THE ROLE OF FETAL CALF SERUM IN THE PRIMARY IMMUNE RESPONSE IN VITRO*

By HANS-G. OPITZ, UTA OPITZ, HILMAR LEMKE, GUY HEWLETT, WOLFGANG SCHREML, AND HANS-D. FLAD

(From the Department of Clinical Physiology, University of Ulm, Ulm; the Department of Microbiology and Sonderforschungsbereich 111, University of Kiel, Kiel; and the Institute for Immunology and Oncology, Bayer AG, Wuppertal, Federal Republic of Germany)

2-Mercaptoethanol (2-ME)[1] has a beneficial effect on various cell culture systems. It has been shown to act as a growth-promoting agent for several cell lines (1), it enhances erythropoiesis in vitro (2), and it is able to promote mixed lymphocyte culture reactions in serum-free cultures (3, 4). 2-ME has also been shown to enhance the primary immune response in vitro, to substitute functionally for macrophages in this immune response, and to increase cell viability (5, 6). Recently it was reported that 2-ME can convert a fetal calf serum (FCS) that is unable to support an in vitro primary immune response (deficient serum, FCS_{def}), into an effective FCS (good serum, FCS_{good}).[2] It was assumed that 2-ME activates a serum component which is normally present in FCS. Moreover, it was demonstrated that 2-ME in association with FCS exerts a strong mitogenic activity on T cells (7). Experimental evidence was presented suggesting that 2-ME in the presence of FCS can functionally substitute for macrophages in the primary immune response in vitro, because of this strong mitogenicity for T cells. In this report we will demonstrate that 2-ME does indeed activate a component of FCS. This 2-ME-activated FCS component acts on lymphocytes independently of free 2-ME and is able to substitute functionally for macrophages and complete FCS.

Materials and Methods

Animals. Female BALB/c mice (Bomholtgaard, Ry, Denmark) weighing 20–25 g were used throughout the experiments. The animals received standard diet and water ad libitum.

Reagents. Three different batches of FCS were investigated. One (lot 136; Seromed, Munich, Germany) was sufficient in supporting primary immune responses in vitro. Two other batches (lot 108 and lot 140; Seromed) were not able to support the primary immune responses of normal mouse spleen cells.

2-ME was obtained from Merck-Schuchard, Darmstadt, Germany, (2-ME for synthesis, 99% pure by gas chromatography). Stock solutions, diluted 1:100 with saline were kept at 4°C. Final dilutions were prepared for each experiment.

Antigen. Sheep red blood cells (SRBC) obtained from Behringwerke AG., Marburg, Germany,

* Supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 112, Zellsystemphysiologie).

[1] *Abbreviations used in this paper:* BSS, balanced salt solution; FCS, fetal calf serum; MaSF, 2-ME-activated serum factor; 2-ME, 2-mercaptoethanol; PFC, plaque-forming cells.

[2] Lemke, H., and H. G. Opitz. Manuscript submitted for publication.

were stored in sterile Alsever's solution. SRBC were washed twice in balanced salt solution (BSS) before use. 10^7 SRBC in BSS were added to each culture dish.

Culture of Spleen Cells for Primary Immune Responses. Spleens were removed under sterile conditions and teased with forceps in ice-cold BSS. After brief sedimentation to remove coarse particles, the cell suspension was washed twice in 20 ml of cold BSS and subsequently suspended in culture medium (RPMI-1640 medium; Seromed), supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 5% FCS. Cell counts and viability testing by the dye exclusion method (0.5% trypan blue) were performed in a Neubauer hemocytometer.

The primary immunization of cultured spleen cells with SRBC was performed according to the method originally described by Mishell and Dutton (8). The cultures were kept stationary.

Removal of Macrophages. Macrophages were removed from spleen cells by the carbonyl iron method described by Lundgren et al. (9). Carbonyl iron powder (General Aniline & Film Corp., New York) was added to 5 ml spleen cell suspension (2×10^7 nucleated cells/ml) in RPMI-1640 medium supplemented with 10% FCS in Petri dishes (65 mm diameter, Greiner tissue culture dishes). Cells were incubated at 37°C for 30 min in an atmosphere containing 5% CO_2 and those adherent to or containing iron particles were removed with a magnet. The cells were subsequently washed twice in BSS and suspended in culture medium. Macrophage depletion with the iron treatment reduced the number of macrophages by 90–95% (8).

Preparation of 2-ME-Activated Serum Factor (MaSF). FCS was incubated with 10^{-3} M 2-ME for 24 h at room temperature and lyophilized in a WKF L 05 lyophilisator at −20°C. Lyophilized FCS (5 ml) dissolved in 2 ml of distilled water was fractionated on a 0.8 × 60 cm column of Sephadex G-100 equilibrated with 0.05 M Tris-HCl/0.1 M NaCl, pH 7.5 and eluted at a flow rate of 15 ml/h. The elution profile of this material was measured by optical density at 280 nm. Ferritin (mol wt 460,000), bovine serum albumin (mol wt 67,000), ovalbumin (mol wt 45,000), chymotrypsin (mol wt 25,000), and myoglobin (mol wt 17,800) served as markers. Fractions of 5.7 ml were lyophilized, dissolved in 2 ml of distilled water, dialyzed against RPMI-1640 medium, and sterilized by Millipore filtration before use in tissue culture. Various volumes of the single fractions were added to the spleen cell cultures and the concentration expressed as percentage of the final volume of the culture.

Absorption of MaSF. Packed spleen cells or SRBC (0.5 ml) were incubated with 2 ml of the fraction containing MaSF for 1 h at 4°C. After centrifugation the supernates were tested for MaSF activity in the Mishell-Dutton system.

Assay of Antibody Synthesis. On day 5 of culture a modified hemolytic plaque assay was used to quantitate the antibody production in vitro (10). 0.6 ml of 0.5% agar (Difco Bacto Agar; Difco Laboratories, Detroit, Mich.) in BSS with 0.05% DEAE dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) was kept at 46°C, and 0.05 ml of packed SRBC diluted 1:8 in BSS, 0.1 ml of washed spleen cells harvested from the culture, and 0.05 ml of guinea pig serum (diluted 1:4 in BSS) were added. The mixture was plated on plastic Petri dishes and incubated for 2 h at 37°C. The data obtained are expressed as plaque-forming cells (PFC) per culture.

Results

Effect of 2-ME on Antibody Synthesis In Vitro. The experiments summarized in Table I show the effect of three different FCS batches on the primary immune response of cultured normal and macrophage-depleted spleen cells. FCS_3 is able to support the anti-SRBC-PFC response, whereas FCS_1 and FCS_2 fail to stimulate the immune response of normal spleen cells. However, when 2-ME at a final concentration of 5×10^{-5} M is added to the cultures an immune response with FCS_1 and FCS_2 is also obtained. The yield of anti-SRBC-PFC obtained with FCS_3 is improved after addition of 2-ME. In macrophage-depleted spleen cell cultures no immune response is obtained, regardless of the FCS used. After addition of 2-ME to the macrophage-depleted spleen cell cultures an immune response is recovered with all three FCS batches, although it appears that the achievable PFC level is dependent upon the quality of the FCS.

Activation of a Serum Component by 2-ME. To decide whether 2-ME acts

TABLE I

Effect of Three Different FCS on the Primary Anti-SRBC Response of Spleen Cell Cultures

Type of spleen cells	Addition of 2-ME	Anti-SRBC PFC/culture							
		FCS_1		FCS_2			FCS_3		
		Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Normal spleen cells	–	<100	<100	<100	<100	<100	3,970±180	4,100±264	4,400±310
Normal spleen cells	+	2,170±140	1,830±220	3,350±210	3,800±264	2,900±230	5,260±370	6,120±290	5,850±420
Macrophage-depleted spleen cells	–	<100	<100	<100	<100	<100	<100	<100	<100
Macrophage-depleted spleen cells	+	1,730±195	1,590±160	2,760±184	3,270±260	2,040±310	4,950±240	5,350±430	5,200±530

The anti-SRBC PFC response was measured on day 5. The anti-SRBC PFC response of unstimulated cultures did not exceed 300 PFC per culture. The data represent the mean of triplicate cultures ± SE.

TABLE II

Interaction between 2-ME and FCS

Treatment of FCS before use in the Mishell-Dutton system	Anti-SRBC PFC/culture	
	FCS_3	FCS_2
Untreated (1)	<100	<100
Incubated with 2-ME (2)	5,450 ± 470	2,970 ± 360
Untreated, lyophilized (3)	<100	<100
Incubated with 2-ME, lyophilized (4)	4,970 ± 620	2,570 ± 480

FCS (5%) untreated or incubated with 10^{-3} M 2-ME for 24 h was used either directly or after removal of free 2-ME by lyophilization. PFC response was measured on day 5 in spleen cell cultures depleted of adherent cells. The data represent the mean of triplicate cultures ± SE.

directly on spleen cells or indirectly via a serum component, FCS was incubated with 10^{-3} M 2-ME for 24 h at room temperature. This concentration was found to be optimal for the activation of FCS, at 10^{-2} M strong inhibition occurred while at 10^{-5} M no activation was observed. Thereafter, one half of the FCS containing 2-ME was tested directly while the other one was lyophilized before use in the primary immune response in vitro. Unbound 2-ME is completely removed by the lyophilization procedure. The lyophilized material was dissolved in distilled water to its initial volume and its effect on the antibody synthesis of macrophage-depleted spleen cells was examined. The results are shown in Table II. The same FCS not treated with 2-ME was not sufficient for an antibody synthesis by macrophage-depleted spleen cells, whether it was lyophilized or not. The ability of 2-ME-treated FCS to support a primary immune response in macrophage-depleted spleen cells, however, remains unchanged after lyophilization. This shows that no free 2-ME is necessary for the cell culture once it has reacted with FCS.

Fractionation of 2-ME-Treated FCS on Sephadex G-100. Gel filtration of 2-ME-treated FCS on Sephadex G-100 resulted in the elution profile shown in Fig. 1. The area containing the active component is marked by a horizontal line. Untreated FCS, when separated over Sephadex G-100, did not show a macrophage-replacing activity in any fraction. Table III shows the effect of different concentrations of the active fraction on the antibody synthesis of macrophage-depleted spleen cells. Increasing amounts of the factor lead to increased antibody synthesis which reached a plateau at 20% MaSF. Moreover, MaSF im-

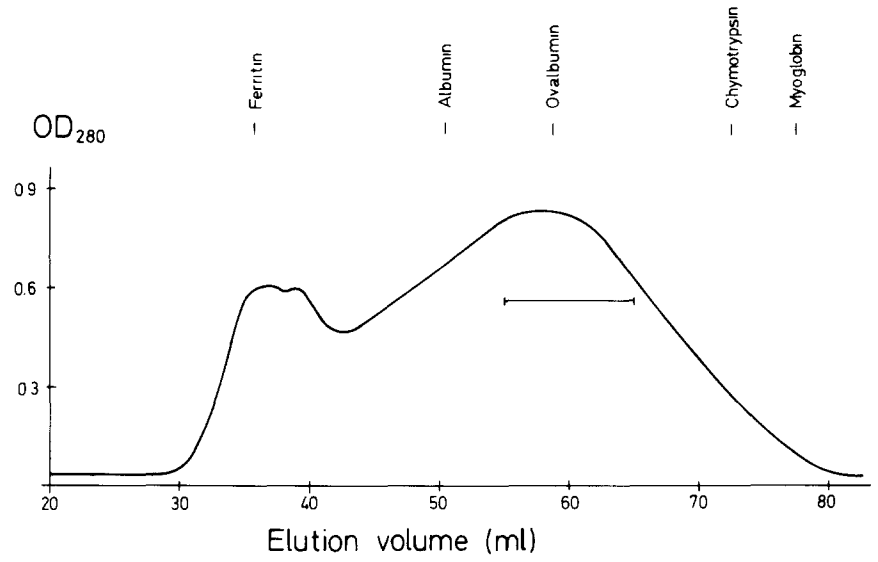

FIG. 1. Elution profile of 2-ME-treated FCS, separated on Sephadex G-100. The horizontal line indicates the area containing MaSF.

TABLE III

Dose Response of the Sephadex G-100 Separated 2-ME-Activated Serum Component

Addition of Sephadex G-100 fraction	Anti-SRBC PFC/culture		No. viable cells/culture (× 10^5)	
	Serum factor		Serum factor	
	Activated	Nonactivated	Activated	Nonactivated
%				
1	160 ± 45	<100	12	<10
5	180 ± 38	<100	14	<10
10	4,780 ± 620	<100	24	<10
20	7,450 ± 530	<100	39	16
30	6,970 ± 480	<100	42	17
Control				
5% FCS	<100			
5% FCS + 2-ME (10^{-5} M)	5,370 ± 540			

FCS_2 treated with or without 2-ME (10^{-3} M) was separated on Sephadex G-100 column. Different concentrations of active fraction were added to 10^7 macrophage-depleted spleen cells. PFC and viability were determined on day 5. The results indicate the mean of triplicate cultures ± SE.

proves cell viability in the cultures and resembles in that respect, the effect of 2-ME added to spleen cell cultures directly. Additional experiments showed that the serum component activated by 2-ME is resistant to heating to 56°C for 30 min. A temporary variation of the pH value to pH 4 had also no effect on activity. No loss of activity was observed after storage at 4°C for more than 2 wk.

Determination of MaSF in Three Different Batches of FCS. The following experiment was designed to determine whether a correlation exists between the efficacy of an FCS in the Mishell-Dutton culture system and its content of

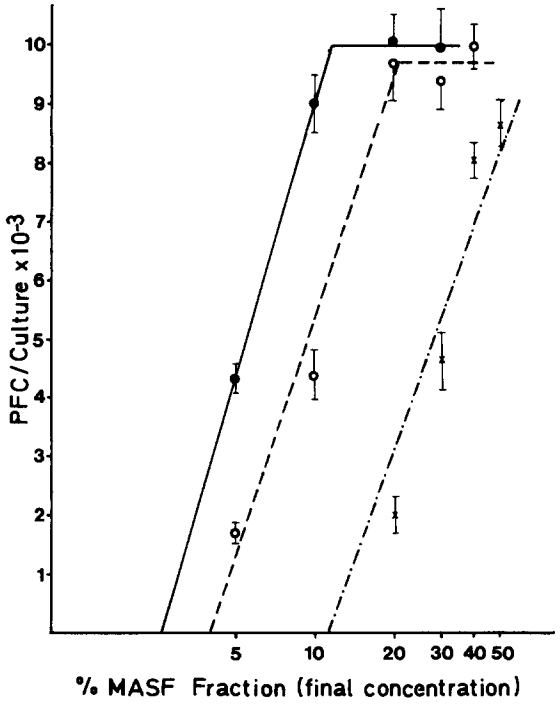

FIG. 2. Dose-response curves of MaSF preparations from two deficient FCS (FCS_1, FCS_2) and one good FCS (FCS_3): FCS_1, (×-·-×); FCS_2 (O---O); FCS_3 (●—●). The experimental data were analyzed for linear regression on a Wang 600 calculator; correlation coefficients were: FCS_1, 0.98; FCS_2, 0.98; and FCS_3, 0.95.

MaSF. In order to assess MaSF concentrations, identical volumes of each batch of 2-ME-treated FCS were chromatographed over a Sephadex G-100 column. The MaSF-containing fractions were added to macrophage-depleted spleen cell cultures in final concentrations as shown in Fig. 2. In these experiments the spleen cell cultures were supplemented with 5% FCS. The doses resulting in maximal stimulation for MaSF-FCS_1 was 40% (vol/vol), for MaSF-FCS_2 20% (vol/vol), and for MaSF-FCS_3 10% (vol/vol). The number of PFC per culture was similar for all three MaSF preparations at these concentrations, i.e., higher concentrations did not increase the response.

Activity of MaSF in the In Vitro Humoral Immune Response in the Presence or Absence of FCS. In the previous experiment 5% FCS was present in the culture medium in addition to the MaSF-containing fraction, and the cultures were fed daily with a FCS-containing mixture as described by Mishell and Dutton (8). The following experiments were performed to investigate the activity of MaSF in normal and macrophage-depleted spleen cell cultures in the absence of additional FCS. As shown in Table IV, MaSF isolated from FCS_2 and FCS_3 gave an optimal response when present at a concentration of 20% vol/vol or 10% vol/vol, in the presence or absence of additional homologous FCS. Further addition of 2-ME to the MaSF-containing fraction had no effect on the antibody synthesis. This experiment demonstrates that the MaSF-containing serum fraction is able to support the in vitro primary humoral immune response against

TABLE IV

Effect of MaSF on the PFC Response of Normal and Macrophage-Depleted Spleen Cells Without Additional FCS

Type of spleen cells	Culture conditions				
	FCS_2	FCS_2 + 2-ME	MaSF + FCS_2	MaSF	MaSF + 2-ME
Normal spleen cells	<100	3,350 ± 410	7,670 ± 1,320	8,180 ± 520	7,950 ± 1,020
Macrophage-depleted spleen cells	<100	2,760 ± 395	9,320 ± 860	7,770 ± 670	7,560 ± 840
	FCS_3	FCS_3 + 2-ME	MaSF + FCS_3	MaSF	MaSF + 2-ME
Normal spleen cells	2,890 ± 410	4,260 ± 370	6,350 ± 530	6,960 ± 470	7,260 ± 730
Macrophage-depleted spleen cells	<100	3,870 ± 420	5,970 ± 460	6,330 ± 560	5,760 ± 370

Normal or macrophage-depleted spleen cells were incubated with 5% FCS, 5% FCS + 2-ME (10^{-5} M), 20% MaSF + 5% FCS, or 20% MaSF with or without addition of 2-ME (10^{-5} M). FCS-containing cultures were fed with FCS while MaSF-containing cultures were fed with MaSF. The number of PFC of unstimulated cultures did not exceed 320 ± 45 PFC per culture. PFC response was measured on day 5. The data represent the mean of triplicate cultures ± SE.

SRBC without additional FCS in the culture medium and also to replace the function of macrophages.

MaSF Addition to, or Removal from Macrophage-Depleted Spleen Cell Cultures at Various Times. The following experiments were designed to investigate the period of contact between MaSF and macrophage-depleted spleen cells required for an optimal immune response. The cultures received an optimal dose of MaSF at various times after initiation of the culture. PFC were determined on day 5. Table V shows that MaSF has to be added during the first 24 h of culture in order to obtain a PFC response. At later intervals the addition of MaSF becomes ineffective. The viability of cultured cells determined by dye exclusion also correlates with the time of addition of MaSF. The duration of contact between MaSF and spleen cells required for an optimal PFC response was studied by removing MaSF at various times after initiation of the culture. Control cultures were subjected to the same washing procedure, but were immediately reconstituted with the original MaSF-containing medium. As can be seen from Table VI, MaSF has to be present during the first 72 h of culture to obtain an optimal PFC response; if MaSF is removed within 72 h, no, or a suboptimal, response is observed.

Absorption of MaSF-Activity by Spleen Cells. After incubating a MaSF-containing fraction with packed spleen cells or SRBC the supernate was tested for its MaSF activity in macrophage-depleted spleen cell cultures. As shown in Table VII, MaSF activity is reduced to about 30% of the original activity by packed spleen cells. No activity is lost after absorption with SRBC.

Discussion

In the present study, the mode of action of 2-ME as a macrophage substitute in the primary immune response against SRBC in vitro was further analyzed. The following results were obtained:

(*a*) It is confirmed that 2-ME can support a PFC response in cultures supplemented with deficient FCS in normal or macrophage-depleted spleen cell cultures. FCS and 2-ME are both essential (Table I).

(*b*) The active component is not 2-ME itself but a serum factor activated by 2-

TABLE V

Effect of Adding MaSF to Macrophage-Depleted Spleen Cells at Various Times after Initiation of Culture

	Time of adding MaSF (hours after initiation of culture)				
	0	24	48	72	Not added
Anti-SRBC PFC/culture					
Exp. 1	8,160 ± 260	7,090 ± 430	510 ± 80	<100	<100
Exp. 2	7,690 ± 570	7,480 ± 370	815 ± 140	<100	<100
Viable cell recovery at day 5					
Exp. 1	46%	37%	22%	10%	<1%

MaSF/FCS_2, 20% vol/vol was added to macrophage-depleted spleen cell cultures at various times. All cultures were supplemented with 5% FCS_2 from the beginning. The data represent the mean of triplicate cultures ± SE.

ME (Table II). The serum factor is altered by 2-ME in such a way that it can completely replace the function of macrophages in terms of supporting a primary anti-SRBC PFC response and promoting good cell viability in the culture (Table III). MaSF can be separated from the bulk of serum proteins of FCS by gel filtration on Sephadex G-100. The factor appears to have a molecular weight in the order of that of ovalbumin (Fig. 1).

(*c*) FCS_{good} can be distinguished from FCS_{def} by their different concentrations of MaSF. From the slopes of the dose-response curves (Fig. 2) it can be seen that the quality of the response is similar in all three FCS lots. From this result it can be deduced that only the concentration of this factor is critical for the efficacy of a particular FCS lot in a primary humoral immune response in vitro.

(*d*) FCS is no longer necessary when the cell culture medium is supplemented with optimal amounts of MaSF. The response cannot be increased by further addition of FCS or 2-ME to the cell culture. Therefore an in vitro immune response can be elicited from a macrophage-depleted, FCS-free system, i.e. from a system consisting of spleen lymphocytes, SRBC, and MaSF. An inherent feature of MaSF-supplemented cultures is the improved viability of spleen lymphocytes. MaSF also reduces the variability of the anti-SRBC PFC response normally found in the Mishell-Dutton culture system.

(*e*) MaSF needs to be added within the first 24 h of culture and should remain in contact with the spleen cells for at least 72 h to enable spleen cells to synthesize antibodies. When MaSF is incubated with SRBC no loss of activity is observed, whereas a loss of activity does occur after incubation of MaSF with spleen cells. This suggests a specific interaction of MaSF with spleen cells.

Recently it was reported that 2-ME could not replace the function of macrophages when they were removed by passage through columns of Sephadex G-10 or by high concentrations of carbonyl iron (11, 12). We have investigated the depletion of macrophages by carbonyl iron in detail (7). In our hands carbonyl iron reduced the concentration of macrophages in spleen cells by 90–95%. Even in the presence of silica, macrophage-depleted spleen cells exhibited a PFC response to SRBC when 2-ME was added. The difference between these findings

TABLE VI
Effect of Removing MaSF from the Spleen Cells at Various Times of Culture

Treatment	Time of treatment (hours after culture) anti-SRBC PFC/culture				
	0	24	48	72	96
Removal of MaSF	0	0	1,680 ± 460	4,320 ± 530	6,760 ± 420
Remove and add back MaSF immediately	5,780 ± 610	6,120 ± 310	4,860 ± 280	5,430 ± 660	6,780 ± 430

Macrophage-depleted spleen cells stimulated with SRBC were cultured with MaSF/FCS_2 (20% vol/vol) on day 0 and then transferred at various times to fresh medium + 5% FCS_2. The data represent the mean of triplicate cultures ± SE.

probably results from the methods used: it is possible that by certain methods, other cells in addition to macrophages are removed and therefore the PFC response is inhibited in a way that cannot be abrogated by 2-ME.

Macrophages have been considered to be essential for the induction of a primary immune response in vitro against thymus-dependent antigens (6, 13, 14), although direct contact between macrophages and B cells is not necessary (15). It has also been shown that macrophage-derived factors are able to replace this macrophage function (16–18), but only in the presence of FCS.

MaSF permits a humoral immune response in macrophage-depleted spleen cultures, i.e., MaSF can completely replace macrophage function in these cultures. On the basis of similar results with 2-ME Chen and Hirsch suggested that a "2-ME-like substance" was the active factor enabling macrophages to activate lymphocytes in the Mishell-Dutton culture system (6). Based on our experimental data it is suggested that 2-ME or macrophages act on lymphocytes indirectly, i.e., through the activation of a serum component. The following observations support this conclusion. It has been shown that the addition of 2-ME to the Mishell-Dutton cultures permits an optimal immune response; the presence or absence of suboptimal or optimal numbers of macrophages in the culture is irrelevant (6), probably 2-ME is able to activate the serum factor faster and more efficiently than macrophages. This effect of 2-ME is also seen in its ability to activate FCS_{def} in normal Mishell-Dutton cultures,[2] whereas macrophages permit a response only if a "good" serum is present, i.e., one that contains a high concentration of nonactivated serum factor (Fig. 2). A further argument in favor of this model is the finding that in the primary in vitro antibody response both 2-ME and macrophages act on T cells (7, 19).

Many factors have been described that, like MaSF, can replace macrophages in in vitro antibody synthesis (16–18). These factors can be distinguished from MaSF: they are actively synthesized by macrophages and are released into the medium, they can be produced in the absence of FCS, and can be absorbed by SRBC (16). The fact that they can functionally substitute for macrophages in in vitro antibody synthesis only in medium containing FCS suggests that these factors may be activators of the serum factor described in this report.

The activity of 2-ME, macrophages or macrophage factors in the in vitro antibody synthesis may be explained by the hypothesis that they are able to activate a component of FCS which, in its activated form, is essential for the in vitro humoral immune response.

TABLE VII
Absorption of MaSF by Spleen Cells or SRBC

Treatment of MaSF	Addition of supernate (%, vol/vol)	Anti-SRBC PFC/culture
MaSF fraction absorbed with spleen cells	20	1,080 ± 240
	40	3,120 ± 330
MaSF fraction absorbed with SRBC	20	5,630 ± 720
	40	8,790 ± 810
MaSF fraction control	20	5,020 ± 470
	40	10,410 ± 620

2 ml MaSF/FCS_1 were incubated with 0.5 ml packed spleen cells or SRBC at 4°C for 1 h. Different concentrations of MaSF were added to macrophage-depleted spleen cells. Cultures did not receive additional FCS. PFC response was measured on day 5. The data represent the mean of triplicate cultures ± SE.

Summary

The mode of action of 2-mercaptoethanol (2-ME) on the primary immune response in vitro was investigated. Fetal calf serum (FCS) was preincubated with 2-ME and lyophilized to remove free 2-ME. This 2-ME-treated FCS was able to substitute the function of adherent cells in the primary immune response against sheep red blood cells (SRBC) in vitro. Fractionation of 2-ME-treated FCS on a Sephadex G-100 column showed that 2-ME acted on a high molecular serum component which after activation, could substitute for macrophages. In order to obtain a humoral immune response against SRBC in vitro, spleen cells require selected FCS. These "good" sera could be distinguished from "deficient" sera by their higher content of this 2-ME-activated factor. The height of the in vitro immune response to SRBC was dependent on the amount of activated factor added to the culture medium. FCS normally required in the culture medium could be completely replaced by the factor-containing fraction without deleterious effect on the culture. The factor should be added to the spleen cells during the first 24 h of culture and remain there for 72 h in order to obtain an optimal immune response. The factor could be partially absorbed by spleen cells but not by SRBC. The relationship between macrophage, 2-ME, and FCS in eliciting an in vitro primary immune response is discussed.

We gratefully acknowledge the excellent technical assistance of Mrs. G. Kepes and the helpful suggestions of Dr. H. D. Schlumberger in the preparation of this manuscript.

Received for publication 9 September 1976.

References

1. Broome, J. D., and M. W. Jeng. 1973. Promotion of replication in lymphoid cells by specific thiols and disulfides in vitro. *J. Exp. Med.* 138:574.
2. Iscove, N. N., and F. Sieber. 1975. Erythroid progenitors in mouse bone marrow detected by macroscopic colony formation in culture. *Exp. Hematol. (Copenh.)*. 3:32.
3. Heber-Katz, E., A. B. Peck, and R. E. Click. 1973. Immune response in vitro. II.

Mixed leucocyte interaction in a protein-free medium. *Eur. J. Immunol.* 3:379.
4. Peck, A. B., E. Heber-Katz, and R. E. Click. 1973. Immune response in vitro. IV. A comparison of the protein-free and mouse serum supplemented mouse mixed lymphocyte interaction assays. *Eur. J. Immunol.* 3:516.
5. Click, R. E., L. Benck, and B. J. Alter. 1972. Enhancement of antibody synthesis in vitro by mercaptoethanol. *Cell Immunol.* 3:156.
6. Chen, Ch., and J. G. Hirsch. 1972. The effect of mercaptoethanol and of peritoneal macrophages on the antibody-forming capacity of nonadherent mouse spleen cells in vitro. *J. Exp. Med.* 136:604.
7. Lemke, H., and H. G. Opitz. 1976. Function of 2-mercaptoethanol as a macrophage substitute in the primary immune response in vitro. *J. Immunol.* 117:388.
8. Mishell, R., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* 126, 423.
9. Lundgren, G., Ch. F. Zukoski, and G. Möller. 1968. Differential effects of human granulocytes and lymphocytes on human fibroblasts in vitro. *Clin. Exp. Immunol.* 3:817.
10. Bullock, W. W., and E. Möller. 1972. Spontaneous B-cell activation due to loss of normal mouse serum suppressor. *Eur. J. Immunol.* 2:514.
11. Ly, I. A., and R. I. Mishell. 1974. Separation of mouse spleen cells by passage through columns of Sephadex G-10. *J. Immunol. Methods.* 5:239.
12. Lee, K. Ch., R. E. Langman, V. H. Paetkau, and E. Diener. 1975. The cellular basis of cortisone-induced immunosuppression of the antibody response studied by its reversal in vitro. *Cell. Immunol.* 17:405.
13. Mosier, D. E., 1967. A requirement for two cell types for antibody formation in vitro. *Science (Wash. D. C.).* 158:1573.
14. Shortman, K., and J. Palmer. 1971. The requirement for macrophages in the in vitro immune response. *Cell. Immunol.* 2:399.
15. Calkins, C. E., and E. S. Golub. 1972. Direct demonstration of lymphocyte-macrophage cooperation in the absence of physical contact between the two cell types. *Cell. Immunol.* 5:579.
16. Hoffman, M., and B. W. Dutton. 1971. Immune response restoration with macrophage culture supernatants. *Science (Wash. D. C.).* 172:1047.
17. Calderon, J., and E. Unanue. 1975. Two biological activities regulating cell proliferation found in cultures of peritoneal exudate cells. *Nature (Lond.).* 253:359.
18. Möller, G., H. Lemke, and H. G. Opitz. 1976. The role of adherent cells in the immune response. *Scand. J. Immunol.* 5:269.
19. Erb, P., and M. Feldman. 1975. The role of macrophages in the generation of T helper cells. III. Influence of macrophage-derived factors in helper cell induction. *Eur. J. Immunol.* 5:759.