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T Cells and the Anti-trinitrophenyl Antibody Response to Fetal Calf Serum and 2-Mercaptoethanol (41711)

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Abstract. Unprimed murine lymphocytes maintained in culture medium containing fetal calf serum (FCS) and 2-mercaptoethanol (2-ME) developed very high levels of anti-trinitrophenyl (TNP) plaque forming cells (PFC). Both FCS and 2-ME contributed to the response. The development of anti-TNP PFC during culture was accompanied by a 10-fold expansion in the number of immunoglobulin-secreting cells, indicating polyclonal stimulation. However, the number of anti-TNP PFC was disproportionately high and not accompanied by a similar increase in plaques specific for sheep red blood cells. The TNP-specific plaques were not artifacts of the plaque assay since they were 98% inhibited by specific antigen. The *in vitro* induction of anti-TNP PFC by FCS and 2-ME was common to a number of mouse strains, although some genetic variation occurred. Nylon–wool-separated B cells, nude mouse spleen cells, and bone marrow cells all produced high levels of anti-TNP after culture with medium containing FCS and 2-ME. The addition of T cells to B-cell cultures increased the numbers of anti-TNP PFC by 1.5- to 2.5-fold. The presence of a TNP-cross-reacting antigen in FCS probably contributed to the unexpectedly high anti-TNP response. The response to the antigen in FCS was potentiated by the enhancing activity of 2-ME.

Chen and Hirsch (1, 2) found that macrophages could be replaced by 2-mercaptoethanol (2-ME) in the *in vitro* induction of primary plaque-forming cell (PFC) assays. The use of 2-ME has expanded due to its ability to enhance a variety of *in vitro* murine lymphocyte responses (3–7). Most of the assays require that 2-ME be used in combination with fetal calf serum (FCS) (1–5, 7). A 2-ME-modified FCS component has been suggested (8, 9), however, more recent evidence has suggested that the two components function independently (10). Two effects of 2-ME on murine lymphocytes have been distinguished, a direct stimulatory activity on mature lymphocytes and an enhancing activity for other stimulators which can affect both mature and immature cells (11). The direct stimulatory activity of 2-ME acts on B cells and to a lesser extent T cells (6) and the enhancing activity affects both B- (1, 2, 5, 8) and T-cell (3, 4) functions. Fetal calf serum is also stimulatory in culture (10). While wide-spread in use, FCS and 2-ME have introduced unknown variables to culture systems.

We have found that culture of murine lymphocytes in medium containing FCS and 2-ME without specific antigen generated high levels of anti-TNP antibody, due, in part, to the presence of a TNP-cross-reactive antigen in FCS (12). We report here that the anti-TNP levels generated were not artifacts of the assay system and were too high to be accounted for by the stimulatory activities of FCS and 2-ME. The response was T-independent, but was enhanced by T cells. Both mature and immature cells were induced to form anti-TNP suggesting that the enhancing function of 2-ME was critical to the response.

Materials and Methods. *Animals.* Male BALB/c and female C57BL/6, AKR, and C3H mice were obtained from Charles River Breeding Laboratory (North Wilmington, Mass.). Unless otherwise designated, 2- to 6-month-old BALB/c mice were used in all experiments. Congenitally athymic nude (nu/nu) and heterozygote (nu/+) mice with a Swiss–Webster background were obtained from the National Center for Toxicological Research (Jefferson, Ark.).

Cell culture. Cell suspensions were prepared by pressing spleens through a stainless-steel screen (60 mesh), triturating with a pasteur pipet, and filtering through a gauze pad to

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remove connective tissue and cell clumps. In most cases, spleen cells from two to five mice were pooled, washed twice, and cultured in 60 × 15-mm culture dishes at 1×10^7 cells in 5 ml medium per dish. The medium used throughout was RPMI 1640 containing L-glutamine (2 m*M*), penicillin (200 units/ml) and streptomycin (100 μg/ml). Unless otherwise designated, the medium was supplemented with 5% FCS (GIBCO Laboratories, Grand Island, N.Y.), 5×10^{-5} *M* 2-ME (Sigma Chemical Co., St. Louis, Mo.) and essential vitamins (Microbiological Associates, Walkersville, Md.). Cultures were maintained for 4, 7, or 10 days at 37°C in 5% CO_2.

Mouse bone marrow cell cultures were prepared from BALB/c femoral bone marrow using the same procedures described for spleen cells for preparing cell suspensions.

Nylon-wool columns were used to separate T and B lymphocytes from spleen cell suspensions as described by Handwerger and Schwartz (13). The nonadherent cell population was 90–95% non-Ig-bearing cells as determined by specific fluorescein-labeled anti-Ig staining. The nylon-wool-adherent cell population, which could be recovered, was 85–95% positive for surface Ig.

Plaque-forming cell (PFC) assay. The direct PFC (IgM) were assayed by a slide modification of the Jerne and Nordin plaque assay (14). Indicator cells were SRBC, SRBC coupled with TNP as described by Rittenberg (15), or SRBC coupled with protein A (Pharmacia, Piscataway, N.J.) as described elsewhere (16, 17). An amount of 0.1 ml 10% indicator cells and 0.1 ml of dilutions of the lymphocyte culture were added to 0.4 ml melted 0.6% agarose (Sigma Chemical Co., St. Louis, Mo.) in Hanks' balanced salt solution (Microbiological Associates, Walkersville, Md.), pH 7.4, at 47°C. After mixing, suspensions were poured onto agarose-coated slides and incubated at 37°C for 90 min. Slides were then flooded with diluted guinea pig complement (Microbiological Associates, Walkersville, Md.) and incubated for 45 min before counting. To determine indirect PFC (IgG), diluted rabbit anti-mouse immunoglobulin (Miles Laboratories, Elkhart, Ind.) was used to flood the slides during the first 90-min incubation period of the assay. Results were reported as the number of anti-TNP PFC per 10^6 viable cells. The averages of duplicate PFC assays were reported as is, without statistics, or were used to compute the mean and standard error of triplicate cultures.

Inhibition of plaques. To verify the specificity of TNP-specific plaques, inhibition studies of the PFC assay were performed. Specific anti-TNP PFC could be inhibited by dinitrophenyl (DNP) antigen which was either incorporated into the agar gel or used to flood the slides during plaque assay. Various amounts of DNP–keyhole limpet hemocyanin (DNP–KLH), prepared as described previously (12), in 0.05 ml of phosphate-buffered saline (PBS) were incorporated into the 0.4-ml melted 0.6% agarose and before TNP–SRBC and spleen cells were added. Alternatively, various amounts of DNP–KLH in 1 ml Hanks' BSS were used to flood each slide during the first 90-min incubation period.

Results. *Induction of anti-TNP among cells maintained in FCS and 2-ME.* Unprimed mouse spleen cells, maintained for 4 days in culture medium containing FCS and 2-ME, developed unusually high levels of anti-TNP PFC without exposure to antigen (Table I). Only low levels of anti-SRBC PFC were stimulated under the same culture conditions. Culture in the presence of FCS and 2-ME caused the total number of Ig-secreting cells to increase 10-fold over that of spleen cells not maintained in culture. Anti-TNP production represented 7.6% of all Ig-secreting cells maintained in culture, while anti-SRBC production represented less than 0.5% of Ig-secreting cells. When spleen cells were assayed

TABLE I. PLAQUE-FORMING CELL (PFC) RESPONSES OF UNPRIMED LYMPHOCYTES CULTURED WITHOUT ANTIGEN

	PFC/10^6 spleen cells[a]				
	Anti-SRBC		Anti-TNP		
	IgM	IgG	IgM	IgG	Total Ig secreting[c]
Without culture	0	0	0	0	1,192
4 Days culture[b]	54	0	688	255	11,977

[a] Mean of duplicate plaque-forming cell assays.

[b] Cells were maintained in RPMI 1640 containing 5% FCS and 5×10^{-5} *M* 2-ME.

[c] Protein A-coated SRBC were used as indicator cells in the plaque assays.

TABLE II. SPECIFIC INHIBITION OF ANTI-TNP PLAQUES

	Inhibitor[a] DNP-KLH (μg/slide)	IgM anti-TNP PFC/10^6 spleen cells[b]	Percentage inhibition[c]
DNP-KLH-primed cells without culture	0	65	—
	1	38	41
	10	12	81
	100	2	96
Unprimed cells[d] 4 days in culture	0	913	—
	3	289	68
	10	228	75
	60	19	98

[a] DNP-KLH was used at 1 ml/slide to flood slides during the plaque assay of primed cells. DNP-KLH was incorporated into the agarose for the plaque assays of cultured, unprimed cells.
[b] Mean of duplicate plaque-forming cell assays.
[c] (Control − experimental)/control × 100.
[d] Unprimed spleen cells were cultured 4 days in the presence of 5% FCS and 5×10^{-5} *M* 2-ME.

without maintenance in culture, neither SRBC-specific nor TNP-specific plaques were detected. Cells maintained in 0.5% mouse serum, instead of FCS, showed only low levels of anti-TNP and Ig-secreting cells. Thus, culture in the presence of FCS and 2-ME induced polyclonal stimulation of spleen cells, as shown by increased numbers of anti-SRBC, anti-TNP, and Ig-secreting PFC. However, the increase in anti-TNP PFC was disproportionately high.

Since such unusually high levels of anti-TNP PFC were generated, it was important to determine whether these plaques were actually due to anti-TNP production or were artifacts of the assay system. To determine the specificity of the plaques, an inhibition assay was performed. Specific antigen was incorporated into the agarose or used to flood the slides prior to developing the plaques (Table II). Spleen cells from mice primed with DNP-KLH were inhibited 96% from forming TNP plaques by exposing the slides to 100 μg DNP-KLH/slide. The inhibition of TNP-plaques by DNP-KLH correlated with our previous observations of cross-reacting immune responses to DNP and TNP epitopes. Unprimed cells cultured for 4 days in the presence of FCS and 2-ME generated high levels of TNP plaques, which were 98% inhibited by 60 μg DNP-KLH/slide. Plaque inhibition was antigen dose dependent. Each plaque contained a single lymphocyte at its center. The plaques generated by culture in FCS and 2-ME were indeed due to specific anti-TNP secreting cells.

To be sure that FCS and 2-ME induction of such stimulation was not a peculiarity of BALB/c mice, we also tested unprimed spleen cells from C57BL/6, AKR, and C3H mouse strains (Table III). Spleen cells from all four strains generated very high levels of anti-TNP PFC in culture. Cells from C3H mice were the most active and cells from C57BL/6 were slightly lower than BALB/c spleen cells.

Since FCS has been shown to vary by lot in its ability to support *in vitro* antibody induction, we examined various lots of FCS in culture with 2-ME (Table IV). FCS lots, from three different suppliers, with some variation, induced uniformly high levels of anti-TNP plaques. Newborn calf serum did not interact with 2-ME to induce anti-TNP.

Relative T-independence of anti-TNP induction by FCS and 2-ME. 2-ME or FCS and 2-ME have been reported to stimulate mito-

TABLE III. RESPONSES TO CULTURE WITH FCS AND 2-ME BY SPLEEN CELLS FROM VARIOUS MOUSE STRAINS

Mouse strains	Anti-TNP PFC/10^6 spleen cells[a]	
	IgM	IgG
BALB/c	880 ± 84	403 ± 40
C57BL/6	808 ± 102	302 ± 69
C3H	3373 ± 216	1939 ± 108
AKR	1907 ± 174	1035 ± 83

[a] Normal spleen cells from various mouse strains were cultured 4 days in the presence of 5% FCS and 5×10^{-5} *M* 2-ME. Results are reported as the mean ± SE of triplicate samples.

TABLE IV. STIMULATORY ACTIVITY OF DIFFERENT LOTS OF SERUM

Fetal calf serum (lots)	Anti-TNP PFC/10^6 cells[a] IgM	IgG
Irvine Scientific Co.		
1	1045	391
2	440	144
3	1123	435
Grand Island Biological Co.		
1	937	417
2	549	166
Microbiological Associates		
1	529	347
2	630	302
Newborn calf serum	0	0
None	0	0

[a] Spleen cells from normal BALB/c mice were cultured with 2-ME and various batches of 5% serum. Anti-TNP plaque assays were performed on Day 4 and are reported as the mean of duplicate assays.

genesis of B cells (6) and T cells (18) and enhance induced B- (1, 5) and T-cell (3) activities. We next examined whether the induction of anti-TNP PFC in the presence of FCS and 2-ME was due strictly to B-cell stimulation, B- and T-cell stimulation, or only T-helper-cell stimulation. Unprimed mouse spleen cells were separated on nylon-wool columns and the nylon-adherent and nonadherent populations were cultured for 4 days in FCS and 2-ME before plaque assays were performed (Fig. 1). The nylon-adherent population (B cells), which was made up of 85–95% surface Ig-bearing cells, was stimulated in culture to produce high levels of anti-TNP PFC. As expected, few or no anti-TNP PFC were generated in the nonadherent population (T cells), which was 90–95% non-Ig-bearing cells. Adding T cells to the B-cell population increased IgM anti-TNP PFC stimulation by an average of 38% and IgG by an average of 76%. Since the B-cell population contained 5–15% non-Ig-bearing cells, which might have provided T-helper function, we examined spleen cells from athymic nude mice (Fig. 2). Spleen cell cultures prepared from nude mice and maintained in medium containing FCS and 2-ME generated high levels of anti-TNP PFC, particularly IgM PFC. Spleen cell cultures prepared from thymus-normal heterozygous littermates generated anti-TNP PFC, where IgM PFC were 44% higher and IgG PFC were 160% higher than cells from the athymic mice. The IgM anti-TNP PFC response of nude mouse

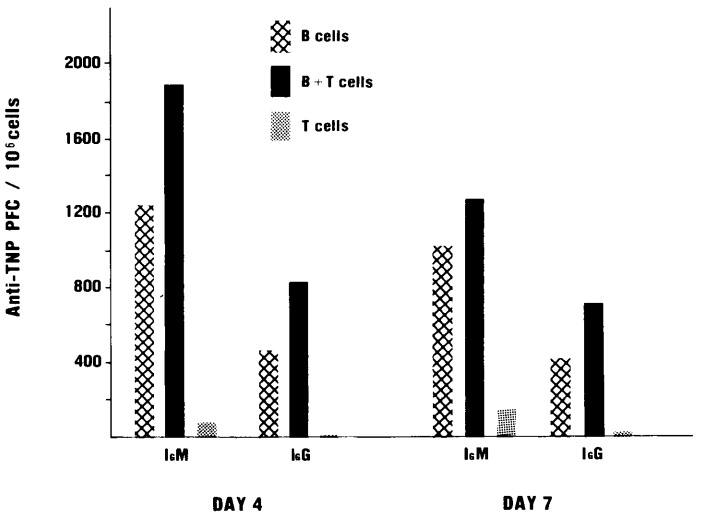

FIG. 1. Anti-TNP responses of nylon-wool-separated lymphocytes to culture in FCS and 2-ME. Spleen cells, pooled from three unprimed mice, were separated on nylon wool columns. Nonadherent cells (T) were 90–95% negative when stained with fluorescein-labeled anti-Ig. Nylon-adherent cells (B), which could be recovered from the columns, were 85–95% positive for surface Ig. The T cells, B cells, or equal mixtures of T and B cells were cultured for 4 or 7 days in medium containing 5×10^{-5} *M* 2-ME and 5% FCS. Duplicate direct (IgM) and indirect (IgG) plaque assays were performed.

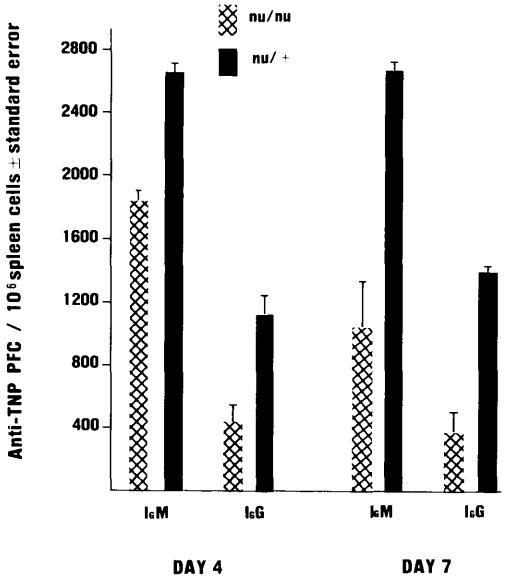

FIG. 2. Anti-TNP responses of congenitally athymic mouse lymphocytes to FCS and 2-ME. Spleen cell suspensions were prepared from unprimed athymic nude (nu/nu) mice or thymus-normal heterozygous (nu/+) mice. Cells were maintained in culture medium containing 5 $\times$ 10^{-5} *M* 2-ME and 5% FCS. After 4 or 7 days, direct (IgM) and indirect (IgG) plaque assays were performed in duplicate. Vertical bars represent the means and standard error of triplicate cultures.

cells declined from 4-day cultures to almost half on continued culture for 7 days. The normal spleen cells sustained high PFC levels through 7 days.

Since 2-ME is nonstimulatory to immature lymphocytes, but can enhance their responses to other agents (11), it was of interest to find out whether only mature lymphocytes could be induced to form anti-TNP PFC by culturing in medium containing FCS and 2-ME. Mouse femoral bone marrow cells were maintained in culture for 4, 7, or 10 days in the presence of FCS and 2-ME, but in the absence of antigen (Fig. 3). Without added T cells, the bone marrow cells generated both direct and indirect anti-TNP PFC. The bone marrow cells generated lower levels of PFC than spleen cells, over a longer culture period. Only background levels of anti-TNP PFC were generated from bone marrow after 4 days in culture, a time at which spleen cell PFC were reaching peak levels. Bone marrow PFC increased through 10 days in culture.

Discussion. The culture medium components, FCS and 2-ME, are frequently used for the *in vitro* maintenance of murine lymphocytes. It has been reported that these medium components produce lymphocyte mitogenesis (3, 4, 6, 11), are necessary for the *in vitro* induction of anti-SRBC PFC (17, 19, 20), B-cell stimulation with anti-IgM (5), and enhanced proliferative responses to mitogens (21). We found that *in vitro* maintenance of unprimed murine lymphocytes in medium containing FCS and 2-ME caused the induction of an extraordinarily high number of anti-TNP PFC without exposure to antigen. Both FCS and 2-ME were required to be present. Identical culture conditions led to relatively slight increases in anti-SRBC plaques and a 10-fold increase in the number of Ig-secreting cells. Corresponding with other reports (6), FCS and 2-ME in culture stimulated a polyclonal expansion of antibody producing cells. The anti-TNP response, however, was 16-fold greater than the anti-SRBC response and could account for 7.6% of all of the Ig-producing cells.

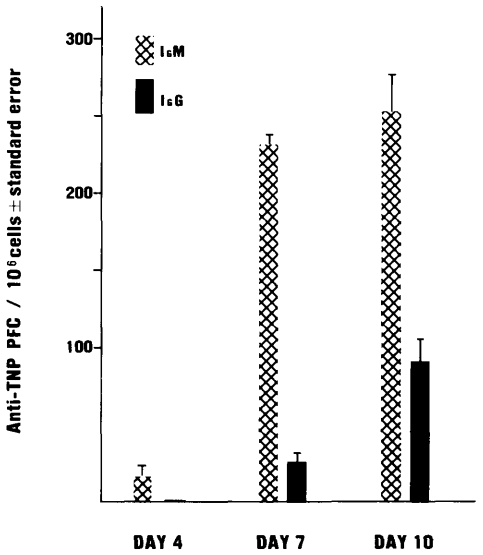

FIG. 3. Anti-TNP responses of bone marrow cells to FCS and 2-ME. Bone marrow was collected from the femur bones of three unprimed mice and cell suspensions were prepared and pooled. Cells were maintained in culture medium containing 5 $\times$ 10^{-5} *M* 2-ME and 5% FCS. After 4, 7, or 10 days, direct (IgM) and indirect (IgG) plaque assays were performed in duplicate. Vertical bars represent the means and standard error of triplicate cultures.

The very large TNP-specific antibody response nonspecifically induced by culture in the presence of FCS and 2-ME was not an artifact of the plaque assay system. Each plaque had a single lymphocyte at its center and anti-TNP plaques were 98% inhibited by specific antigen. Each of seven different lots of FCS from three distributers induced high levels of anti-TNP in the presence of 2-ME. Every strain of mouse tested generated very high levels of anti-TNP PFC. The C3H mouse strain was the most sensitive to culture in FCS and 2-ME, generating 4-fold more anti-TNP PFC than BALB/c or C57BL/6.

The culture components stimulated the differentiation of TNP-specific B cells without T-cell help. The induction of anti-TNP PFC, particularly IgM anti-TNP, could occur in the absence of T cells and to that extent, stimulation resembled a T-independent response. Nylon-wool-separated B cells (85–95% sIg^+), congenitally athymic nude mouse spleen cells, and bone marrow cells all developed high levels of anti-TNP PFC after culture in FCS and 2-ME. The TNP-specific response, also showed some T-dependent characteristics, since the presence of T cells enhanced the induction of anti-TNP, particularly IgG anti-TNP. The presence of T cells in culture increased the anti-TNP response by 1.5- to 2.5-fold, increasing IgG consistently more than IgM. Goodman and Weigle (6) reported that B cells proliferated in response to 2-ME in the absence of serum and that both B and T cells proliferated in the presence of 2-ME and FCS. We found a definitive requirement for FCS in the induction of anti-TNP antibody by 2-ME. Only very low levels of anti-TNP were generated by 2-ME in the absence of serum or in the presence of mouse serum, rabbit serum, newborn calf serum, or bovine serum (data not shown). T cells have been shown to enhance the activity of the B-cell mitogens, lipopolysaccharide and pokeweed mitogen (22–24). The response to the T-cell mitogen, phytohemagglutinin, was also enhanced when B and T cells were present together (23). The induction of anti-TNP antibody by FCS and 2-ME was similar to responses to mitogens in that B cell responses were enhanced by the presence of T cells. A synergy between B and T cells has been reported for 2-ME stimulation of anti-SRBC PFC (25). The maximum levels of anti-SRBC PFC reported to be induced by 2-ME (25) were not different from the anti-SRBC PFC that we found and were substantially lower than the anti-TNP levels reported here. The anti-TNP PFC response was likely mediated, in part, by such a polyclonal mitogenic activity from FCS and 2-ME, but such stimulation was not sufficient to account for the very high anti-TNP response. The presence of an antigen in FCS which cross-reacts with TNP which we reported (12), probably contributed to the very high anti-TNP response to FCS and 2-ME.

Both mature and immature B cells were induced to differentiate and produce antibody. Bone marrow cells generated anti-TNP PFC due to culture in 2-ME and FCS, but after a longer period in culture and the levels reached were lower than those induced with spleen cells. Goodman, *et al.* (11) reported that only more mature cells were stimulated by 2-ME, but that 2-ME could enhance the ability of immature cells to respond to other stimulating agents. The bone marrow response observed may have been due to mature B cells present in the marrow (26), but the longer induction time required for the generation of anti-TNP PFC suggested that less mature cells were stimulated. Alternatively, the bone marrow cells might have spontaneously developed sensitivity to the mitogenic activity of 2-ME during the culture period. Immature cells from the fetal liver were shown to develop such sensitivity, but only after 29 days in an adoptive host (11). Since 2-ME does not directly activate immature cells (11) and since such a large proportion of the Ig response was TNP-specific, it is suggested that the function of 2-ME in inducing anti-TNP production was due to enhancing activity, rather than simply direct stimulatory activity. With spleen cells, direct stimulation by 2-ME may have augmented the enhancing activity, generating the larger responses observed. The 2-ME probably acted to enhance the anti-TNP response to the FCS component which we reported (12) to be antigenically cross-reactive with TNP.

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