

## NONSPECIFIC ACTIVATION OF MURINE LYMPHOCYTES

### I. Proliferation and Polyclonal Activation Induced by 2-Mercaptoethanol and $\alpha$ -Thioglycerol\*

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The addition of 2-mercaptoethanol (2-ME)<sup>1</sup> to culture media has been found to produce profound effects in a number of different situations: (a) it is essential for the establishment and propagation of several murine lymphoma cell lines (1); (b) it enhances the DNA synthetic response of lymphocytes to mitogens (1, 2), (c) and to allogeneic lymphocytes in mixed lymphocyte cultures (MLC) (3, 4); (d) it enhances the primary immune response in vitro (5); (e) it functionally replaces macrophages in the primary immune response (6); (f) it enhances the generation of cytotoxic thymus-dependent lymphocytes in the MLC (4, 7, 8), and (g) it is essential for the culture and proliferation of clones of B lymphocytes (9, 10).

In studying the mechanism by which antigens activate lymphocytes to proliferate and synthesize antibody, the small number of lymphocytes specific for any particular antigen has proven to be a major problem. Therefore, the use of nonspecifically activating substances, or mitogens, has gained popularity as a practical model for the study of lymphocyte activation (11-13). To dissect the mechanism involved, the actions of the mitogen must first be dissected. Bacterial lipopolysaccharide (LPS), for example, is known to affect a large number of biological parameters (cited in 14), some of which may<sup>2</sup> (15) or may not (16) be dissociable. Therefore, the simpler the probe used, the clearer is the interpretation of the results obtained.

In this paper, data are presented which indicate that 2-ME and  $\alpha$ -thioglycerol

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<sup>1</sup> *Abbreviations used in this paper.* ATS, rabbit anti-mouse thymocyte serum; Con A, concanavalin A; FCS, fetal calf serum; GRBC, goat red blood cells; LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; MLC, mixed lymphocyte culture; NW, nylon wool; PFC, plaque-forming cell; Poly IC, polyinosinic-polycytidilic acid;  $\alpha$ TG,  $\alpha$ -thioglycerol.

<sup>2</sup> Parks, D. E., M. V. Doyle, and W. O. Weigle. Induction of responsiveness and unresponsiveness to HGG in C57BL/6J athymic mice. Manuscript in preparation.

( $\alpha$ TG), two- and three-carbon molecules, respectively, are able to enhance DNA synthesis, blast transformation, and polyclonal antibody production in cultures of murine spleen cells. Cellular targets and requirements for this response are also examined.

### Materials and Methods

*Mice.* C3H/St male mice, 8–12 wk of age, were obtained from the L. C. Strong Laboratory, Del Mar, Calif. C3H/HeJ male mice, 8–12 wk of age, were obtained from The Jackson Laboratory, Bar Harbor, Maine. Congenitally athymic (nude) mice, generously provided by Dr. Norman D. Reed at Montana State University, Bozeman, Mont., were subsequently bred onto a C57BL/6J background at Scripps Clinic and Research Foundation, La Jolla, Calif. All mice were maintained on Wayne Lab-Blox F6 pellets (Allied Mills, Inc., Chicago, Ill.) and chlorinated water acidified to a pH of 3.0 with HCl (17).

*Mitogens.* *Escherichia coli* K235 LPS, prepared by a phenol-water extraction procedure, was generously supplied by Abbott Laboratories, North Chicago, Ill., through Dr. Floyd C. McIntire, School of Dentistry, University of Colorado Medical Center, Denver, Colo. Concanavalin A (Con A) was obtained from Miles-Yeda, Ltd., Rehovot, Israel. Polyinosinic-polycytidilic acid (Poly IC) double stranded sodium salt was purchased from P-L Biochemicals, Inc., Milwaukee, Wis.

*Culture Reagents.* Constituents of the serum-containing culture medium employed have been previously described (18), except that RPMI-1640 was substituted for Eagle's medium. Serum-free medium was prepared as follows: 100 ml contained 95.9 ml RPMI-1640 (Flow Laboratories, Inc., Rockville, Md.), 0.1 ml of  $100 \times$  glutamine, 1.0 ml of  $100 \times$  Na pyruvate, 1.0 ml of  $50 \times$  nonessential amino acids, and 1.0 ml of 1.0 M HEPES buffer (Microbiological Associates, Bethesda, Md.). 2-ME (Matheson, Coleman, & Bell, Los Angeles, Calif.) and  $\alpha$ TG (Sigma Chemical Co., St. Louis, Mo.) were diluted in phosphate-buffered saline and sterilized by filtration. Further dilution in complete medium was used for microcultures.

*Lymphocyte Suspensions.* Spleen cell suspensions were prepared as described previously (19). Spleen cells enriched for T lymphocytes were prepared by passage through nylon wool (NW) columns, as described previously (19). B-cell-enriched populations were prepared by treating  $10^8$  spleen cells with a 1:80 dilution of rabbit anti-mouse thymocyte serum (ATS) (Lot 15038, Microbiological Associates), absorbed 6 times with a 1:10 ratio of a surface immunoglobulin-positive, nonsecreting myeloma cell line (XS-63), and absorbed one time at a 1:10 ratio with mouse erythrocytes after removal of the buffy coat cell layer. All absorptions were carried out for 30 min at 4°C. The treated cells were then spun at 280 g for 10 min, the serum removed, and the cells resuspended in a 1:6 dilution of C3H-RBC-absorbed guinea pig complement at 37°C for 45 min. Cells were then washed and cultured as above.

Macrophages were depleted by incubation of spleen cells with a suspension of carbonyl iron (GAF Corp., New York) as described by Sjöberg et al. (20) and modified by Lee et al. (21). In other experiments, spleen cells were allowed to adhere to plastic Petri dishes (Falcon Plastics, Oxnard, Calif.) at 37°C in 5% CO<sub>2</sub> for 3 h. Nonadherent cells were transferred to a second dish and allowed to adhere for an additional hour (21). Nonadherent cells from this dish were counted and cultured as described.

*Lymphocyte Cultures.* Lymphocytes were cultured in plastic tubes (No. 2054, Falcon Plastics) at a cell density of  $2.0 \times 10^6$  viable cells/ml in a vol of 1.0 ml. Tubes were incubated at an angle of 5°, at 37°C, in a humidified atmosphere of 5% CO<sub>2</sub> in air. In other experiments, cultures were incubated in microculture plates (No. 3040, Falcon Plastics) at a density of  $5 \times 10^5$  viable cells/well in a vol of 0.1 ml. These were also incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Culture tubes were fed daily with 120–150  $\mu$ l of nutritional cocktail; microcultures were fed daily with 10–20  $\mu$ l of cocktail (22).

*Measurement of DNA Synthesis.* During the final 24 h of culture, cells were radiolabeled with 1.0  $\mu$ Ci of [<sup>3</sup>H]thymidine (TdR) per culture (6.7 Ci/mM, New England Nuclear, Boston, Mass.). Cells from culture tubes were then washed twice with cold phosphate-buffered normal saline and precipitated with cold 5% TCA. TCA-insoluble precipitates were filtered, dried, and counted for tritium as previously described (19). Microcultures were harvested on a MASH-II unit (Microbiological Associates). Results are expressed as the arithmetic mean of triplicate or quadruplicate cultures  $\pm$  the standard error.

*Enumeration of Blast Cells.* Histological preparations were generated on microscopic slides from individual lymphocyte cultures with the aid of a cytocentrifuge. Slides were stained with the methyl green-pyronin Y technique (23) to simplify the enumeration of pyroninophilic blast cells. Results are expressed as the arithmetic mean of quadruplicate cultures  $\pm$  the standard error.

*Assay of Plaque-Forming Cells (PFC).*  $1.5 \times 10^7$  spleen cells/ml were incubated in 3 ml with or without 2-ME or  $\alpha$ TG in serum-free or 5% fetal calf serum (FCS)-containing media. PFC's to sheep red blood cells (SRBC) and trinitrophenyl (TNP) (24) were assayed at day 2 of culture (25) using a modification of the hemolytic plaque assay of Jerne and Nordin (26) as previously described (22). The number of TNP-specific plaques was calculated by subtracting the PFC response to goat red blood cells (GRBC) from the response to TNP-GRBC. Results are expressed as the arithmetic mean of triplicate cultures  $\pm$  the standard error.

## Results

*Dose Dependency of the DNA Synthetic Response to 2-ME and  $\alpha$ TG.* The ability of 2-ME and  $\alpha$ TG to activate DNA synthesis was investigated in cultures of spleen cells from LPS-responsive (C3H/St) and LPS-resistant (C3H/HeJ) mice. Spleen cells were incubated in serum-free or 5% FCS containing medium with variable concentrations of 2-ME. Resultant DNA synthesis was measured at 3 days of culture after incubation with [ $^3$ H]TdR for the final 24 h of culture. The results shown in Figs. 1, 2, and 3 indicate that these thiol compounds do indeed promote the incorporation of [ $^3$ H]TdR into DNA in a dose-dependent manner. [ $^3$ H]TdR incorporation was much greater in medium containing 5% FCS than in serum-free medium in both control and experimental cultures. However, the stimulation index (E/C) in serum-free medium was greater than that in medium with 5% FCS due to low background [ $^3$ H]TdR incorporation under serum-free conditions. In each strain assayed, peak [ $^3$ H]TdR incorporation occurred at a concentration of  $5 \times 10^{-5}$  M for either thiol tested. Lower concentrations induced suboptimal DNA synthesis, while higher concentrations appeared to be inhibitory.

*Ability of 2-ME to Induce Blastogenesis.* To further establish the stimulatory effect of these thiol compounds, the ability of 2-ME to induce blast transformation was investigated in spleen cell cultures from C3H/St mice. Spleen cells were cultured in the absence of serum with variable concentrations of 2-ME. After 3 days of incubation, each culture was transferred to a slide by means of a cytocentrifuge, and stained as described in Materials and Methods. Pyroninophilic blast cells were enumerated under the microscope. The results, shown in Fig. 4, indicate that 2-ME is able to cause blast transformation. The dose-dependency and optimal concentration of 2-ME for this effect parallel those seen with incorporation of [ $^3$ H]TdR into DNA.

*Kinetics of the Response to 2-ME.* The time at which the maximal 2-ME effect occurred was assessed in C3H/St and C3H/HeJ mice. Spleen cells were cultured either with or without serum in the presence or absence of  $5 \times 10^{-5}$  M 2-ME. Subsequently, one set of cultures was harvested daily, after a 24-h pulse of [ $^3$ H]TdR. In both strains, maximal [ $^3$ H]TdR incorporation into DNA occurred at day 3 and declined thereafter (Figs. 5 and 6). In another experiment, complete dose-response curves, assayed at days 2 and 3, confirmed the day 3 kinetic peak. Kinetic experiments with  $\alpha$ TG gave results parallel to those seen with 2-ME.

*Effect of 2-ME on Cell Viability.* A number of other investigations have gathered evidence that the various effects of 2-ME studied were not simply

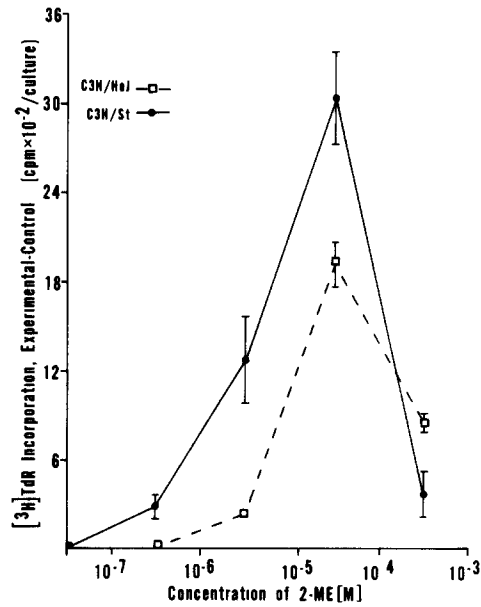


FIG. 1. Dose-response profile of 2-ME in serum-free cultures.  $2 \times 10^6$  viable C3H/St or C3H/HeJ spleen cells were cultured for 3 days in 1.0 ml of serum-free medium in the presence of various concentrations of 2-ME. Cells were pulsed with 1.0  $\mu$ Ci of [ $^3$ H]Tdr (0.1 Ci/mM) for the final 24 h of culture. Results are expressed as the arithmetic mean of triplicate cultures containing 2-ME minus triplicate control cultures  $\pm$  the sum of the standard errors

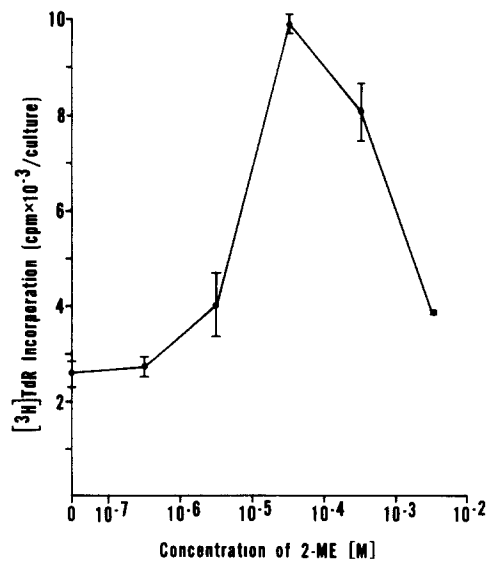


FIG. 2. Dose-response profile of 2-ME in serum containing cultures.  $2 \times 10^6$  viable C3H/St spleen cells were cultured for 3 days in 1.0 ml of medium containing 5% FCS, in the presence of various concentrations of 2-ME. Cells were pulsed with 1.0  $\mu$ Ci of [ $^3$ H]Tdr (0.1 Ci/mM) for the final 24 h of culture. Results are expressed as the arithmetic mean of triplicate cultures  $\pm$  the standard error

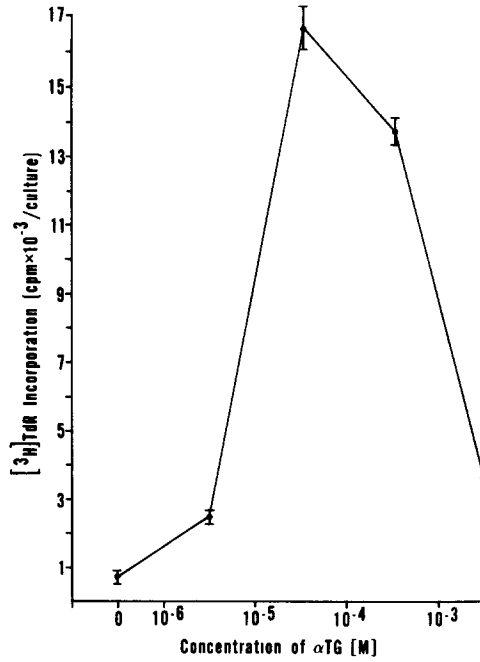


FIG. 3. Dose-response profile of  $\alpha$ TG in serum-free culture.  $5 \times 10^5$  viable C3H/St spleen cells were cultured in microculture wells in 0.1 ml of serum-free medium, in the presence of various concentrations of  $\alpha$ TG. Cells were pulsed with 1.0  $\mu$ Ci of [ $^3$ H]Tdr (6.7 Ci/mM) for the final 24 h of culture. Results are expressed as the arithmetic mean of quadruplicate cultures  $\pm$  the standard error.

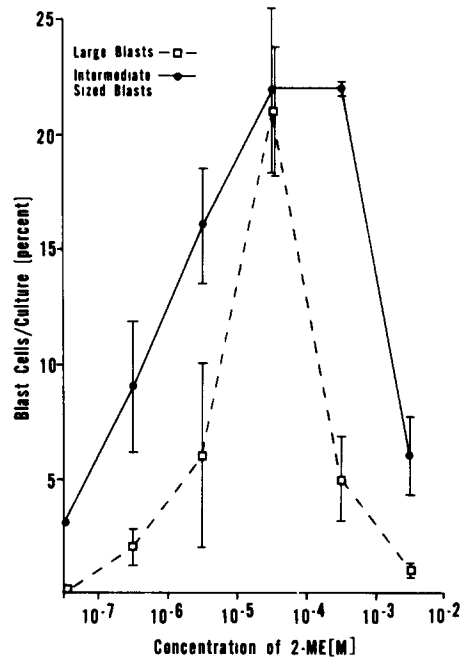


FIG. 4. The blastogenic response to 2-ME in serum-free cultures.  $5 \times 10^5$  viable C3H/St spleen cells were cultured in 0.1 ml of serum-free medium with various concentrations of 2-ME. After 3 days of culture, the cells were transferred to microscopic slides with a cytocentrifuge and stained with methyl green-pyronin Y stain. Blast cells were enumerated under the microscope. Results are expressed as the arithmetic mean of quadruplicate cultures  $\pm$  the standard error.

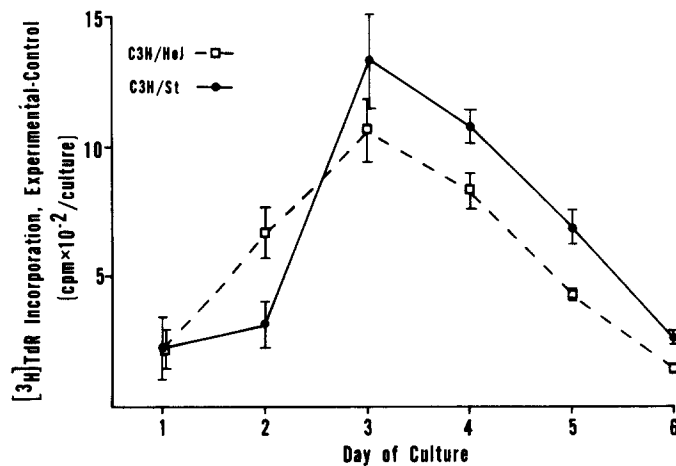


FIG. 5. Kinetic profile of the proliferative response to 2-ME in serum-free cultures  $2 \times 10^6$  viable C3H/St or C3H/HeJ spleen cells were cultured in 1.0 ml of serum-free medium in the presence or absence of  $5 \times 10^{-5}$  M 2-ME. On each day, one set of cultures was harvested after a 24-h pulse of  $1.0 \mu\text{Ci}$  of  $[^3\text{H}]\text{TdR}$  ( $0.1 \text{ Ci/mM}$ ). Results are expressed as the arithmetic mean of  $[^3\text{H}]\text{TdR}$  incorporation of triplicate cultures containing 2-ME minus that of triplicate control cultures  $\pm$  the sum of the two standard errors.

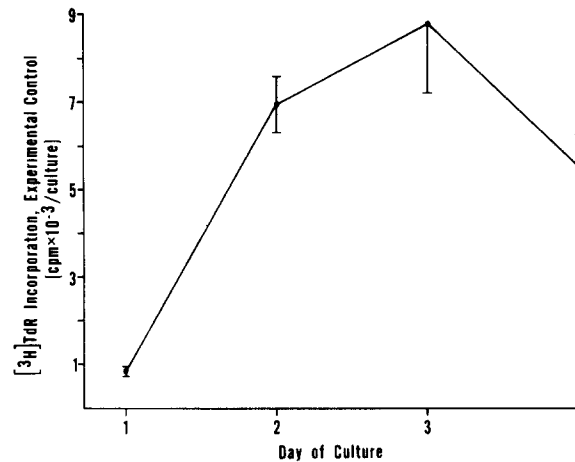


FIG. 6. Kinetic profile of the proliferative response to 2-ME in serum-containing cultures  $2 \times 10^6$  viable C3H/St spleen cells were cultured in 1.0 ml of 5% FCS-containing medium in the presence or absence of  $5 \times 10^{-5}$  M 2-ME. On each day, one set of cultures was harvested after a 24-h pulse of  $1.0 \mu\text{Ci}$  of  $[^3\text{H}]\text{TdR}$  ( $0.1 \text{ Ci/mM}$ ). Results are expressed as the arithmetic mean of  $[^3\text{H}]\text{TdR}$  incorporation of triplicate cultures containing 2-ME minus that of triplicate control cultures  $\pm$  the sum of the two standard errors.

artifacts of enhanced cellular viability (1, 3). It is of paramount importance to determine whether improved viability could be a factor as early as day 3 of culture, and whether this accounts for the increased level of  $[^3\text{H}]\text{TdR}$  incorporation seen in cultures incubated with 2-ME. Therefore, C3H/St spleen cells were cultured under serum-free conditions with either  $5 \times 10^{-5}$  M 2-ME or an equal volume of RPMI-1640 as a control. As can be seen in Table I, no significant

TABLE I  
Effect of 2-ME on Cell Viability\*

| Day of culture | 2-ME | Viable cells $\times 10^{-6}$ /culture $\ddagger$ |
|----------------|------|---|
| 1              | -    | 1.29 $\pm$ 0.18                                   |
|                | +    | 1.18 $\pm$ 0.03                                   |
| 2              | -    | 1.01 $\pm$ 0.10                                   |
|                | +    | 1.08 $\pm$ 0.02                                   |
| 3              | -    | 0.65 $\pm$ 0.05                                   |
|                | +    | 0.77 $\pm$ 0.04                                   |

\*  $2 \times 10^6$  C3H/St spleen cells were cultured in 1 ml of serum-free medium in the presence or absence of  $5 \times 10^{-5}$  M 2-ME. Viable cells/culture were determined by exclusion of trypan blue dye at days 1, 2, and 3.

$\ddagger$  Results are expressed as the arithmetic mean of triplicate cultures  $\pm$  standard error.

difference in viabilities occurs during the first 3 days of culture. This result is in agreement with that of Broome and Jeng (1), who found no significant effect on viability before day 4. We have consistently seen no difference in viability between 2-ME treated and control cultures, for both C3H/St and C3H/HeJ mice, whether cultured in FCS-containing or serum-free medium.

*Ability of 2-ME to Promote DNA Synthesis in Cultures from Congenitally Athymic Mice.* Attention next focused upon whether a molecule as small as 2-ME is capable of enhancing DNA synthesis in both bone marrow-derived (B) lymphocytes and thymus-dependent (T) lymphocytes, or whether it is restricted to one of these populations. Spleen cells from congenitally athymic (*nu/nu*) C57BL/6J mice and from their heterozygous (*nu/+*) littermates were cultured in the presence of varying doses of 2-ME, in medium containing 5% FCS. In addition, the response to a known B-lymphocyte mitogen, Poly IC, was elicited for purposes of comparison (27). Fig. 7 illustrates that in both the C57BL/6J nude and its heterozygous littermate, 2-ME evokes a dose-response profile similar to that seen in the C3H/St and C3H/HeJ, with peak [ $^3$ H]TdR incorporation occurring at a concentration of  $5 \times 10^{-5}$  M 2-ME. However, two additional points should be noted. First, the response of the nude was only about 60% of that of the heterozygous littermate. Second, in order to evaluate whether this result was representative of depressed reactivity of nude B cells to agents capable of inducing DNA synthesis, the response to a maximally stimulatory dose of Poly IC was elicited. In this case, however, the nude response was 160% of that mounted by its heterozygous littermate. That this response was greater than normal may well have been a reflection of the greater preponderance of B lymphocytes in the nude cultures. In any case, nude B-cell responsiveness to an established B-cell mitogen does not appear to be depressed. This raises the possibility that the difference between the *nu/nu* and *nu/+* responses may have been attributable to an effect of 2-ME on T lymphocytes. The data presented below support such a conclusion.

*Response of Separately Cultured B-Enriched and T-Enriched Lymphocytes to 2-ME and  $\alpha$ TG.* C3H/St spleen cell populations enriched for B cells by treat-

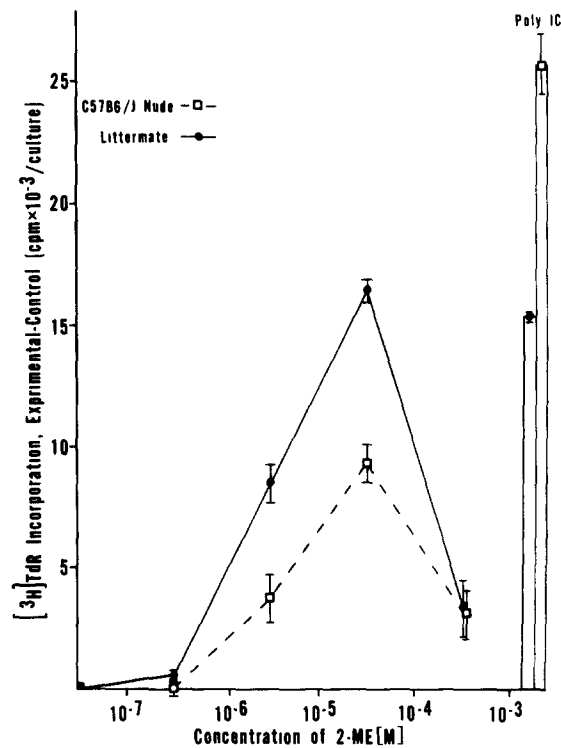


FIG. 7. The response of *nu/nu* and *nu/+* spleen cells to 2-ME.  $2 \times 10^6$  spleen cells from C57BL/6J *nu/nu* or *nu/+* mice were cultured for 3 days in 1.0 ml of 5% FCS-containing medium in the presence of various concentrations of 2-ME. Cells were pulsed with 1.0  $\mu$ Ci of [ $^3$ H]TdR (0.1 Ci/mM) during the final 24 h of culture. The response to Poly IC is shown for comparison. Results are expressed as the arithmetic mean of triplicate cultures containing 2-ME minus triplicate control cultures  $\pm$  the sum of the two standard errors

ment with rabbit anti-thymocyte serum and guinea pig complement were cultured under serum-free conditions with optimal doses of 2-ME,  $\alpha$ TG, K235 LPS, or Con A. As can be seen in Table II, the responses to 2-ME and  $\alpha$ TG are retained, while the insignificant response to Con A indicates marked depletion of T cells. This provides strong confirmatory evidence that B cells are 2-ME responsive. T-lymphocyte-enriched populations prepared by nylon column passage and then cultured under serum-free conditions were not capable of a significant response to any of the above stimulatory substances. Therefore, the experiment was repeated under more optimal culture conditions using medium containing 5% FCS. The results of such experiments (Table III) demonstrate that both B lymphocytes and T lymphocytes can proliferate in response to 2-ME. When the two enriched populations are combined, a definite synergistic effect results (15,361 cpm/co-culture vs. 8359 cpm/average culture of T and B cells).

*The Response to 2-ME and  $\alpha$ TG is Not Adherent-Cell Dependent.* Since 2-ME has long been considered to have the capability of replacing macrophages in vitro (6, 28), an experiment was designed to determine whether the proliferative response to 2-ME and  $\alpha$ TG is mediated through macrophages or is independent of them. C3H/St spleen cells were allowed to adhere to plastic Petri dishes for 3



TABLE II  
Effect of 2-ME and  $\alpha$ TG on ATS + C'-Treated Spleen Cells in Serum-Free Culture\*

| Cell treatment | Stimulator added | cpm/culture‡ | ±SE    | E/C§   | $\frac{\text{cpm ATS}\parallel}{\text{cpm normal}}$ |
|----------------|------------------|--------------|--------|--------|---|
| None           | Complete medium  | 235          | ±25    | 1.00   |   |
|                | 2-ME             | 12,373       | ±1,074 | 52.61  |   |
|                | $\alpha$ TG      | 17,020       | ±458   | 72.36  |   |
|                | LPS              | 72,912       | ±2,909 | 310.00 |   |
|                | Con A            | 129,139      | ±7,331 | 549.06 |   |
| ATS + C'       | Complete medium  | 683          | ±105   | 1.00   | 2.91  |
|                | 2-ME             | 19,286       | ±1,152 | 28.24  | 1.56  |
|                | $\alpha$ TG      | 13,908       | ±441   | 20.36  | 0.82  |
|                | LPS              | 62,158       | ±1,385 | 91.01  | 0.85  |
|                | Con A            | 1,395        | ±151   | 2.04   | 0.01  |

\*  $5 \times 10^6$  normal or ATS + C'-treated C3H/St spleen cells were cultured in 0.1 ml of serum-free medium for 3 days in the presence of various stimulating substances. Cells were pulsed with 1  $\mu$ Ci of [ $^3$ H]TdR for the final 24 h of culture.

‡ Results are expressed as the arithmetic mean of quadruplicate cultures  $\pm$  standard error.

§ E/C represents the index of stimulation.

|| The ratio of the treated response: normal response reflects the fraction of the original response left intact after treatment.

TABLE III  
Effect of 2-ME on B-Cell Enriched Cultures, T-Cell Enriched Cultures, and on B + T Cell Cultures in 5% FCS-Containing Medium\*

| No cells cultured $\times 10^{-6}$ |          |    | Stimulator added | cpm/culture‡ | ±SE    | E/C§   | $\frac{\text{cpm treated}\parallel}{\text{cpm normal}}$ |
|------------------------------------|----------|----|------------------|--------------|--------|--------|---|
| Normal                             | ATS + C' | NW |                  |              |        |        |   |
| 2                                  | —        | —  | RPMI-1640        | 2,193        | ±53    | 1.00   |   |
| 2                                  | —        | —  | 2-ME             | 11,939       | ±130   | 5.44   |   |
| 2                                  | —        | —  | LPS              | 29,281       | ±2,100 | 13.35  |   |
| 2                                  | —        | —  | ConA             | 57,724       | ±7,194 | 26.32  |   |
| —                                  | 2        | —  | RPMI-1640        | 2,073        | ±410   | 1.00   | 0.95  |
| —                                  | 2        | —  | 2-ME             | 11,335       | ±453   | 5.47   | 0.95  |
| —                                  | 2        | —  | LPS              | 33,421       | ±2,235 | 16.12  | 1.14  |
| —                                  | 2        | —  | ConA             | 910          | ±200   | 0.44   | 0.02  |
| —                                  | —        | 2  | RPMI-1640        | 218          | ±28    | 1.00   | 0.10  |
| —                                  | —        | 2  | 2-ME             | 5,383        | ±449   | 24.69  | 0.45  |
| —                                  | —        | 2  | LPS              | 2,551        | ±434   | 11.7   | 0.09  |
| —                                  | —        | 2  | ConA             | 33,634       | ±2,744 | 154.29 | 0.58  |
| —                                  | 1        | 1  | RPMI-1640        | 1,561        | ±43    | 1.00   | 0.71  |
| —                                  | 1        | 1  | 2-ME             | 15,361       | ±300   | 9.84   | 1.29  |
| —                                  | 1        | 1  | LPS              | 21,348       | ±904   | 13.68  | 0.73  |
| —                                  | 1        | 1  | ConA             | 54,442       | ±3,538 | 34.88  | 0.94  |

\*  $2 \times 10^6$  normal, ATS + C' treated, or nylon wool-passaged C3H/St spleen cells were cultured in 1.0 ml of 5% FCS-containing medium for 3 days, in the presence of various stimulatory substances. Cells were pulsed with 1  $\mu$ Ci of [ $^3$ H]TdR for the final 24 h of culture.

‡ Results are expressed as the arithmetic mean of triplicate cultures  $\pm$  standard error.

§ Same as Table II.

|| Same as Table II.

h, after which time the nonadherent cells were transferred to fresh plastic Petri dishes and allowed to adhere for an additional hour (21). Nonadherent cells obtained by this procedure were then incubated at 37°C with 80 mg/ml of carbonyl iron for 45 min, with agitation every 5 min to discourage depletion of

lightly adherent (B) cells. After three 3-min passes over a magnet, the remaining cells were cultured with 2-ME,  $\alpha$ TG, LPS, Con A, or complete medium as a control. Control cells for cultures depleted of macrophages by adherence were incubated in plastic Petri dishes and transferred to fresh Petri dishes after resuspension of adherent cells with a rubber policeman. Cells obtained by this procedure were incubated for 45 min without carbonyl iron before initiation of cultures as a control for macrophage depletion by treatment with carbonyl iron. Cell preparations depleted of macrophages by a combination of adherence followed by carbonyl iron treatment were unresponsive to 2-ME,  $\alpha$ TG, LPS, or Con A for unknown reasons. Therefore, cells were treated either by adherence to plastic twice or by carbonyl iron incubation, but not by both. Table IV shows that adherent cell-depleted populations respond well to 2-ME and  $\alpha$ TG. Table V illustrates the response to spleen cells treated with carbonyl iron. All responses are significantly diminished, but the 2-ME and  $\alpha$ TG responses are not preferentially reduced relative to the LPS response.

*Ability of 2-ME and  $\alpha$ TG to Induce Polyclonal Activation.* Since mitogenicity and polyclonal B-cell activation appear to be very closely linked (29, Goodman, M. G., unpublished observation), the ability of 2-ME and  $\alpha$ TG to promote polyclonal B-cell expansion was evaluated.  $4.5 \times 10^7$  spleen cells were cultured in either serum-free or 5% FCS-containing media. Experimental cultures were made  $5 \times 10^{-5}$  M with 2-ME or  $\alpha$ TG, while control cultures received an equal volume of RPMI-1640. The data presented in Table VI indicate that both 2-ME and  $\alpha$ TG are effective polyclonal activators. In all cases,  $\alpha$ TG was a more potent polyclonal activator than 2-ME. As expected, the anti-TNP PFC response in 5% FCS exceeded that produced under serum-free conditions. The reasons why the anti-SRBC PFC response was not greater in 5% FCS than in serum-free medium are unclear.

### Discussion

Addition of either 2-ME or  $\alpha$ TG to cultures of murine spleen cells results in a dramatic increase in incorporation of [ $^3$ H]TdR. This phenomenon occurs in a dose-related fashion, such that suboptimal doses result in suboptimal [ $^3$ H]TdR incorporation and supraoptimal doses cause an inhibition of incorporation. The shape of the dose-response curve is reminiscent of those seen with the murine lymphocyte mitogens, LPS and tetravalent Con A (18, 30). It may be tempting, therefore, to classify 2-ME and  $\alpha$ TG with surface-active mitogens. However, since other sites of action are also compatible with the present data such a classification would be premature. The conclusion that 2-ME is able to effect increased [ $^3$ H]TdR incorporation is in conflict with that of some investigators (1) whose data, however, appear contrary to their conclusion. Other authors have noted enhanced DNA synthesis due to 2-ME while investigating various actions of this thiol compound (3, 31). In any event, with the exception of one other report,<sup>3</sup> a stimulatory effect of 2-ME on lymphocytes has not been studied in depth, nor has evidence for a similar effect induced by  $\alpha$ TG yet been reported.  $\alpha$ TG was shown to be a more potent inducer of lymphocyte proliferation and

<sup>3</sup> During preparation of this manuscript, a paper with similar results was published by H. Lemke and H-G. Opitz (28)

TABLE IV  
Effect of 2-ME and  $\alpha$ TG on Adherent Cell-Depleted Cultures\*

| Cell treatment | Stimulator added | cpm/culture‡ | ±SE    | E/C§  | cpm treated |
|----------------|------------------|--------------|--------|-------|-------------|
|                |                  |              |        |       | cpm normal  |
| None           | Complete medium  | 270          | ±24    | 1.00  |             |
|                | 2-ME             | 4,425        | ±187   | 16.41 |             |
|                | $\alpha$ TG      | 5,440        | ±1,141 | 20.15 |             |
| Adherence      | Complete medium  | 265          | ±18    | 1.00  | 0.98        |
|                | 2-ME             | 4,568        | ±389   | 17.24 | 1.03        |
|                | $\alpha$ TG      | 5,735        | ±525   | 21.64 | 1.05        |

\*  $5 \times 10^5$  normal or adherent cell-depleted C3H/St spleen cells were cultured in 0.1 ml of serum-free medium for 3 days in the presence of various stimulating substances. Cells were pulsed with  $1 \mu\text{Ci}$  [ $^3\text{H}$ ]TdR for the final 24 h of culture.

‡ Results are expressed as the arithmetic mean of quadruplicate cultures  $\pm$  standard error.

§ Same as Table II.

|| Same as Table II.

TABLE V  
Effect of 2-ME and  $\alpha$ TG on Cultures Depleted of Macrophages by Treatment with Carbonyl Iron\*

| Cell treatment | Stimulator added | cpm/culture‡ | ±SE    | E/C§  | cpm treated |
|----------------|------------------|--------------|--------|-------|-------------|
|                |                  |              |        |       | cpm normal  |
| Exp. 1         |                  |              |        |       |             |
| None           | Complete medium  | 194          | ±9     | 1.00  |             |
|                | 2-ME             | 5,230        | ±448   | 26.91 |             |
|                | $\alpha$ TG      | 8,781        | ±768   | 45.18 |             |
| Carbonyl iron  | Complete medium  | 171          | ±23    | 1.00  | 0.88        |
|                | 2-ME             | 2,455        | ±142   | 14.37 | 0.45        |
|                | $\alpha$ TG      | 4,065        | ±521   | 23.80 | 0.45        |
| Exp. 2         |                  |              |        |       |             |
| None           | Complete medium  | 537          | ±78    | 1.00  |             |
|                | 2-ME             | 5,892        | ±179   | 10.97 |             |
|                | $\alpha$ TG      | 9,083        | ±687   | 16.91 |             |
|                | LPS              | 38,718       | ±4,477 | 72.10 |             |
| Carbonyl iron  | Complete medium  | 414          | ±58    | 1.00  | 0.77        |
|                | 2-ME             | 3,618        | ±259   | 8.79  | 0.61        |
|                | $\alpha$ TG      | 4,053        | ±308   | 9.79  | 0.45        |
|                | LPS              | 10,319       | ±918   | 24.93 | 0.27        |

\*  $5 \times 10^5$  normal or macrophage-depleted C3H/St spleen cells were cultured in 0.1 ml of serum-free medium for 3 days in the presence of various stimulatory substances. Cells were pulsed with  $1 \mu\text{Ci}$  [ $^3\text{H}$ ]TdR for the final 24 h of culture.

‡ Same as Table II

§ Same as Table II

|| Same as Table II.

polyclonal activation than 2-ME. The only structural difference between 2-ME ( $\text{CH}_2\text{OH}-\text{CH}_2\text{SH}$ ) and  $\alpha$ TG ( $\text{CH}_2\text{OH}-\text{CHOH}-\text{CH}_2\text{SH}$ ) is the -CHOH- moiety interposed between the two carbons of the 2-ME molecule. Therefore, it seems reasonable to assume that the two thiols act by means of one common mecha-

TABLE VI  
*Ability of 2-ME and  $\alpha$ TG to Induce Polyclonal Activation\**

| Culture con-<br>ditions | Stimulator<br>added | Anti-SRBC $\ddagger$<br>PFC/culture | $\pm$ SE    | Anti-TNP $\ddagger$ , $\S$<br>PFC/culture | $\pm$ SE    |
|-------------------------|---------------------|-------------------------------------|-------------|---|-------------|
| 5% FCS                  | RPMI-1640           | 30                                  | $\pm$ 15.0  | 55  | $\pm$ 15.0  |
|                         | 2-ME                | 177                                 | $\pm$ 33.5  | 383                                       | $\pm$ 78.9  |
|                         | $\alpha$ TG         | 253                                 | $\pm$ 122.5 | 570                                       | $\pm$ 205.0 |
| Serum free              | RPMI-1640           | 118                                 | $\pm$ 33.3  | 32  | $\pm$ 6.0   |
|                         | 2-ME                | 530                                 | $\pm$ 93.1  | 167                                       | $\pm$ 25.2  |
|                         | $\alpha$ TG         | 708                                 | $\pm$ 52.0  | 283                                       | $\pm$ 20.9  |

\*  $4.5 \times 10^7$  normal C3H/St spleen cells were cultured in 3.0 ml of serum-free or 5% FCS-containing medium for 2 days in the presence of various stimulating substances.

$\ddagger$  Results are expressed as the arithmetic mean of triplicate cultures  $\pm$  standard error

$\S$  The anti-TNP PFC response was calculated by subtracting the anti-GRBC response from the anti-TNP-GRBC response.

nism, and that the greater effect of  $\alpha$ TG is due to its extra hydroxyl group. This hydroxyl group may well give  $\alpha$ TG an advantage over 2-ME by enhancing the affinity of the thiol for its target structure, very possibly through hydrogen bonding.

The stimulation of blast transformation of lymphocytes by 2-ME further supports the role of 2-ME as a lymphocyte activator. It is known that spleen cell cultures, upon incubation with certain mitogens, synthesize and extrude DNA into the culture medium (32, 33). Rogers et al. (32) showed that many cells synthesizing DNA did not undergo blast transformation. It was therefore of importance to establish whether the enhanced incorporation of [ $^3$ H]TdR seen upon culture with 2-ME was due only to increased uptake of DNA precursors for extrusion into the medium, or was a reflection of morphological blast transformation. Both the number of blasts present in culture and their state of activation (intermediate vs. large blasts with copious pyroninophilic cytoplasm) correlated with the dose of 2-ME used. Again, the peak effect occurred at a dose of  $5 \times 10^{-5}$  M, paralleling that operant for peak incorporation of [ $^3$ H]TdR. This data, then, would imply that blast cells not present in control cultures develop more completely as the optimal stimulatory dose of 2-ME is approached.

Since the kinetics of 2-ME and  $\alpha$ TG differ markedly from those seen with antigen-induced proliferation (34), it appears that the proliferative responses to these thiol compounds, like those to lymphocyte mitogens, may be of a nonspecific nature. Peak incorporation of [ $^3$ H]TdR into DNA, stimulated by either 2-ME or  $\alpha$ TG, occurs between 48 and 72 h of culture, in accord with the results of Lemke and Opitz (28). Identical kinetic patterns were observed in both C3H/St and in C3H/HeJ mice. The rapid rise and decline is similar to that seen with the B-cell mitogens, LPS (18), PPD (35), and Poly IC (27), and also with some T-cell mitogens, such as Con A (30).

The ability of 2-ME to enhance [ $^3$ H]TdR incorporation is not an artifact of improved culture conditions and resultant prolonged cellular viability. The possibility that the effects of 2-ME on mixed lymphocyte reactivity and propagation of certain lymphoid tumor lines was due to enhanced viability was entertained and discounted by Heber-Katz and Click (3) and by Broome and Jeng (1),

respectively. Similarly, as demonstrated by the present data, no significant difference in viability between 2-ME-treated and control cells was noted over the 3-day culture period. Other experiments indicated a significant improvement in viability due to 2-ME by day 5, but by this time the 2-ME-induced proliferative response had practically disappeared. The above investigation of the kinetic effects of 2-ME on cell viability is critical to an interpretation of its mechanism of action because computation of cpm/10<sup>6</sup> viable cells (28) biases the result by failing to eliminate counts of [<sup>3</sup>H]TdR taken up by cells that subsequently lost viability.

In probing the mechanism of action of 2-ME and  $\alpha$ TG, it is important to determine whether the target cell is a B cell, a T cell, or both. An indication of the involvement of B cells may be gained by examining the response of congenitally athymic (*nu/nu*) spleen cells to 2-ME. The results of stimulation of *nu/nu* spleen cells in 5% FCS-containing medium indicate that these cells, presumably bone marrow-derived, are indeed responsive to 2-ME. The 2-ME data support the observation of Lemke and Opitz (28). However, the latter authors believe that because the nude response was of low magnitude, the effect of 2-ME on B cells must be minimal. The present data make the point that the stimulation seen in nude spleen cells is 60% of that seen in the *nu/+* littermate. The ability of *nu/nu* cells to respond to B-cell mitogens is made clear by a normal response to Poly IC. Thus it would appear that the difference between nude and littermate stimulation by 2-ME may be due to a T-cell component. The repertoire of *nu/nu* B-cell responses, however, may be different from that of B cells from normal mice. This possibility is supported by other work from this laboratory suggesting a B-cell defect in LPS responsiveness in the nude mouse.<sup>4</sup> Therefore, the observation that normal B cells, prepared by treatment of spleen cells with ATS + C', are highly responsive to 2-ME and  $\alpha$ TG is of particular significance. The fact that B-cell-enriched populations reacted at an equal (in 5% FCS) or greater (serum-free) level than untreated cells under identical conditions would indicate that B-cell responsiveness is equal to or greater than that of T cells. This latter point is reinforced by the ability of B cells obtained by treatment of spleen cells with ATS + C' to be stimulated to incorporate [<sup>3</sup>H]TdR by 2-ME in medium with 5% FCS to a greater degree than T cells obtained by NW passage. There should be no confusion, however, about the nature of the lymphocyte populations under consideration: they are enriched for a particular cell type, but not purified. Since contaminating cells in enriched populations cannot be totally eliminated, the exact role of T and B cells in the 2-ME response cannot be precisely delineated.

A collaborative interaction involving T cells, B cells, and possibly macrophages could well prove to be the mechanism of the 2-ME-induced proliferative response. When an equal number of NW passaged cells (T cells) and ATS + C'-treated cells (B cells) were co-cultured, definite synergy of stimulation occurred. Such synergism bears on the question of whether B cells and/or T cells are stimulated by 2-ME, and may cause misinterpretation of experiments examining the effect of 2-ME on cell populations enriched for a single cell type. Weksler and Kuntz recently noted synergy of human B and T cells in the response to phytohemagglutinin and Pokeweed mitogen (36). This was shown to

<sup>4</sup> See footnote 2.

result from a permissive effect of T cells on B-cell proliferation, and did not represent T-cell proliferation or release of mitogenic factors.

Although not conclusive, the experimental data fail to implicate macrophages in activation of lymphocytes by 2-ME and  $\alpha$ TG. Drastic reduction of macrophages in culture is not accompanied by a concomitant decrease in uptake of [ $^3$ H]TdR. The results of such experiments corroborate those of Lemke and Opitz (28). In the present study, the effect of depletion of macrophages on the proliferative response to 2-ME and  $\alpha$ TG was assessed using two different techniques: adherence to plastic and ingestion of carbonyl iron. Neither of these procedures totally eliminates macrophages from culture, although a marked diminution of carbon-ingesting cells was seen in the depleted populations.

Polyclonal B-cell activation induced by either 2-ME or  $\alpha$ TG was found to be associated with cellular proliferation, as is the case for B-cell mitogens. It would appear that either both of these phenomena are modulated through the same triggering mechanism, or the requirements for triggering at two discreet points are very nearly identical.

The concept of a two- or three-carbon molecule activating cells to blast transformation and polyclonal antibody production is a novel one. Several intriguing questions concerning the mechanism of action must be considered. First, is 2-ME or  $\alpha$ TG stimulating resting cells alone, or is it acting in concert with other substances, possibly contaminants, in the culture medium? The possibility that 2-ME acts solely by amplifying mitogens present in FCS (13, 37) is obviated by the response that occurs under serum-free conditions. The objection that submitogenic concentrations of endotoxin may be responsible for the effect is negated by the observation that the C3H/HeJ mouse, a classical endotoxin-resistant strain (34, 38), mounts a respectable response to 2-ME. Another possible mechanism for 2-ME would be that it preferentially permits cells that were prestimulated *in vivo* to survive and proliferate *in vitro*. If this were the case, the mitogenic-type kinetic profile showing a rapid rise and decline would not be what one would expect to see. However, more definitive work must be done to resolve the matter one way or the other. Other experiments are in progress to directly test the capacity of 2-ME and  $\alpha$ TG to activate resting populations of lymphocytes. An additional possibility is that 2-ME counteracts a normal growth control mechanism (39).

Another question that demands attention concerns the site of action of 2-ME and  $\alpha$ TG. Classical lymphocyte mitogens are considered to trigger cells by interaction with mitogen-specific cell-surface receptors. It is certainly possible that 2-ME is active at the cell surface where it may directly or indirectly trigger mitogen receptors or surface Ig. Alternately, it may initiate a cascade of molecular interactions at the cell surface, eventuating in mitogenesis and polyclonal stimulation. These possible models, together with the observed T-cell-B-cell synergism of stimulation by 2-ME and  $\alpha$ TG, suggest that these thiols may nonspecifically react with protein or sugar constituents and may thereby alter cell-cell interactions. A second possible site of action for 2-ME and  $\alpha$ TG is an intracellular one. It is conceivable that 2-ME or  $\alpha$ TG traverses the cell membrane to act at the level of the cytoplasm, short-circuiting the chain of transmission for activating stimuli perceived at the cell surface en route to the nucleus. The other obvious intracellular site would be at the nucleus itself. The mode of

activation of lymphocytes by 2-ME and  $\alpha$ TG could then occur either by virtue of an actively stimulatory effect, or by inhibition of a negative feedback growth control mechanism, resulting in derepression. Either triggering or disinhibition could account for the phenomena observed.

In conclusion, it must be remarked that the addition of 2-ME and  $\alpha$ TG, two- and three-carbon molecules, respectively, to our armamentarium of lymphocyte activators gives us the exciting advantage of having an easily manipulatable probe capable only of a limited number of reactions. Further investigations into both the cellular and chemical mechanisms of action are in progress in this laboratory.

### Summary

The effect of 2-mercaptoethanol (2-ME) and  $\alpha$ -thioglycerol ( $\alpha$ TG) on proliferation and polyclonal activation of lymphocytes was studied in cultures of spleen cells from C3H mice. Inclusion in serum-free or serum-containing medium of the optimal concentration ( $5 \times 10^{-5}$  M) of either 2-ME or  $\alpha$ TG resulted in highly significant uptake and incorporation of tritiated thymidine ( $^3\text{H}$ ]TdR) into DNA and in morphological blast transformation. These phenomena were dose-dependent, with both lower and higher doses causing less marked effects. The kinetic peak of these responses was found to occur at day 3 of culture. Improved cellular viability could not explain these results, because by day 3 there was no significant difference in viability between cells cultured in the presence or absence of 2-ME. 2-ME evoked a proliferative response in cultures of congenitally athymic (*nu/nu*) spleen cells that exhibited a similar but lower dose-response profile compared with that of heterozygous (*nu/+*) littermates. Cultures of bone marrow-derived (B) lymphocytes, generated by treatment of spleen cells with rabbit antithymocyte serum and complement, incorporated  $^3\text{H}$ ]TdR to a degree at least equal to that of normal spleen cell cultures. Thymus-dependent (T) cells did not support significant 2-ME,  $\alpha$ TG, or Concanavalin A responses in the absence of serum. However, when cultured in 5% fetal calf serum, definite T-cell responses occurred, though always of a lower magnitude than B-cell responses in this system. When the enriched B-cell and T-cell preparations were co-cultured, a synergistic response was noted. Macrophage dependency of the 2-ME and  $\alpha$ TG effect was shown to be minimal. It is likely that the greater effectiveness of  $\alpha$ TG relative to 2-ME is due to differences in the chemical structure of these two thiol compounds. The advantages of utilizing 2-ME and  $\alpha$ TG as probes in the study of lymphocyte activation are evaluated and their possible mechanisms of action are discussed.

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