1.5 |
| 5% FCS; C57BL/6 + BALB/c | 77,744 | 96 | 100 |
| 0.8% NMS; C57BL/6 + C57BL/6M | 623 | 30 | 0 |
| 0.8% NMS; C57BL/6 + BALB/C | 18,854 | 35 | 4.1 |
| No serum; C57BL/6 + C57BL/6M | 221 | 22 | 0 |
| No serum; C57BL/6 + BALB/C | 861 | 24 | 0 |

* Cultures contained in 1 ml of medium 2 × 10^6 viable-responding and mitomycin-C-treated stimulating cells. All contained 60 μM 2-ME.
† Expressed on a per culture basis.

hemagglutinin (PHA) in the 4-h assay to provide close contact between cells leads to an increase in cytotoxicity on either target. Thus it appears that the background cytotoxic cells are directed against antigens not present on the target cell.

The enhancement of in vitro immune responses by 2-ME probably comes about simply by improving culture conditions. That thiols improve the medium for the growth of some lymphomas (10) in the absence of any accessory cells or antigenic stimulation is supporting evidence for this.

SUMMARY

Mouse spleen cells which have been depleted of adherent cells do not respond to allogeneic lymphocytes in vitro. Their cytotoxic response can be restored by inclusion of mercaptoethanol in the medium. Mercaptoethanol is shown to have a stimulatory effect also on the response of normal (unseparated) spleen cells to alloantigens. The enhancement of the DNA-synthetic and cytotoxic response is similar, varying from 3.5-15-fold. Cytotoxic cells also appear in unmixed lymphocyte cultures in the presence of mercaptoethanol and fetal calf serum. The specificity of these background cytotoxic cells is not known.

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