

THE EFFECTS OF MERCAPTOETHANOL AND OF PERITONEAL
MACROPHAGES ON THE ANTIBODY-FORMING CAPACITY OF
NONADHERENT MOUSE SPLEEN CELLS IN VITRO*

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The immune response in vitro of mouse spleen cells to sheep erythrocytes (SRBC)¹ requires the presence of macrophages or adherent spleen cells (1, 2). The function of the adherent cells in this system is still unknown. Various roles for them have been suggested, including: (a) antigen-macrophage interaction (1, 2), leading to production of informational RNA (3), antigen-RNA complex (4), or some other forms of "processed" antigen; (b) solubilization of the particulate antigen, with release of immunogenic materials into the culture supernatant (5, 6); (c) "cluster" formation (2, 7), perhaps facilitating interaction between various lymphoid cells on macrophages; antigen may be presented on the surface of macrophages, as proposed by Cruchaud and Unanue (8).

Click (9) reported that addition of mercaptoethanol to the Mishell-Dutton culture system (10) greatly enhanced the in vitro immune response. The mechanism of the mercaptoethanol action is unknown. In a preliminary report (11) we showed that inclusion of mercaptoethanol in the medium led to restoration of the plaque-forming capacity of nonadherent mouse spleen cells. It therefore appeared that mercaptoethanol somehow substituted for the function of macrophages. We now report further studies on the relations between lymphocytes, macrophages, and mercaptoethanol in the Mishell-Dutton system. The results suggest that macrophages and mercaptoethanol exhibit some sort of trophic influence, exact nature not yet defined, on the medium and/or on the lymphoid cells, resulting in markedly improved lymphocyte viability as well as increased number of cells producing antibody to SRBC.

Materials and Methods

Animals.—Normal DBA/2J and B6D2F₁ mice, 3–6 months old, were obtained from Jackson Laboratory, Bar Harbor, Maine.

Spleen Cells.—Mice were killed by cervical dislocation. Spleens were removed and teased in cold Hanks' balanced salt solution. The cell suspension was then passed through a 100 gauge

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¹ *Abbreviations used in this paper:* PFC, plaque-forming cells; SRBC, sheep erythrocytes.

stainless steel sieve, centrifuged (200 *g*, 5 min), and resuspended in supplemented Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.) with 10% fetal calf serum (Reheis Chemical Co., Chicago, Ill.), plus 10,000 units of penicillin and 10 mg of streptomycin/100 ml of medium. Cell viability was assessed by trypan blue exclusion.

Nonadherent Cells.—The technique of Mosier (1) with some modification was used. 10×10^7 spleen cells in 8 ml of medium described above were incubated at 37°C in a 100 × 20 mm Falcon plastic tissue culture dish (Falcon Plastics, Div. of B-D Laboratories, Inc., Los Angeles, Calif.) without rocking for 2 hr in an atmosphere of 7% O₂, 10% CO₂, and 83% N₂. The dish was then gently shaken, and the cells in suspension were transfixed to another dish and incubated again for 1 hr under the same conditions. The nonadherent cells were centrifuged and resuspended in fresh medium.

Peritoneal Macrophages.—3 ml of medium were injected into the mouse peritoneal cavity and then withdrawn after 1 min. The cells were counted and diluted with medium to the desired concentration. Usually 40–50% of these peritoneal cells were macrophages. 1 ml of the cell suspension was placed in a Falcon 35 × 10 mm culture dish and incubated with the same gas mixture described above at 37°C without rocking for 24 hr. The nonadherent lymphoid cells were then washed away and the adherent cells, essentially all macrophages, were cultured together with spleen nonadherent cells and antigen.

Antigen.—SRBC in Alsever's solution (Microbiological Associates, Inc., Bethesda, Md.) were washed three times with saline. 5×10^6 cells were added per culture.

Spleen Cell Culture.—The method described by Mishell and Dutton (10) was used. 5×10^6 viable spleen nonadherent cells, with or without peritoneal macrophages, or 1×10^7 viable unseparated spleen cells were placed in a Falcon 35 × 10 mm tissue culture dish and incubated with gentle rocking for 4 or 5 days. Nutritional mixture and fetal calf serum were added daily.

Mercaptoethanol.—2-Mercaptoethanol (Matheson, Coleman & Bell, Norwood, Ohio) was diluted 1:1000 with saline and kept as a stock solution. This solution was stable for several months in the cold. Immediately before use, the stock solution was further diluted six times with medium, and 25 μl were added to each culture to make the final concentration of mercaptoethanol 5×10^{-5} M. Unless specified, mercaptoethanol was added at the initiation of culture.

Hemolytic Plaque Assay.—The direct hemolytic plaque assay of Jerne et al. (12) was used. The mean number of plaque-forming cells (PFC) was computed from triplicate cultures. Variation in an experimental group was always less than 20%.

Viable Cell Recovery.—At the time of plaque assay, an aliquot of the cultured cell suspension was washed, resuspended in medium, and mixed with $\frac{1}{5}$ volume of freshly filtered 0.4% trypan blue solution in saline, and the dye-negative cells were counted directly using a hemacytometer. The recovery was the percentage of cells that remained viable after various periods of culture, as compared with the number of viable cells at the start of the culture. Most of the macrophages remained adherent to the culture dish and were damaged during the harvesting of culture by scraping the culture dish. The few viable macrophages were full of ingested red cells and debris, allowing ready differentiation from lymphoid cells. These macrophages were not included with viable cell count.

Enumeration of Macrophages.—The residual, small number of macrophages or macrophage precursors in the nonadherent spleen cell populations were impossible to enumerate accurately. On culture for 2 or more days without rocking, however, these matured into typical macrophages and became adherent and well spread on the culture dish. Using an inverted microscope the number of macrophages was counted on the entire dish or on a defined area of the dish. In some instances a suspension of carbon (Pelikan, Günther-Wagner, Hanover, Germany) 20 μl/culture was added for 48 hr, which resulted in heavy labeling of the macrophages with black granules, confirming their phagocytic nature and making them easy to enumerate.

RESULTS

Effect of Adding Peritoneal Macrophages and Mercaptoethanol on Survival and Plaque-Forming Response of Nonadherent Spleen Cells.—Spleen cells were separated into adherent and nonadherent cell populations. 5×10^6 nonadherent spleen cells were cultured with various numbers of peritoneal macrophages;

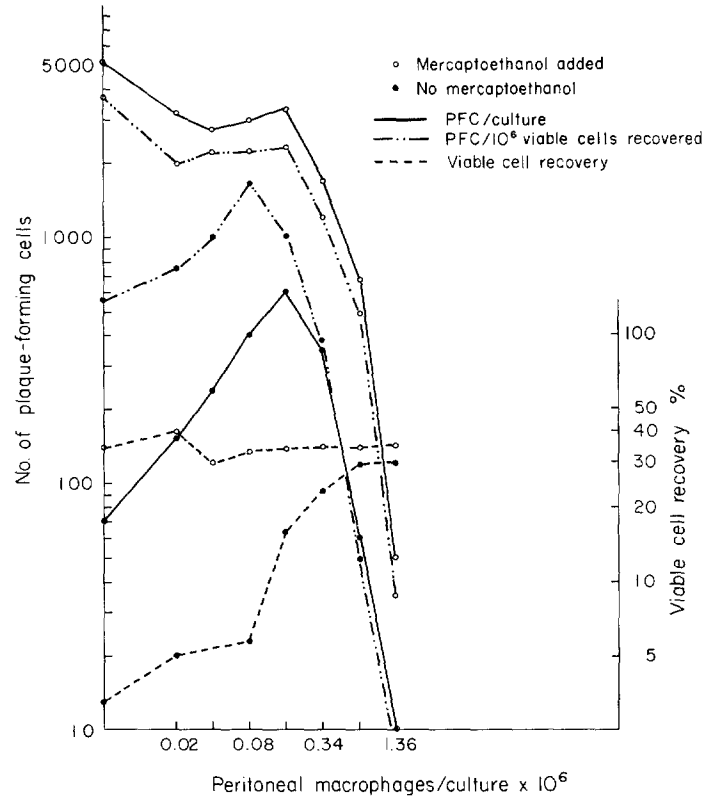


FIG. 1. Assessment on day 5 of culture of viable cell recovery, PFC/culture, and PFC/ 10^6 viable cells recovered of mouse spleen nonadherent cells as influenced by the addition of various numbers of peritoneal macrophages in the presence or the absence of added mercaptoethanol.

5×10^6 SRBC were added per culture as antigen. Mercaptoethanol at a final concentration of 5×10^{-5} M was added to some cultures. The numbers of PFC after 5 days of culture are shown in Fig. 1. In the absence of mercaptoethanol, the response per culture was maximal at approximately 1.7×10^5 peritoneal macrophages per culture. Lower or higher numbers of macrophages resulted in fewer PFC per culture, as has been shown before by Hoffmann (13). In the presence of mercaptoethanol, large numbers of PFC developed in the nonadherent spleen cell cultures without the addition of peritoneal macrophages. In the

cultures containing mercaptoethanol, addition of peritoneal macrophages at various concentrations ranging up to 1.7×10^5 per culture resulted in no instance in enhancement of the PFC response. Also notable is the fact that in the presence of mercaptoethanol the PFC response was approximately fourfold higher than the peak response with optimal numbers of macrophages but no mercaptoethanol. High concentrations of macrophages still inhibited the PFC response in the presence of mercaptoethanol.

It thus seemed that macrophages exerted at least two influences in the Mishell-Dutton system: (a) facilitating the plaque-forming response of non-adherent spleen cells when the number of macrophages was small, with an optimal response at approximately 1.7×10^5 macrophages per culture; this macrophage influence can be replaced by mercaptoethanol, and (b) inhibiting the response when the number of macrophages was high, with essentially complete suppression of plaque formation at macrophage concentrations of 1.8×10^6 per culture or higher; this inhibitory effect of excess macrophages was not altered when mercaptoethanol was present.

The facilitating function of small numbers of macrophages might be related to some trophic effect on the cultural conditions. When lymphoid cell recovery was assessed at the time of plaque assay on day 5 (Fig. 1), the nonadherent cell culture exhibited very poor viability, with only 0–2.5% viable lymphoid cells remaining. The number of viable lymphoid cells recovered on day 5 increased with increasing number of macrophages in the culture. At the level of macrophages (1.7×10^5 per culture) giving an optimal PFC response, the recovery of lymphoid cells was 15%. With larger numbers of macrophages there occurred a sharp falloff in PFC, but the recovery of lymphoid cells continued to improve, reaching a plateau of 30%.² When the results are plotted as PFC per 10^6 viable cells recovered (Fig. 1), the maximum was at 0.8×10^5 macrophages per culture, with a sharp decline at higher numbers of macrophages and a gradual decline with fewer macrophages. In the presence of mercaptoethanol, the viable cell recovery at day 5 was uniformly near 35%, regardless of the number of macrophages added, and the curve for PFC per 10^6 viable cells recovered was essentially identical to that for PFC per culture.

The Effects of Mercaptoethanol on Nonadherent Spleen Cell Viability and PFC Response at Various Times during the 5 Day Culture Period.—The PFC response and viable cell recovery of the nonadherent cells with or without mercaptoethanol were evaluated daily during the 5 day incubation period, as shown in Fig. 2, and compared with that of unseparated spleen cells, without mercaptoethanol. At day 1 there was only a small difference in the viable cell recovery for the three cultures. The nonadherent cells in the absence of mercaptoethanol then showed a rapid exponential decrease in viable cell recovery, reaching 11%

² An improvement in viability of nonadherent cells in Mishell-Dutton cultures upon addition of adherent cells has also been reported in a preliminary communication by D. D. Wood (33).

at day 2, 5% at day 3, 1.5% at day 4, and 0.2% at day 5. In contrast, in the presence of mercaptoethanol, the viable cell recovery of spleen nonadherent cells remained near the 40% day 1 level, suggesting little or no further cell death. The unseparated spleen cells, containing macrophages but no mercaptoethanol, showed a behavior intermediate between these two extremes, with a gradual fall in viable cell recovery, reaching approximately 15% at day 5. The PFC/culture

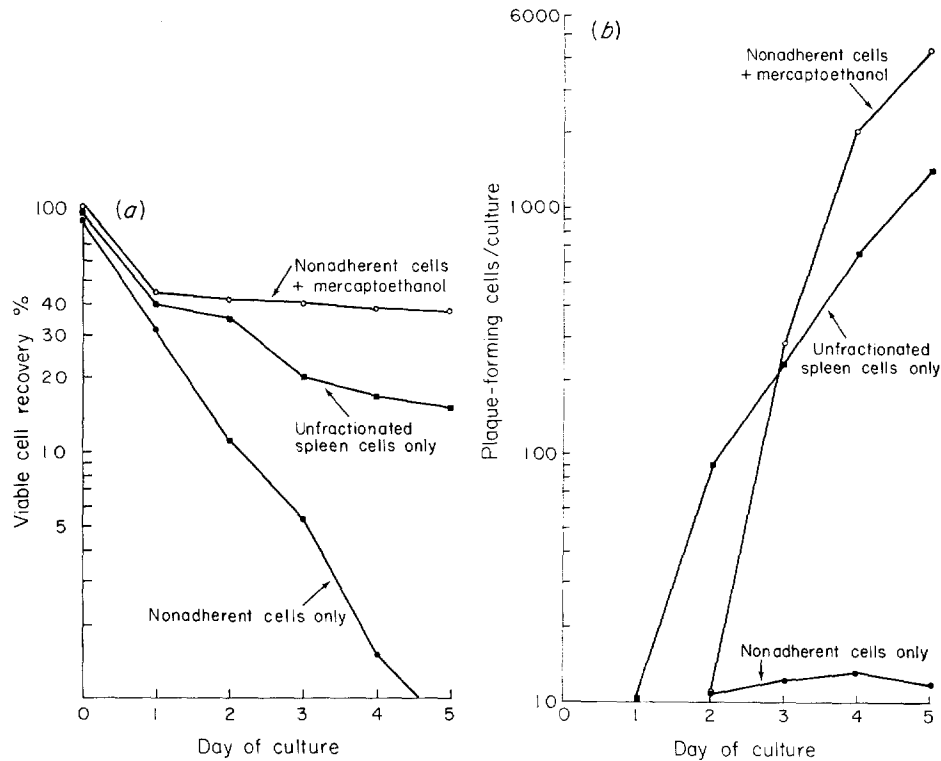


FIG. 2. Viable cell recovery (a), and PFC per culture (b) of nonadherent spleen cells (●), unseparated (adherent and nonadherent) spleen cells (■), and nonadherent spleen cells plus 5×10^{-5} M mercaptoethanol (○) assayed daily during the culture period.

developing in the nonadherent cells maintained without mercaptoethanol was very low (10 or less), whereas with mercaptoethanol these cells exhibited an exponential increase in PFC between day 2 and day 5 of culture. The unseparated spleen cells cultured without mercaptoethanol showed a similar but less rapid increase in number of PFC. There was thus a general correlation between viability of lymphoid cells and plaque-forming capacity at day 4 or 5 of the culture.

Mercaptoethanol was also added to cultures of nonadherent spleen cells at different times after the initiation of culture, with the addition of antigen in all

cases on day 0 of culture, and the PFC response was then evaluated on day 4. As is shown in Table I, addition of mercaptoethanol at 24 hr of culture resulted in a partial preservation of plaque-forming capacity, and a similar partial presentation of lymphoid cell viability; thus the PFC per 10^6 cells recovered was not changed in comparison with cultures containing mercaptoethanol from the outset. Adding mercaptoethanol to the cultures at 48 hr or later was completely ineffective in restoring viability of plaque-forming capacity.

TABLE I
The Effect of Adding Mercaptoethanol to Nonadherent Spleen Cells at Various Times after Initiation of Culture

Experiment	Time of adding mercaptoethanol (hr after initiation of culture)			
	0	24	48	Not added
I. PFC/culture at 96 hr	465	280	0	0
II. PFC/culture at 96 hr	2450	955	0	0
Viable cell recovery at 96 hr	32.2%	15.4%	1.0%	0.16%
PFC/ 10^6 viable cells recovered at 96 hr	3000	2274	0	0
III. PFC/culture at 96 hr	2010	805	0	0
Viable cell recovery at 96 hr	30.0%	6.6%	0.8%	<0.1%
PFC/ 10^6 viable cells recovered at 96 hr	1182	2415	0	

TABLE II
Effect of Removing Mercaptoethanol from the Culture Medium at Various Times of the Culture

Treatment	Time of treatment (hr after culture) PFC/culture at day 5			
	0	24	48	72
Remove mercaptoethanol*	0	0	220	315
Remove and add back mercaptoethanol immediately	2250	2010	2800	2490

* Nonadherent spleen cells plus SRBC were cultured with mercaptoethanol on day 0 and then were transferred at various times to fresh medium without mercaptoethanol.

Results of experiments in which mercaptoethanol was present at the outset of culture but removed at various times thereafter are shown in Table II. When cells were cultured for 24 hr in the presence of mercaptoethanol and then transferred to a tube, centrifuged, resuspended, and cultured up to 5 days in fresh medium without mercaptoethanol, the PFC response was no better than that seen in cultures not exposed to mercaptoethanol at all. Washing to remove the mercaptoethanol after 2 or 3 days of culture resulted in 8 and 13%, respectively,

of the numbers of PFC seen in cultures handled similarly but placed in media containing mercaptoethanol after washing. There was in general a corresponding decrease in viable cell recovery on day 5 of culture in those cells washed and replaced in media free of mercaptoethanol.

Stability of the Mercaptoethanol Activity in the Culture.—Our observations did not seem to fit with the speculation that mercaptoethanol was only stable for a short period of time (9) and affected the early events occurring perhaps within 12 hr of culture. It was desirable to have additional information on stability of mercaptoethanol activity in the culture. Fresh medium containing 5×10^{-5} M mercaptoethanol in plastic dishes was incubated at 37°C for 3 days in the same gas mixture as that used for the cell cultures. This medium was effective in restoring the PFC response of nonadherent spleen cells (Table III), suggesting that mercaptoethanol was stable to the condition of response or that the restoring activity was due to a stable oxidation product of mercaptoethanol. When

TABLE III
Stability of Mercaptoethanol Activity

Medium used	PFC/culture at day 4
No mercaptoethanol	0
Mercaptoethanol freshly added	560
Mercaptoethanol + medium, preincubated 3 days	700
Mercaptoethanol + cell culture, 3 day supernatant	1250
Cell culture supernatant alone	5

Nonadherent spleen cells, 5×10^6 per dish plus SRBC, were cultured in 1 ml of the medium prepared as above.

mercaptoethanol was added to a culture of nonadherent cells and incubated for 3 days, the culture supernatant was also fully active in restoring new nonadherent cells, whereas the supernatant of whole spleen cell culture without mercaptoethanol was ineffective. Thus it appeared likely that mercaptoethanol or a related product was capable of exerting an effect for an extended period in the culture system.

Effect of Further Reducing the Numbers of Residual Macrophages on the Nonadherent Cell Population.—Since there were still small numbers of macrophages (500–2000 per culture) in the nonadherent spleen cell population, mercaptoethanol might be working by promoting the function of these residual macrophages. It was also difficult to rule out the possibility that small numbers of macrophages were still needed for some functions which could not be replaced by mercaptoethanol. Table IV shows the results of efforts to deplete the cultures further of macrophages. Nonadherent cells were incubated at 5×10^6 per culture dish without rocking for 24 hr in the absence of antigen and mercaptoethanol, allowing time for additional maturation and attachment of monocytes and macrophages. The nonadherent cells were then collected, washed, and

resuspended in the same volume of fresh medium. Antigen (SRBC) and mercaptoethanol were then added and the cultures were continued in new dishes for further 4 days. The numbers of macrophages counted as cells sticking to dishes or engulfing carbon particles (see Materials and Methods) during the entire incubation period were less than 100–200 per dish, which was 0.003% of the cell population. In another experiment, nonadherent cells prepared at day 0 were cultured for 24 hr as above, then the nonadherent cells were collected, centrifuged, and resuspended in medium containing mercaptoethanol, and incubated for an additional 24 hr in new dishes without rocking. It was necessary to add mercaptoethanol during this second incubation to avert further loss of viability of the lymphoid cells. The nonadherent cells were again collected and transferred to new dishes; antigen was finally added and the cultures were incubated for 4 days. In these cultures only 10–20 macrophages were found per dish. When

TABLE IV
Effect of Further Depletion of Macrophages

Treatment	No. of macrophages counted per culture on day 4	PFC per culture on day 4
Add SRBC day 0, mercaptoethanol day 1	500–2000	285
Deplete 24 hr*, add SRBC and mercaptoethanol day 1	100–200	280
Deplete 24 hr*, add mercaptoethanol, deplete further 24 hr, add SRBC	10–20	235
No mercaptoethanol added	500–2000	0

* Nonadherent spleen cells, all 5×10^6 per dish at day 0, were washed after 24 hr, transferred to same number of dishes; plaque assay was done 4 days after adding SRBC.

compared with cultures of the original nonadherent cells in which antigen was added on day 0 and mercaptoethanol was added on day 1, cultures severely depleted of macrophages could still respond fully. These experiments showed that in the presence of mercaptoethanol, macrophages could be reduced to very low numbers indeed without affecting the immune capability of the lymphoid cells. The extremely small numbers of macrophages in these cultures made it highly unlikely that these cells were playing an essential role in antigen handling or otherwise in this system.

DISCUSSION

In the present work the interactions between mercaptoethanol, macrophages, and lymphoid cells were studied in the Mishell-Dutton antibody-forming system *in vitro*. The viability of mouse spleen nonadherent cells declined rapidly after 1 day in culture, and this rapid death could be prevented by adding macrophages

or mercaptoethanol, with concomitant restoration of PFC response. It is reasonable to speculate that macrophages may produce some substance with a function similar to mercaptoethanol. We found that the preservation of viability or of PFC response of nonadherent cells by supernatants of macrophage cultures did not occur reproducibly. Since aggregation of lymphocytes around macrophages has been observed (2, 7, 14) and this aggregation seemed necessary for *in vitro* response (2, 7, 14), perhaps the macrophages transfer some mercaptoethanol-like substance directly to lymphoid cells rather than secreting it into the culture medium. We have never succeeded in replacing macrophages, in terms of PFC response or viability, with mouse fibroblasts. The role of macrophages in this system appears therefore to be quite specific, not merely a general "feeder-layer" phenomenon.

The specific requirement for macrophages may be an *in vitro* artifact, due to the fact that rodent lymphoid cells do not survive well in culture, or it may have significance *in vivo* as well. Macrophages are found associated with lymphocytes in all lymphoid organs, and they may serve to provide a suitable microenvironment for lymphoid cells in these organs as they apparently do *in vitro*. Macrophages may of course play other roles as well in the immune response, such as antigen trapping (15) or antigen destruction (16, 17). The requirement *in vivo* for "accessory cells," presumably macrophages, for optimal immune response to SRBC in mice under certain conditions has been established (18). Further studies on the nature of interaction between lymphocytes and macrophages may yield interesting biochemical, cytological, or developmental biological information.

The plaque-forming capacity of nonadherent cells can be restored completely by mercaptoethanol (11). In fact, addition of mercaptoethanol to nonadherent cultures regularly results in higher numbers of PFC than does the addition of optimal numbers of peritoneal macrophages or of adherent spleen cells. When residual macrophages in the original nonadherent cells are exhaustively depleted by repeated adsorption and incubations, the addition of antigen and mercaptoethanol still leads to a full plaque-forming response. Recently, by applying a new separation method (M. Hoffmann, personal communication) using a Sephadex column (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden), the macrophages could sometimes be eliminated completely (no macrophages detectable at the conclusion of the culture). The lymphoid cells completely depleted of macrophages exhibited, with added mercaptoethanol, a normal plaque-forming response to whole SRBC antigen, further supporting the notion that the requirement for macrophages in this system can be completely eliminated by addition of mercaptoethanol. The other proposed macrophage cell-mediated functions, mentioned in the introduction of this paper, would thus appear not to be obligatory steps in the *in vitro* immune response to SRBC.

We have no evidence that mercaptoethanol "solubilizes" or otherwise alters the SRBC antigen (11). Preliminary results indicate that thymus-derived cells as well as bone marrow-derived cells are required in the presence of mercapto-

ethanol, therefore the requirement *in vitro* for T-B cell interaction for the antibody response to SRBC (19-21) is apparently not altered by mercaptoethanol. We cannot determine whether mercaptoethanol alone "stimulates" lymphoid cells, as do the nonspecific mitogens, since both mercaptoethanol and heterologous serum (fetal calf serum) are needed for the optimal survival of mouse lymphoid cells and stimulation might be wholly or in part due to the foreign serum. Mercaptoethanol does not appear to stimulate human peripheral lymphocytes maintained in a medium containing autologous serum (unpublished observation).

In the presence of mercaptoethanol the PFC per 10^6 viable cells recovered did not vary significantly upon addition of peritoneal macrophages provided the number of added macrophages was not too high, whereas in the absence of mercaptoethanol the PFC per 10^6 viable cells was usually lower when less than optimal number of macrophages were added. There are two possible explanations: first, at low macrophage concentration the number of surviving cells was less, thus resulting in less chance for essential cell-cell interactions; and second, full immune response may require not only survival, but optimal condition for cell function as well.

Click et al. (9) reported that with whole spleen cells, mercaptoethanol added at the start of the culture or as late as 48 hr after initiation of culture gave a similar response in terms of PFC/ 10^6 viable cells recovered. The viability of the spleen cells was reported to be the same during this early period whether mercaptoethanol was present or not. In our study the nonadherent cells alone did not survive in appreciable number for 48 hr, so that addition of mercaptoethanol at 48 hr was completely ineffective. Perhaps in Click's spleen cell culture the concentration of macrophages was adequate to maintain the viability up to 48 hr but not sufficient to permit an optimal immune response. Difference in age and strains of mice, rearing methods and environment, and methods of preparing spleen cells all may influence the number and function of macrophages in spleen.

Pierce and Benacerraf showed that macrophages were only needed for the first 24 hr of culture (2), whereas others recently have found that the requirement for macrophages in the primary *in vitro* response may be longer than 48 hr (22). Perhaps the activated or blast-transformed lymphoid cells are relatively independent of macrophages. In the present study the PFC response of nonadherent cells initially cultured in a medium containing mercaptoethanol was suppressed if the cells were transferred to a medium without mercaptoethanol as late as 72 hr after culture. Thus, mercaptoethanol appears to be required throughout the culture period, whereas macrophages are needed only during the first 48 hr, at least in some laboratories. This difference may or may not reflect a different basic mechanism of action. Perhaps the lymphoid cells, once adapted to the medium containing mercaptoethanol, are damaged on transfer to a mercaptoethanol-free environment.

The finding of poor survival of nonadherent cells alone raises a problem in

relation to reports that some antigens like polymerized bacterial flagellin (23) and sonicated SRBC (24) can elicit good *in vitro* antibody response in the Marbrook culture system in the absence of macrophages. It is possible that these antigens foster lymphoid cell survival, or that activation of immunocompetent cells in the case of these antigens may involve mechanisms different from those involved with intact sheep red cells. We found that concanavalin A can transform purified lymphoid cells to blast cells, and sustain their survival without macrophages or mercaptoethanol, as reported by others for human lymphocytes stimulated by phytohemagglutinin (25), suggesting that lymphoid cells can respond to certain stimuli and then maintain their own viability. The antibody response to polymerized flagellar antigen is thymus independent (19), as is the response to sonicated SRBC (cited in the discussion of Feldmann and Basten [19]), whereas antibody response to whole SRBC is thymus dependent (19–21). It will be interesting to investigate whether or not mercaptoethanol can increase the response to these “macrophage independent” antigens.

The inhibitory action of large numbers of macrophages on PFC response was not due to killing of lymphoid cells. In fact, in the absence of mercaptoethanol, the viable cell recovery with excess macrophages was nearly as high as that observed in the presence of mercaptoethanol. Were it not for the inhibitory action of macrophages, the PFC/culture in the absence of mercaptoethanol would perhaps continue to rise with increasing numbers of macrophages, instead of reaching a peak at 1.7×10^5 macrophages per culture with a PFC response approximately one-fourth of that seen in the presence of mercaptoethanol. Restoring the antibody-forming capacity of nonadherent cells with macrophages alone therefore depends upon the balance between the facilitating and inhibitory effects of macrophages in this system. Perturbation of macrophages by carbon (26), carageenan (27), antimacrophage serum (6), actinomycin (28), etc. may either decrease the facilitating activity or increase the inhibitory activity of macrophages in the culture, thereby decreasing the final immune response. Mercaptoethanol reconstituted the PFC response of nonadherent spleen cells in a more reproducible manner, since it promoted lymphoid cell viability and functions but did not exhibit inhibiting effects at the concentration (5×10^{-5} M) used.

The inhibitory activity of large numbers of macrophages has been observed also in several other systems (16, 17, 29–34). The exact mechanism is still unknown. *In vivo*, excess macrophages destroy immunogen (16, 17). In the present study, after 5 days of culture, there were still large numbers of free SRBC present, thus destruction of antigen is not likely to be the main reason for the suppression of immune response. Inhibition of blast transformation (13, 30, 31) or plasma cell formation (32) by excess macrophages have been observed. Whether this phenomenon is an experimental artifact or has any regulatory function *in vivo* remains to be elucidated.

SUMMARY

Nonadherent mouse spleen cells exhibited poor viability and little or no capacity to form antibodies to sheep red cells in the Mishell-Dutton culture system. Viability and antibody-forming capacity could be restored by addition to these cultures of low concentrations of mercaptoethanol (10^{-4} – 10^{-5} M), or by addition of appropriate numbers of mouse peritoneal macrophages. Macrophage concentrations lower than optimal resulted in lower lymphoid cell viability and correspondingly fewer plaque-forming cells, whereas excess macrophages resulted in marked inhibition of antibody formation despite good viability of the lymphocytes. Restoration of the nonadherent cells with mercaptoethanol was thus much simpler and more reproducible than it was with macrophages; furthermore, the number of plaque-forming cells developed in cultures restored with mercaptoethanol was approximately fourfold higher than it was in cultures restored with optimal numbers of macrophages.

In the presence of mercaptoethanol, the plaque-forming capacity of the nonadherent spleen cells was not increased when small numbers of macrophages were added to the system, nor was it decreased when the few macrophages present in the nonadherent cells were further reduced or eliminated. Excess macrophages inhibited antibody formation in the cultures containing mercaptoethanol as they did in control cultures.

Optimal restoration of plaque-forming capacity to the nonadherent spleen cells with mercaptoethanol required the reducing agent to be present throughout the 4 or 5 day culture period. Addition of mercaptoethanol 1 or more days after initiation of culture, or transfer of the cells to a medium free of mercaptoethanol before completion of the culture resulted in a reduction in the numbers of plaque-forming cells.

The results suggest that mouse lymphoid cells do not require macrophages in order to form antibodies to sheep red cells *in vitro*, provided mercaptoethanol is present in the culture medium. The mechanism of action of mercaptoethanol under these conditions is not completely clear, but one of its effects is to promote the viability of lymphoid cells in the cultures.

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