

## NONSPECIFIC ACTIVATION OF MURINE LYMPHOCYTES

### VI. Mediation of Synergistic Interaction between T and B Lymphocytes by a Cell-Associated, Reciprocally Acting Lymphocyte Proliferation Helper\*

BY MICHAEL G. GOODMAN‡ AND WILLIAM O. WEIGLE

*From the Department of Immunopathology, Scripps Clinic and Research Foundation,  
La Jolla, California 92037*

Induction of cellular proliferation in lymphocyte cultures by 2-mercaptoethanol (2-ME)<sup>1</sup> is a complex process involving both bone marrow-derived (B) and thymus dependent (T) lymphocytes (1, 2). The activation process is not limited to cellular proliferation, but extends to differentiative events, culminating in the induction of specific cellular function. Thus, polyclonal immunoglobulin secretion is exhibited by B cells, and polyclonal cytotoxicity by T cells stimulated with optimal concentrations of 2-ME (1, 3, 4). This thiol compound has been demonstrated to transform small, resting cells into blasts (5), acting preferentially on a subpopulation of mature splenic lymphocytes which is not represented in the repertoire of the CBA/N mouse (6).

Earlier work demonstrated that the mitogenic response to 2-ME is essentially independent of the presence of macrophages and occurs both in separated B-cell-enriched and T-cell-enriched populations (1, 3), suggesting the possibility that B cells as well as T cells are activated in a direct manner by simple contact with 2-ME. It has been observed, however, that B and T lymphocytes respond to 2-ME synergistically (1).<sup>2</sup> The existence of synergy implies an interaction between the cooperating cells to amplify the response. Observation of synergy, then, contradicts a simple, one-hit mechanism and supports a more complex mode of action involving cellular collaboration.

We have previously shown that responsiveness of murine spleen cell cultures to 2-ME depends upon the concentration at which cells are cultured, and that synergy in this response occurs between B and T lymphocytes without the participation of macrophages.<sup>2</sup> This situation was contrasted to the mitogenic response to lipopolysac-

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\* Publication 1622 from the Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, Calif. Supported in part by U. S. Public Health Service grant AI-07007, American Cancer Society grant IM42-G, and Biomedical Research Support Program grant (RRO-5514).

‡ Recipient of National Institutes of Health grant AI-15284 and a Fellowship from the Arthritis Foundation.

<sup>1</sup> *Abbreviations used in this paper:* ATS, anti-mouse thymocyte; B cell, bone-marrow-derived lymphocyte; BSS, balanced salt solution; C', guinea pig complement; Con A, concanavalin A; [<sup>3</sup>H]TdR, tritiated thymidine; LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; poly IC, polyinosinic-polycytidylic acid; PPD, purified protein derivative of tuberculin; RALPH, reciprocally acting lymphocyte proliferation helper; T cell, thymus-dependent lymphocyte.

<sup>2</sup> 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.* In press.

charide (LPS), in which case T cells do not synergize with responding B cells. Furthermore, it was shown that T cells augmented 2-ME-induced B-cell function (polyclonal antibody generation). These data, then, lend support to the thesis that both direct and indirect mechanisms are involved in lymphocyte activation by 2-ME.

The studies reported in the present paper were undertaken to determine whether intercellular communication between B and T lymphocytes occurred via soluble agents released into the culture supernate, or was mediated by direct cell contact, and then to investigate the mechanism by which such synergy operates.

### Materials and Methods

*Mice.* C3H/St male mice, 8–12 wk of age, were obtained from the L. C. Strong Laboratory, Del Mar, Calif., and the mouse breeding facility 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 (7).

*Mitogens.* *Escherichia coli* K-235 LPS, prepared by phenol-water extraction procedure, was generously supplied by Abbott Laboratories, Chicago, Ill., through Dr. Floyd C. McIntire, School of Dentistry, University of Colorado Medical Center, Denver, Colo. Polyinosinic-polycytidilic acid (poly IC) double stranded sodium salt was purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Purified protein derivative (PPD) extracted from *Mycobacterium tuberculosis* RT33 was obtained from Statens Serum Institute, Copenhagen, Denmark. Concanavalin A (Con A) was purchased from Miles Yeda, Ltd., Rehovot, Israel. 2-ME (Matheson, Coleman, Bell, East Rutherford, N. J.) was dissolved in phosphate-buffered saline and sterilized by filtration. Further dilution of all mitogens in complete medium was used for microculture.

*Culture Reagents.* Dissociated spleen cells were cultured in serum-free medium, whose constituents have been specified in previous publications (6). Serum-containing culture medium consisted of the same ingredients as the serum-free medium with the substitution of 5% fetal calf serum (lot A846523, Microbiological Associates, Baltimore, Md.) for an equivalent volume of RPMI-1640. Constituents of the nutritional cocktail that were used to feed cultures daily have been detailed elsewhere (8).

*Lymphocyte Suspensions.* Dissociated spleen cell suspensions were prepared as previously described (6). Spleen cells enriched for T lymphocytes were prepared by passage over nylon wool columns by the method of Julius et al. (9). B lymphocyte-enriched populations were prepared by treating  $10^8$  spleen cells with a 1:100 dilution of rabbit anti-mouse thymocyte (ATS) serum (lot 15038, Microbiological Associates), which had been appropriately absorbed (10). These cells were then spun out of the antiserum and incubated with absorbed guinea pig complement and washed as described elsewhere (1).

Nonviable spleen cells were produced by exposing the cells to a source of ultraviolet light at a distance of 25 cm for 15 min, followed by a 30-min incubation at 56°C. The resulting cell population was approximately 98% nonviable as determined by exclusion of trypan blue dye.

*Lymphocyte Cultures.* Lymphocytes were cultured in plastic microculture plates (3040, 3041, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) at a density of  $5 \times 10^5$  viable cells per well in a final vol of 0.1 ml of medium. In some experiments, cells were cultured in modified Marbrook vessels (generously provided by Dr. Edward L. Morgan) partitioned by a detergent-free, EDTA-washed 0.22  $\mu\text{m}$  Millipore membrane (Millipore Corp., Bedford, Mass.).  $5 \times 10^6$  viable lymphocytes were cultured on each side of the membrane in a final vol of 1.0 ml of medium inside the vessel, 4.0 ml outside. Microculture plates and modified Marbrook vessels were incubated at a temperature of 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Microcultures were fed daily with 8  $\mu\text{l}$  of nutritional cocktail, and Marbrook cultures with 30–45  $\mu\text{l}$  of the same cocktail.

*Measurement of DNA Synthesis.* During the final 24 h of culture, cells were radiolabeled with 1.0  $\mu\text{Ci}$  of tritiated thymidine ( $^3\text{H}$ ]TdR) per culture (6.7 Ci/mM, New England Nuclear, Boston, Mass.). The microcultures were harvested with a Brandel cell harvester, model M24V (Biological Research and Development Laboratories, Rockville, Md.) onto glass fiber filter strips (Reeve Angel, Clifton, N. J.). Marbrook cultures were transferred to millipore filters followed by two washes of the entire vessel. The filters were then further washed three times

with balanced salt solution (BSS) on the Millipore manifold. Filter disks were transferred to plastic scintillation vials (Kimble Products Div., Owens-Illinois, Inc., Toledo, Ohio), covered with liquid scintillation cocktail (Scintiverse, Fisher Scientific Co., Pittsburgh, Pa.) and counted in a Beckman LS-230 liquid scintillation counter.

*Preparation of Activated Cell Supernates.* C3H/St spleen cells were cultured at a density of  $10^7$  viable cells per ml in 60-mm plastic Petri dishes (Falcon Plastics) in a 2.0 ml vol of serum-free medium for 24 h in the presence or absence of optimal concentrations of 2-ME. After this incubation, cells were washed three times in BSS at 4°C and recultured for an additional 24 h. At the termination of the second culture period, the cells were pelleted by centrifugation and the supernates removed, sterilized by filtration, and either used immediately or stored at -20°C until further use.

*Preparation of Reciprocally-Acting Lymphocyte Proliferation Helper (RALPH).* C3H/St spleen cells were cultured at a density of  $10^7$  viable cells per ml in 60-mm plastic Petri dishes in a vol of 2.0 ml of serum-free medium in the presence or absence of  $5 \times 10^{-5}$  M 2-ME for 24 h. At the end of this culture period, cells were harvested from the dishes by scraping with a rubber policeman, transferred to tubes, and washed three times. Viable cells were then enumerated by the trypan blue dye exclusion test. These cells were resuspended in 1 ml of complete medium and sonicated on ice for 2 min. The resultant sonicate was then adjusted to the equivalent of  $5 \times 10^6$  viable cells/ml with complete medium, based on the preceding count. After thorough mixing, the preparation was subjected to centrifugation at 30,000 g for 1 h at 4°C. Except as noted specifically, the supernatant fluid was removed after centrifugation and used as medium for lymphocyte culture. The activity of this soluble factor was unaltered by storage at -20°C.

*Enumeration of Blast Cells.* Histological preparations were generated on microscope slides from individual lymphocyte cultures with the aid of a cytocentrifuge. Slides were stained with the methyl green-pyronin Y-technique (1) to simplify the enumeration of pyroninophilic blast cells.

## Results

*Inability of Activated Supernates to Mediate Synergy.* Experiments were undertaken to determine whether synergy is mediated by the secretion of soluble factors from 2-ME-activated lymphocytes into the culture medium. Spleen cells were cultured in Petri dishes in the presence or absence of  $5 \times 10^{-5}$  M 2-ME for 24 h under serum-free conditions. At that time, cells were harvested into tubes and washed three times in sterile BSS. These cells were then cultured at a cell density of  $5 \times 10^6$  viable cells per ml for an additional 24 h period. The culture supernate was then collected, sterilized by filtration, and assayed for its ability to stimulate fresh, syngeneic spleen cells to incorporate [ $^3$ H]TdR. Table I demonstrates that whether assayed after 24, 48, or 72 h of culture, 2-ME-activated cell supernates were unable to elicit activation of fresh spleen cell cultures. The ability of these cultures to respond to  $5 \times 10^{-5}$  M 2-ME is included for purposes of comparison. Parallel experiments using unfiltered supernates yielded identical results. These data demonstrate that the entity mediating cellular collaboration in this system is not a soluble factor secreted into the culture medium.

*Dependence of Synergy on Cell Contact.* To more directly investigate the mechanism of synergy between B and T cells, these cells were cultured in the presence or absence of  $5 \times 10^{-5}$  M 2-ME in modified Marbrook vessels partitioned by a 0.22  $\mu$ m Millipore membrane. B cells, generated from spleen cells by treatment with ATS followed by guinea pig complement, and nylon wool passaged T cells were cocultured in the combinations shown in Fig. 1. The responsiveness of T cells cultured on both sides of the membrane in this system was low, and contrasted with the vigorous response of B cells. When B cells were cultured on one side of the membrane and T cells on the other, the resultant response was less than of B cells cultured on both sides of the

TABLE I  
*Inability of 2-ME-Activated Supernates to Mediate Synergy*

Day of culture	Supernate*	Supernatant-induced [ <sup>3</sup> H]- TdR uptake/culture	2-ME-induced [ <sup>3</sup> H]TdR uptake/culture
		<i>cpm</i> ‡	<i>cpm</i> §
1	Unstimulated	24,200 ± 700	27,600 ± 100
	2-ME-stimulated	23,300 ± 200	28,400 ± 600
2	Unstimulated	1,500 ± 200	1,500 ± 100
	2-ME-stimulated	1,900 ± 300	19,700 ± 200
3	Unstimulated	300 ± 4	700 ± 80
	2-ME-stimulated	500 ± 10	11,700 ± 700

\* 24-h supernates of control or 2-ME-stimulated C3H/St spleen cell cultures were made as described in Materials and Methods and used as culture medium for  $5 \times 10^5$  syngeneic spleen cells per microculture.

‡ Cultures were pulsed with 1.0  $\mu$ Ci [<sup>3</sup>H]TdR (6.7 Ci/mM) for the final 24 h of culture.

§  $5 \times 10^5$  viable C3H/St spleen cells were cultured in serum-free medium in the presence or absence of  $5 \times 10^{-5}$  M 2-ME.

membrane. It was only when the two cell types were mixed together and half cultured on each side of the membrane that synergy was observed. Therefore, direct physical contact is mandatory for the generation of this synergistic response.

*Ability of a Factor Derived from 2-ME-Activated Cells to Transfer Activation to Unstimulated Cells.* Because synergy between B and T cells was not found to be mediated by the presence of soluble factors secreted into the culture medium, it appeared likely that this phenomenon was attributable to cell-cell contact. Preliminary experiments conducted with pulse-activated cells lent support to this possibility. Therefore, soluble and insoluble fractions of nonactivated and 2-ME-activated cells were prepared to investigate this possibility in greater depth. For this purpose, spleen cells were cultured at a concentration of  $10^7$  per ml in the presence or absence of  $5 \times 10^{-5}$  M 2-ME. After a 24 h incubation, cells were harvested from the dishes with rubber policemen, transferred to centrifuge tubes, and washed three times in a large excess of sterile BSS. Viable cells were counted and then resuspended in 1 ml of complete medium. The cells were then sonicated on ice for 2 min. The sonicate was subsequently diluted with complete medium to the equivalent of  $5 \times 10^6$  viable cells/ml on the basis of the preceding cell count. After thorough mixing, the preparation was subjected to centrifugation at 30,000 *g* for 1 h at 4°C. The supernate was then removed and used as the soluble fraction; the pellet was resuspended to the same volume as the supernate, and used as the insoluble fraction. The data presented in Fig. 2 illustrate that crude unfractionated supernatant preparations obtained from 2-ME-activated but not from unstimulated cultures were able to induce uptake of [<sup>3</sup>H]TdR in normal syngeneic spleen cell cultures. This activity resides solely in the soluble fraction of the sonicate and not in the particulate fraction. Thus, it appears that the synergy observed in previous experiments is the result of direct cellular contact and is mediated by a soluble cellular component.

*Dose Dependency of the DNA Synthetic Response to 2-ME-Induced Soluble Factor.* The ability of incremental doses of 2-ME-activated soluble factor to induce [<sup>3</sup>H]TdR uptake in fresh syngeneic spleen cell cultures was investigated by resuspending the cell sonicate to  $2 \times 10^7$  viable cell-equivalents per ml, isolation of the soluble fraction

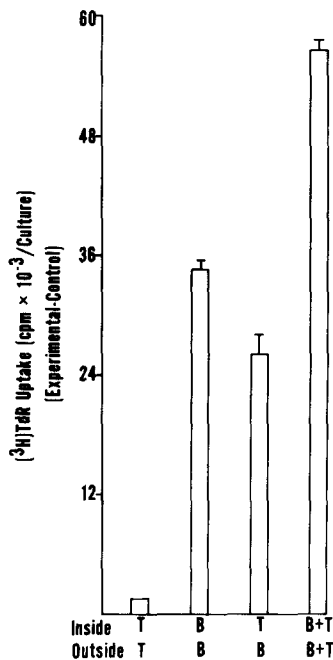


FIG. 1. Dependence of synergy on cell contact.  $5 \times 10^6$  viable C3H/St B-cell-enriched and/or T-cell enriched splenic lymphocytes were cultured on either side of a  $0.22\text{-}\mu\text{m}$  Millipore membrane in modified Marbrook vessels in 5.0 ml of serum-containing medium, in the presence or absence of  $5 \times 10^{-5}$  M 2-ME. Cells were pulsed with  $10 \mu\text{Ci}$  of [<sup>3</sup>H]TdR ( $6.7 \text{ Ci/mM}$ ) for the final 24 h of the 3-d culture period. Results are expressed as the arithmetic mean of three replicate experiments  $\pm$  the standard error of the means.

as described, and resuspending fresh cells in various dilutions of this factor. The results of a typical experiment are presented in Fig. 3. Here it can be seen that optimal stimulation occurs at a concentration of  $5 \times 10^5$  viable cell-equivalents per culture. This is the point on the graph at which the number of cells resuspended in the soluble factor is the same as the number of cells used to generate the factor. Both lower and higher factor concentrations appeared to induce suboptimal uptake of [<sup>3</sup>H]TdR.

*Kinetic Profile of the Response to 2-ME-Induced Soluble Factor.* The time at which maximal factor-induced stimulation occurred was assessed in microcultures of C3H/St spleen cells. These cells were cultured for varying periods of time in the presence of either control or 2-ME-activated soluble factors derived from cultures of B-enriched or T-enriched lymphocytes. Subsequently, one set of cultures was harvested daily, after a 24 h pulse of [<sup>3</sup>H]TdR. The kinetic profiles for these responses are illustrated in Fig. 4. Maximal incorporation of [<sup>3</sup>H]TdR into DNA occurred between days 1 and 2 of culture and declined thereafter. These data, then, demonstrate that upon activation with 2-ME both T and B lymphocytes produce stimulatory soluble factors, and furthermore, that the response of unstimulated cells to these factors peaks at the same time.

*Exclusion of Trivial Mechanisms for the Action of 2-ME-Induced Soluble Factor.* To verify that the observed effect of the 2-ME-induced factor was, in fact, attributable to the interaction of this factor with unstimulated cells, an interaction between the factor itself and the [<sup>3</sup>H]TdR used to label these cultures had to be excluded. Because it is

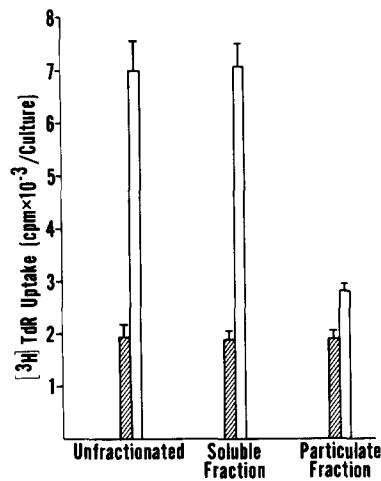


FIG. 2. Ability of a factor derived from 2-ME-activated cells to transfer activation to unstimulated cells.  $5 \times 10^6$  viable C3H/St spleen cells were cultured for 2 d in 0.1 ml of factor generated from 2-ME-activated or control cells, as described in Materials and Methods, and fractionated by high speed centrifugation. Cells were pulsed with  $1.0 \mu\text{Ci}$  of  $[^3\text{H}]\text{TdR}$  ( $6.7 \text{ Ci/mM}$ ) for the final 24 h of culture. Results are expressed as the arithmetic mean of five replicate cultures  $\pm$  the standard error. ▨, control factor; □, 2-ME-induced factor.

easily conceivable that the soluble components of sonicated cells could interact specifically with  $[^3\text{H}]\text{TdR}$  to form aggregates which could adhere to cells as inert carriers, the following experiment was performed. Factors derived from control or 2-ME-activated cells were dispensed into microculture wells. Normal spleen cells were added to one set of factors at the onset of the experiment and labeled from 24 to 48 h of culture. The other set of factors was labeled in identical fashion, but cells were omitted from these cultures until immediately before harvest. Sufficient time was allowed for interaction of the aggregates with the newly added cells. The data presented in Table II demonstrate that the factors cultured in the presence of cells for the entire 48 h period stimulated these cells as has been demonstrated above. However, when soluble factors were incubated with  $[^3\text{H}]\text{TdR}$  in the absence of cells, insignificant trapping of  $[^3\text{H}]\text{TdR}$  resulted.

Other experiments were designed to determine whether 2-ME, loosely bound to cellular components, might be carried over in the soluble fraction and later eluted off into the culture medium during the additional 48 h incubation at  $37^\circ\text{C}$ . Therefore, nonviable cell populations were prepared as described in Materials and Methods. An attempt to induce activity in the soluble fraction of both viable and nonviable cell populations was made by incubating both kinds of cells in the presence of  $5 \times 10^{-5}$  M 2-ME for 24 h. After preparation of soluble factors from both of these populations, the factors were split into two portions, one of which was dialyzed against phosphate-buffered saline for 3 d, with the dialysate being changed twice daily. The final dialysis was performed against complete serum-free medium. The dialyzed and nondialyzed portions of each factor preparation were then compared for their ability to induce stimulation in fresh cultures of C3H/St spleen cells. As shown in Fig. 5, only viable spleen cells incubated in the presence of 2-ME are capable of making an active preparation. The soluble fraction of nonviable cells incubated with the same concen-

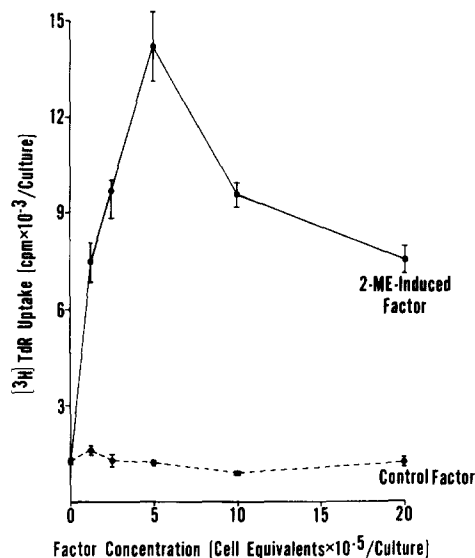


FIG. 3. Dose dependency of the DNA synthetic response to 2-ME-induced soluble factor.  $5 \times 10^6$  viable C3H/St spleen cells were cultured for 2 d in 0.1 ml of control- or 2-ME-induced soluble factor containing an incremental number of cell-equivalents/ml. Cells were pulsed with 1.0  $\mu\text{Ci}$  of [ $^3\text{H}$ ]TdR (6.7 Ci/mM) for the final 24 h of culture. Results are expressed as the arithmetic mean of five replicate cultures  $\pm$  the standard error.

tration of 2-ME is totally inactive. However, because we have previously demonstrated that viable spleen cells bind significantly more 2-ME than nonviable cells (2), dialysis of the resultant factors was essential to definitively rule out differential carry-over of 2-ME. Fig. 5 illustrates the additional point that dialysis of the soluble factor produced by viable cells in no way diminishes its activity. Thus, in this system, the synergy phenomenon appears to be attributable to cell-cell contact mediated by a soluble cell component which is nondialyzable. Moreover, as shown in Table III, exposure to this factor induces morphological blast transformation as well as [ $^3\text{H}$ ]TdR uptake by murine spleen cells.

*Inability of Other Mitogens to Induce Stimulatory Soluble Factors.* To investigate whether other mitogens were capable of amplifying their responses by recruitment of uninvolved cells via factors, viable and nonviable cultures were incubated in the presence of optimal mitogenic concentrations of LPS (100  $\mu\text{g}/\text{ml}$ ), poly IC (500  $\mu\text{g}/\text{ml}$ ), PPD (500  $\mu\text{g}/\text{ml}$ ), Con A (1  $\mu\text{g}/\text{ml}$ ), or 2-ME ( $5 \times 10^{-5}$  M). Soluble cellular fractions were prepared from these cultures as before, and were assayed for their ability to activate unstimulated C3H/St spleen cells. The ability of 2-ME to induce factor-mediated activity in cultures of viable spleen cells is in marked contrast to the inability of the other mitogens to induce similar activities (Fig. 6).

*Specificity of Soluble Factors Isolated from B and T Lymphocytes.* Earlier kinetic experiments demonstrated that both B and T lymphocytes are capable of producing active soluble factors. The next question addressed was whether each factor activated both B and T lymphocytes nonspecifically, or whether there existed some degree of target specificity for the lymphocyte subpopulation activated. Therefore, control and 2-ME-activated factors were prepared from isolated cultures of B or T lymphocytes. Each

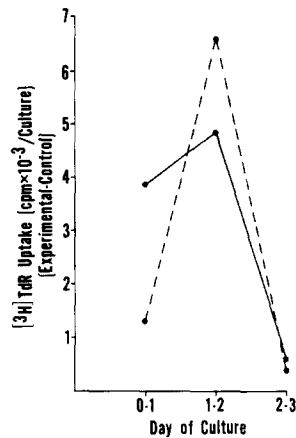


FIG. 4. Kinetic profile of the response to 2-ME-induced soluble factor.  $5 \times 10^5$  viable C3H/St spleen cells were cultured in 0.1 ml ( $5 \times 10^6$  cell-equivalents) of control- or 2-ME-induced soluble factor which was generated in cultures of B-cell-enriched or T-cell-enriched lymphocytes. On each day, one set of cultures was harvested after a 24 h pulse of  $1.0 \mu\text{Ci}$  of  $[^3\text{H}]\text{TdR}$  (6.7 Ci/mM). Results are expressed as the arithmetic mean of quadruplicate experimental cultures - control cultures. —, 2-ME-induced B-cell factor; ---, 2-ME-induced T-cell factor.

factor was then assayed on unstimulated cultures of fresh B and T cells. As illustrated in Fig. 7, activated factors produced by B-cell cultures were stimulatory for T cells. Conversely, activated factors produced from T-cell cultures were found to activate only B-cell cultures. In other experiments (data not shown), factors were produced by stimulation of cultures of whole spleen cells with 2-ME, followed by separation into B-cell and T-cell components just before preparation of soluble fractions. Factors produced in this manner yielded identical results. Because of this reciprocal specificity of T-cell factors for B cells and vice versa, the soluble factor was named RALPH (reciprocally acting lymphocyte proliferation helper). This degree of specificity, of T-cell factors for B cells, and of B-cell factors for T cells, further mitigates against the possibility of 2-ME carry-over as the agent responsible for the activation observed. Thus, it appears that 2-ME-activated B cells have a mechanism designed to allow them to communicate specifically with naive T lymphocytes. Similarly, 2-ME-activated T cells appear to possess the capability of communication specifically with resting B cells.

### Discussion

The dramatic ability of B and T lymphocytes to act synergistically with one another in the proliferative response to 2-ME is mediated by physical contact between these cells. 2-ME-activated B and T cells appear to reciprocally recruit the participation of naive T and B cells, respectively, into this response, with consequent amplification of its magnitude. This type of intercellular communication seems to occur by the direct transfer of RALPH from the activated to the naive cell.

Synergistic interaction between B and T lymphocytes in response to stimulation by 2-ME is not accomplished by the release of soluble mediators into the culture medium. Supernates from lymphocytes activated by 2-ME for either 24 or 48 h were no more able to activate normal lymphocytes than were supernates from control cultures. This



TABLE II  
*Lack of Interaction between Soluble Factors and [<sup>3</sup>H]TdR*

Factor*	Cell added at ‡	[ <sup>3</sup> H]TdR uptake§ cpm/culture ± SE	
Control	Onset	2,270	± 220
2-ME	Onset	9,170	± 410
Control	Harvest	160	± 105
2-ME	Harvest	120	± 40

\*  $5 \times 10^6$  cell-equivalents of control of 2-ME-induced factor was added per culture.

‡  $5 \times 10^5$  viable C3H/St spleen cells were added at the beginning or end of the 2-d culture period.

§ Cultures were pulsed with 1.0  $\mu$ Ci of [<sup>3</sup>H]TdR (6.7 Ci/mM) for the final 24 h of culture.

finding further implies that any cell-bound 2-ME released into the culture medium after washing is too low in amount to provide mitogenic stimulation. Because activated cell supernates are incapable of eliciting proliferative responses from naive lymphocytes, it would appear that synergism in this case is mediated in a manner quite distinct from that demonstrated for blastogenic factors produced in mixed lymphocyte cultures (11, 12). Similarly, this phenomenon would appear to operate by a mechanism different from those by which culture supernates from pokeweed mitogen- of Con A-activated lymphocytes induce B-cell activity (13, 15).

That physical contact between T and B cells is necessary for a synergistic response to 2-ME was demonstrated in experiments in which B cells were separated from T cells by a membrane which permitted free transit of macromolecules but not of cells. In this situation, B cells and T cells were unable to act synergistically in response to 2-ME. Synergy occurred only when B and T cells were mixed together on the same side of the membrane.

The requirement for cell homogenates rather than culture supernates for recruitment of additional cells into the 2-ME response corroborates the observation that cellular contact is necessary for such amplification of this response. Cells which had been activated with 2-ME for 24 h and washed extensively were homogenized by sonication and separated into soluble and particulate fractions. Unseparated, sonicated preparations of 2-ME-activated spleen cells, but not of control spleen cells, were found to be capable of inducing highly significant [<sup>3</sup>H]TdR uptake by fresh lymphocyte cultures. Separation of the sonicate into soluble and particulate fractions by high speed centrifugation demonstrated that the active moiety resided in the soluble (RALPH) fraction only. Therefore, it appears that this active principal either exists in soluble form within the cell or is solubilized by sonication. The fact that culture supernates from cells activated for 24 or 48 h showed no ability to stimulate normal cells indicates that the relevant mediator is retained within the cell and is not released in biologically active quantities into the culture medium.

The dose-response profile for RALPH activity exhibited a peak at  $5 \times 10^6$  cell equivalents/ml, a concentration identical to that at which the spleen cells responding to RALPH were grown in microculture (i.e.,  $5 \times 10^6$  viable cells/ml). This observation implies that communication occurs between one activated and one naive cell, if the

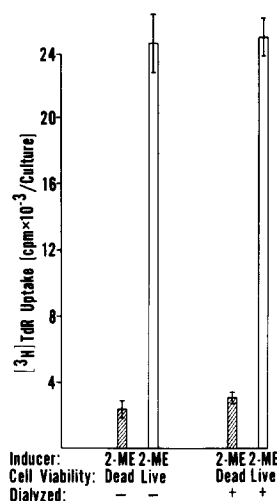


FIG. 5. Exclusion of 2-ME carry-over by 2-ME-induced soluble factor.  $5 \times 10^6$  viable C3H/St spleen cells were cultured for 2 d in 0.1 ml ( $5 \times 10^6$  cell-equivalents) of soluble factor induced by 2-ME in either viable or UV/heat-killed cells. A portion of the factors so generated were dialyzed for 3 d against phosphate-buffered saline with a final change into serum-free medium before use in culture. Cells were pulsed with 1.0  $\mu$ Ci of [<sup>3</sup>H]TdR (6.7 Ci/mM) for the final 24 h of culture. Results are expressed as the arithmetic mean of five replicate cultures  $\pm$  the standard error.

culture conditions used permit near-optimal interaction; if interaction is suboptimal, this 1:1 ratio could represent an artifact due to inefficient transfer of information by the solubilized factor relative to the highly efficient manner in which it is normally transferred by intact living cells. In the latter case, one would expect that a single activated cell would normally transfer its information to several different unstimulated cells. The linearity of the ascending limb of the dose-response profile indicates that the amount of factor present is limiting until it reaches a 1:1 ratio. The diminution of activity beyond the peak implies that all stimutable cells under these *in vitro* conditions have been activated, and further stimulation may transmit an off signal to some of these cells.

RALPH, generated in either T or B lymphocytes, stimulated whole spleen cells with identical kinetic profiles, peaking at day 2 of culture and declining thereafter. As a result, it appears that both B cells and T cells participate in the recruitment of uninvolved cells into the proliferative response to 2-ME.

A consideration of trivial explanations for the activity of 2-ME-induced RALPH was unable to account for the observed phenomenon. Thus, the data could not be explained in terms of binding of [<sup>3</sup>H]TdR to soluble cellular components in the factor preparation, with the cultured cells acting as inert carriers. Moreover, the results obtained were not attributable to 2-ME carryover, as indicated by incubating nonviable and viable cells in the presence of 2-ME and making factors from both preparations, followed by dialysis over a 3-d time period. The fact that dialyzed RALPH produced only from viable lymphocytes was able to stimulate uninvolved cells indicates that the active principal is nondialyzable and is produced solely by viable cells. Furthermore, when the ability of 2-ME-induced RALPH activity to induce blast transformation in lymphocytes was assessed directly, the capacity of this

TABLE III  
*Ability of 2-ME-Induced Soluble Factor to Induce Blast Transformation*

Factor*	Large blasts‡	Intermediate sized blasts	[ <sup>3</sup> H]TdR uptake/culture
	%	%	cpm§
Control	2.23 ± 0.74	3.20 ± 0.07	2,100 ± 150
2-ME-induced	11.90 ± 0.95	10.03 ± 3.93	13,450 ± 550

\*  $5 \times 10^6$  cell-equivalents of control or 2-ME-induced factor was added per culture of  $5 \times 10^6$  viable C3H/St spleen cells.

‡ After 2 d of culture, cells were transferred to microscope 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 ± the standard error.

§ Cultures of  $5 \times 10^6$  viable C3H/St spleen cells were cultured for 2 d in serum-free medium in the presence or absence of  $5 \times 10^{-5}$  M 2-ME. Cells were pulsed with 1.0  $\mu$ Ci of [<sup>3</sup>H]TdR (6.7 Ci/mM) for the final 24 h of culture. Results are expressed as the arithmetic mean of quadruplicate cultures ± the standard error.

preparation to enlist the collaboration of naive cells in the proliferative response to 2-ME became apparent.

RALPH is not a universal factor generated by lymphocyte mitogens. The B-cell mitogens LPS, poly IC, and PPD were unable to induce soluble factors capable of amplifying proliferative responses. Similarly, the T-cell mitogen Con A lacked this capacity. If RALPH is in fact responsible for the synergism observed between B and T lymphocytes, then one might expect its production only in instances in which both B and T cells are stimulated to proliferate by a single mitogen. Thus 2-ME, which activates both B and T lymphocytes and induces synergy between these cell types, is able to elicit the production of RALPH. Furthermore, preliminary observations indicate that pokeweed mitogen, which activates both B and T cells and induces synergy between them as well (15, 16), also evokes a RALPH-like factor which can be extracted from activated cells by sonication, further supporting the role of this factor in the synergy phenomenon.

RALPH activity elicited by 2-ME from T-cell cultures stimulates proliferation among B cells, and such factors produced in B-cell cultures activate naive T cells. Neither control nor activated T-cell factors ever altered the responsiveness of fresh T cells; however, both control and activated B-cell factors increased [<sup>3</sup>H]TdR uptake by unstimulated B-cell cultures, leaving unresolved the question of whether B cells amplify the responses of other B cells in this response. In either case, the specificity of T-cell factor for B cells and of B cell factor for T cells further mitigates against carry-over of 2-ME as the mechanism of factor-induced activation. The activity of separated T and B lymphocyte-derived RALPH factors was more difficult to detect when assayed on separated B and T-cell cultures than when assayed on whole spleen cell cultures. This observation may be indicative of the existence of a positive feedback loop, which cannot operate when one cell type is absent. If such a loop is important in the ultimate production of synergy between B and T cells, one would expect to find responses of considerably lesser magnitude when each cell type is assayed for responsiveness to RALPH separately.

The mechanism whereby the response of B cells to 2-ME is amplified by T cells may be similar to that by which the antigen-specific response of virgin B cells is

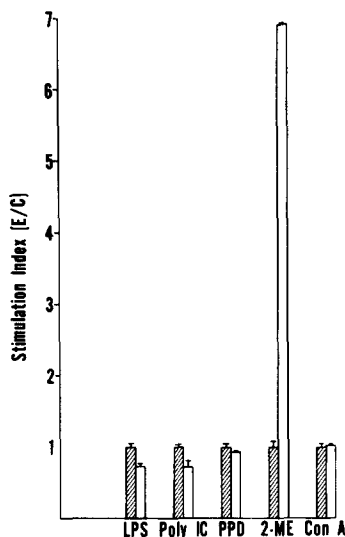


FIG. 6. Relative abilities of an array of mitogens to induce stimulatory soluble factors.  $5 \times 10^6$  viable C3H/St spleen cells were cultured for 2 d in 0.1 ml ( $5 \times 10^6$  cell-equivalents) of soluble factor induced by LPS (100  $\mu\text{g}/\text{ml}$ ), poly IC (500  $\mu\text{g}/\text{ml}$ ), PPD (500  $\mu\text{g}/\text{ml}$ ), 2-ME ( $5 \times 10^{-5}\text{M}$ ), or Con A (1 $\mu\text{g}/\text{ml}$ ) in either viable or UV/heat-killed cells. Cells were pulsed with 1.0  $\mu\text{Ci}$  [ $^3\text{H}$ ]TdR (6.7 Ci/mM) for the final 24 h of culture. Results are expressed as the ratio of the arithmetic means of the stimulation by viable/nonviable factors  $\pm$  the standard error.  $\square$ , nonviable cells;  $\square$ , viable cells.

augmented by T amplifier cells (17, 18). In such a model, amplifier T cells would contact uninvolved antigen-specific B cells, transfer an active principle to them, and so enlist their participation in the response. There are several possible manners in which RALPH could mediate synergy. After its production, it could be packaged (perhaps by the Golgi apparatus) and transferred either to the cytoplasm of the recipient cell or to its cell membrane. Alternately, RALPH could be expressed at the surface of an activated cell and later be transferred to a recipient cell by physical contact. Finally, RALPH could act as a solid phase mitogen adherent to the cell that produced it, triggering the recipient cell through specific receptors without being transferred itself.

The studies presented in this communication provide evidence supporting the existence of B helper cells which augment the mitogenic responsiveness of T cells to 2-ME; and, similarly, of T helper cells which amplify the responsiveness of B cells to this thiol compound. Thus, both cell types produce and respond to appropriate RALPH activity in the amplification of this response. The presence of such factors in other systems, as well as the biochemical properties of these factors, are currently under investigation.

### Summary

The mechanism by which B and T lymphocytes interact synergistically in the proliferative response to 2-mercaptoethanol (2-ME) as a mitogen was investigated in cultures of C3H/St spleen cells. The interaction between these cells required physical contact between the collaborating cell types, and was not mediated by the release of a soluble factor into the culture supernate. Sonicates of spleen cells which had been

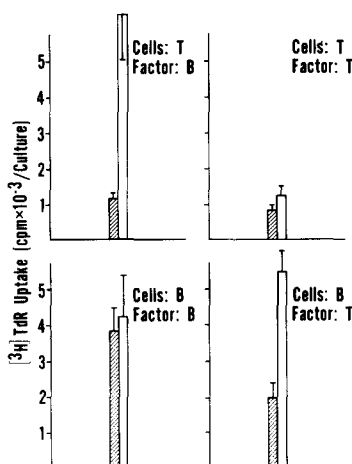


FIG. 7. Specificity of soluble factors isolated from B and T lymphocytes.  $5 \times 10^5$  viable C3H/St T or B lymphocytes were cultured for 2 d in 0.1 ml ( $5 \times 10^5$  cell-equivalents) of 2-ME-induced or control soluble factor generated in cultures of B-cell-enriched or T-cell-enriched lymphocytes. Cells were pulsed with  $1.0 \mu\text{Ci}$  of  $[^3\text{H}]\text{TdR}$  ( $6.7 \text{ Ci/mM}$ ) for the final 24 h of culture. Results are expressed as the arithmetic mean of nine replicate experiments  $\pm$  the standard error of the means.  $\square$ , control factor;  $\square$ , 2-ME-induced factor

activated with optimal concentrations of 2-ME for 24 h and then washed extensively, stimulated the uptake of tritiated thymidine and morphological blast transformation of fresh, unstimulated cells. This activity was found to reside within the soluble fraction of the activated cells, and to activate cells optimally at a ratio of 1 naive cell: 1 activated cell-equivalent. This reciprocally-acting lymphocyte proliferation helper (RALPH) activity was produced by B cells as well as by T cells, with a kinetic peak at 48 h of culture. RALPH activity was produced by viable but not by nonviable cells incubated with 2-ME, and was nondialyzable. It could not be induced by the B-cell mitogens lipopolysaccharide, polyinosinic-polycytidilic acid, or purified protein derivative of tuberculin, or by the T-cell mitogen concanavalin A. RALPH isolated from T cells activated B cells exclusively, while that from B cells acted predominantly upon T cells, possibly with a nonspecific effect on B cells. A model for the cellular interactions involved in the amplification of the proliferative response to 2-ME is described.

The authors wish to express their gratitude to Miss Anne Zumbrun for superb technical assistance, and to Mrs. Chris VANLeeuwen and Mrs. Margaret Stone for their excellent secretarial work in the preparation of the manuscript.

Received for publication 27 September 1978.

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