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# Promotion of Hematopoietic Stem Cell Differentiation In Vitro by a Soluble Mediator, Allogeneic Effect Factor

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This study was designed to investigate the effects of allogeneic effect factor (AEF), a soluble mediator derived from short-term mixed lymphocyte cultures (MLC) of in vitro alloantigen-primed T cells, on cultures of murine bone marrow cells. Cultures established under suboptimal conditions namely, in the absence of a pre-established adherent cell layer as required in conventional Dexter-type cultures–declined and lost their stem cell activity rapidly. In contrast, supplementation of these cultures, at initiation and thereafter, with AEF, but not with T cell growth factor (TCGF), induced cell growth and proliferation for several weeks. Such AEF-supplemented cultures exhibited cellular heterogeneity and stem cell activity for significantly longer periods than the control cultures. Even in conventional Dexter cultures, established under optimal conditions, AEF had a beneficial effect on cellular growth and proliferation and myeloid progenitor cell (CFU-C) activity. Furthermore, cells capable of synergizing with suboptimal numbers of mature T cells in con A-induced mitogenic responses, shown by others to be pre-T cells, were detected in the AEF-supplemented cultures for several weeks.

**Key words: bone marrow, stem cell differentiation, allogeneic effect factor**

The bone marrow is a source of pluripotent hematopoietic stem cells capable of giving rise to all the cellular elements of the blood, including myeloid cells, erythrocytes, and lymphocytes [1]. The study of the pathways and regulatory mechanisms involved in hematopoiesis has greatly benefited from the development of a tissue-culture system that supports the growth and differentiation of pluripotent stem cells for many weeks [2]. Using this system, it has been possible to obtain differentiation of myeloid cells, megakaryocytes,

Abbreviations: AEF, allogeneic effect factor; CFU-C, colony-forming units–culture; CFU-S, colony-forming units–spleen; Con A, concanavalin A; CSA, colony-stimulating activity; $^{3}$HTdR, tritiated thymidine; HS, horse serum; MLC, mixed lymphocyte culture; TCGF, T cell growth factor.

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and erythrocytes from their precursors in vitro [3–6]. However, until very recently, differentiation of lymphoid cells was not observed in these cultures, although cells derived from long-term murine bone marrow cultures were shown to be capable of reconstituting the lymphoid tissues of irradiated recipients and differentiating into immunocompetent lymphocytes [7, 8]. The development of techniques for obtaining differentiation of immunocompetent lymphoid cells from pluripotent stem cells in vitro would greatly facilitate studies of the generation of the immune system and its diversity during ontogenesis.

Very recently, it has been reported that Thy-1-bearing lymphocytes can be generated in murine bone marrow cultures, either without or with a stimulus provided by supernatants of mitogen-stimulated T lymphocytes possessing T cell growth factor (TCGF) activity [9–11]. In all of these studies, these cells were generated in conventional Dexter cultures, which require establishment of an adherent cell layer, followed by a second inoculum of fresh bone marrow cells, in order to obtain long-term maintenance of stem cells [2].

In this communication, we report that allogeneic effect factor (AEF), a soluble mediator derived from short-term secondary mixed lymphocyte cultures (MLC) of in vivo alloantigen-activated murine T cells [12], is capable of supporting the growth of bone marrow stem cells in vitro, in the absence of a pre-established adherent cell layer. AEF, but not TCGF, was highly mitogenic for, and maintained cellular heterogeneity in, such cultures. Furthermore, cells capable of synergizing with suboptimal numbers of mature T cells in concanavalin A (con A)-induced mitogenic responses, shown by others to be pre-T cells [13, 14], could be found in these cultures for several weeks.

## MATERIALS AND METHODS

### Mice

DBA/2, (BALB/c × A/J)$F_1$ ($CAF_1$), or (C57BL/6 × DBA/2)$F_1$ ($B6D2F_1$) hybrid mice were obtained from the Scripps Clinic and Research Foundation breeding colony. (C3H × DBA/2)$F_1$ ($C3D2F_1$) hybrid mice were obtained from the Jackson Laboratory (Bar Harbor, ME).

### Establishment of Bone Marrow Cultures

Donor mice, 2–3 months old, were killed by cervical dislocation and their femurs removed aseptically into cold Eagle's minimal essential medium (MEM). The marrow plugs were expelled by flushing the femurs with cold MEM, and single cell suspensions were obtained by passing the plugs several times through a 20-gauge needle. Cells were added at one femur-equivalent ($1–1.5 \times 10^7$ cells) per 25 $cm^2$ tissue culture flask (Falcon Plastics, Oxnard, CA) in 0.5 ml MEM, and the flasks were supplemented with 6.5 ml of high-glucose Dulbecco's-modified Eagle's medium (DMEM) supplemented with 20% horse serum (HS, Flow Laboratories, Inglewood, CA, lot number 29211044), and 1% each of glutamine, sodium pyruvate (Irvine Scientific Co., Irvine, CA), and gentamicin (Schering Corp., Kenilworth, NJ). Some of the cultures were additionally supplemented with various concentrations of AEF (see below) as described in Results. In some experiments, bone marrow cultures, established in the absence of AEF, were re-inoculated 2–3 weeks later, when an adherent layer became established, with one femur-equivalent of fresh syngeneic bone marrow cells as described by Dexter et al [2]. Cultures were maintained at 33°C in an atmosphere of 10% $CO_2$ in air and fed twice weekly by removing half of the culture medium plus nonadherent cells and replacing it with 3.5 ml fresh supplemented DMEM with or without AEF. At various intervals, as indicated in Results, the harvested cells were counted and assayed in

various assays as described below. In some experiments, the adherent cells were also harvested by treating the flasks (after removing all the non-adherent cells) with a mixture of trypsin and EDTA (Grand Island Biological Co.) and a rubber policeman. The recovered cells (>90% viable) were washed and tested in various functional assays.

### Preparation of AEF

AEF was prepared as described earlier [15, 16]. Briefly, 650 rad-irradiated DBA/2 mice were injected by the intravenous (IV) route with $1 \times 10^8$ each syngeneic thymocytes and 1,500 rad-irradiated semi-allogeneic $C3D2F_1$ spleen cells. The allo-activated T cells, recovered from the spleens of these mice after one week, were cultured for 20 h with irradiated $C3D2F_1$ spleen cells ($1 \times 10^7$/ml each) in serum-free RPMI 1640 supplemented with 2-mercaptoethanol ($1 \times 10^{-4}$ M). The supernatants were harvested by centrifugation, filter-sterilized, and maintained at −70°C until used.

### Colony-Forming Unit-Spleen (CFU-S) Assay

Cells harvested from the bone marrow cultures were washed in MEM and resuspended in the same medium at several dilutions. Cell doses ranging between $2 \times 10^4$ and $1.6 \times 10^5$ were injected IV in 1 ml MEM into groups of three syngeneic recipients irradiated previously with 875 rads. In some experiments, the mice were also injected on the same day and every other day thereafter with a mixture of glucose and gentamicin (27 mg and 100 μg per mouse, respectively) given in 1 ml saline solution intraperitoneally. Spleens were removed from these mice after 7–8 days, fixed in Bouin's solution and macroscopic colonies were enumerated.

### Colony-Forming Unit-Culture (CFU-C) Assay

Progenitors of the granulocyte/macrophage series were assayed by plating $1 \times 10^5$ harvested cells in 35 mm dishes (Falcon Plastics) in 1 ml McCoy's 5a medium (Grand Island Biological Co., Grand Island, NY) supplemented with 15% fetal calf serum (FCS, Irvine Scientific Co.), 0.5% penicillin-streptomycin solution (Irvine Scientific Co.), and 0.3% agar (Difco Laboratories, Detroit, MI), together with 50 μl serum collected from C57BL/6 mice injected 6 h earlier IV with 5 μg E coli lipopolysaccharide as a source of colony-stimulating activity (CSA [17]). Cultures were set in triplicate, and colonies (≥50 cells) were enumerated after 7–8 days of incubation at 37°C using a low power dissecting microscope.

### Thymidine Uptake

Harvested cells were placed in flat-bottom microtiter tissue culture plates (Falcon Plastics) at $1 \times 10^5$ cells per well in 200 μl supplemented DMEM, and pulsed with 1 μCi tritiated thymidine ($^3$HTdR, specific activity 5 Ci/mmole, Amersham Corporation, Arlington Heights, IL). The cultures were harvested after 4 or 16 h on a Skatron cell harvester (Flow Labs., Inc., Rockville, MD) and counted in a liquid scintillation counter (Model LS8000, Beckman Instruments, Inc., Irvine, CA).

### Differential Counts

Cells were resuspended at $4 \times 10^5$/ml in MEM and spun onto microscope slides from a cytocentrifuge. The slides were then stained with Wright-Giemsa stain using an Ames Hema-Tek slide stainer (Miles Laboratories, Elkhart, IN). Slides were mounted using Permount mounting medium (Fisher Scientific Co., Tustin, CA), covered with glass coverslips, and examined under 1,000 × magnification using immersion oil.

TABLE I. The Effects of AEF and TCGF on Growth of Cells in Cultures of CAF, Bone Marrow*

| | Cells/flask × $10^{-5}$ | | | | | | |
|---|---|---|---|---|---|---|---|
| | | AEF-Supplemented | | | TCGF-Supplemented | | |
| Day | Control | 0.1% | 0.5% | 1.0% | 2.5% | 12.5% | 25% |
| 4 | 24 | 33 | 43 | 59 | 26 | 29 | 26 |
| 6 | 8.4 | 15 | 27 | 39 | 7.7 | 7.7 | 8.4 |
| 10 | 3.4 | 37 | 66 | 110 | 4.9 | 6.2 | 7.7 |
| 13 | 1.0 | 21 | 51 | 80 | 1.3 | 1.8 | 3.1 |
| 17 | 0.5 | 32 | 58 | 66 | 1.0 | 1.5 | 1.6 |
| 20 | NT[a] | 28 | 40 | 46 | NT | NT | NT |
| 24 | NT[a] | 20 | 35 | 48 | NT | NT | NT |
| 27 | NT[a] | 9.8 | 18 | 29 | NT | NT | NT |
| 32 | NT[a] | 6.1 | 15 | 15 | NT | NT | NT |

*Cultures were established with one inoculum of $7.5 \times 10^6$ fresh bone marrow cells per flask. TCGF was derived from cultures of rat spleen cells ($2 \times 10^6$/ml) stimulated for 2 days with 5 μg/ml con A in RPMI 1640 + 5% FCS. Several lower concentrations of TCGF were tested and had no significant effect on the cultures (not shown). Data reflect uncorrected, actual cell numbers in the cultures on the given days of feeding.
[a]Not tested.

### Mitogenic Response to Con A

This assay was performed according to Cohen and Fairchild [13, 14]. Various doses of cultured bone marrow cells ($5 \times 10^4$ to $2 \times 10^5$) were cultured in flat-bottom microtiter plates with $5 \times 10^4$ fresh syngeneic lymph node cells in 200 μl RPMI 1640 medium (Irvine Scientific Co.) supplemented with 5% FCS in the absence or presence of 2.5 μg/ml con A (Miles-Yeda, Rehovot, Israel). Controls consisted of bone marrow or lymph node cells cultured separately with or without the mitogen. Cultures were maintained for 48 h at 37°C, pulsed for the final 6 h with 1 μCi $^3$HTdR, harvested, and counted as described above. Results are presented as net $^3$HTdR uptake (Δ cpm) after subtracting the low levels of $^3$HTdR obtained in the absence of con A stimulation.

## RESULTS

### Effects of AEF on Growth and Proliferation of Bone Marrow Cells

Establishment of long-term murine bone marrow cultures possessing stem cell activity normally requires two consecutive inocula of bone marrow cells in culture [2]. The first one results in the establishment of an adherent layer within 2–3 weeks, consisting of several cell types, but lacking any sustained stem cell activity. This adherent layer serves then as a microenvironment allowing the continuous proliferation and maintenance of stem cell activity of a second inoculum of fresh bone marrow cells.

In order to test the effects of AEF on the growth of bone marrow cells in vitro, we deliberately selected suboptimal conditions that led to the rapid decline and loss of function in the control cultures not supplemented with AEF. These conditions included the use of a) only one inoculum of bone marrow cells, and b) a deficient lot of HS, which poorly supported stem cell activity in conventional Dexter cultures. The results in Table I and Figure 1

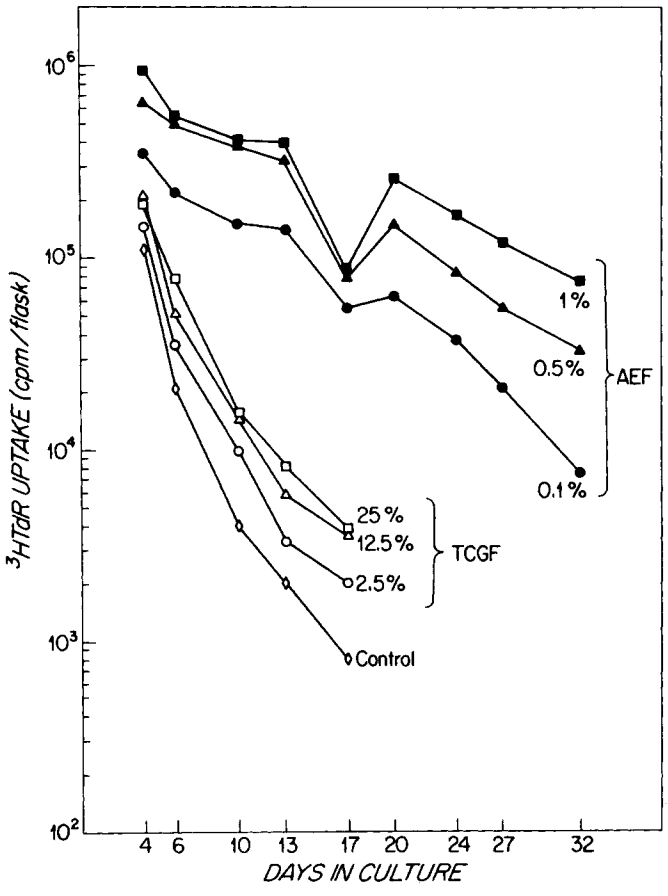


Fig. 1. $CAF_1$ bone marrow cultures were established as described in Table I. Samples of $1 \times 10^5$ cells from each group, harvested on the day of each feeding as shown, were pulsed (in triplicate) in microtiter culture wells with $^3$HTdR and harvested 4 h later. The level of uptake per flask was calculated from the number of cells harvested in each group (see Table I). Data reflect, therefore, $^3$HTdR uptake by non-adherent, recoverable cells.

depict the general effects of AEF on the growth and proliferation of $CAF_1$ bone marrow cells in culture. In addition, we also tested the effect of con A-stimulated rat spleen cell culture supernatants, possessing TCGF activity, on the same cultures. Control cultures without any supplementation or cultures supplemented with three different concentrations of TCGF declined rapidly to low levels, which did not allow manipulation or functional assays beyond 17 days of culture. The number of recoverable, non-adherent cells after 17 days in culture was <1% and 1.3–2.1% of the original inoculum in control and TCGF-supplemented cultures, respectively. These cultures contained sparse monolayers of adherent cells. In contrast, the cultures supplemented with three different concentrations of AEF grew continuously for 32 days, at which time this particular experiment was terminated. Even at this relatively late time-point, the harvested cells constituted 8–20% of the original inoculum.

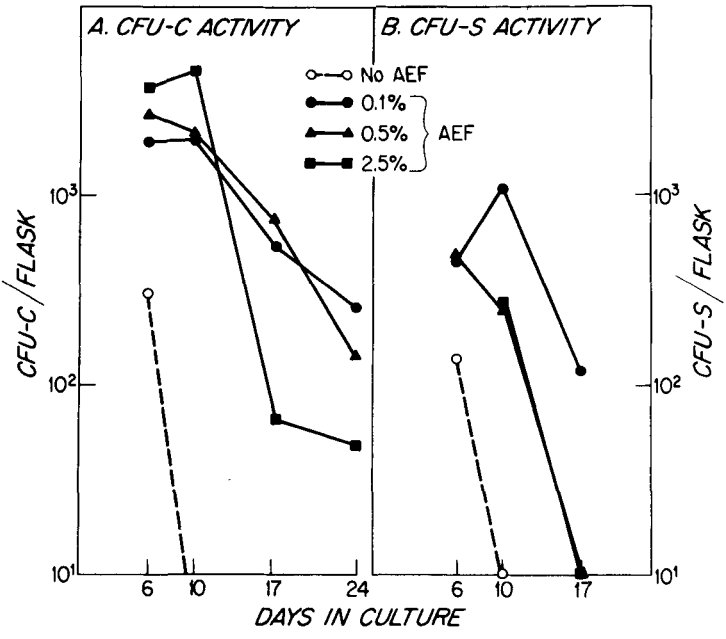


Fig. 2. $CAF_1$ bone marrow cultures were established as described in Table I. CFU-S and CFU-C assays were performed on 3 mice per group and in triplicate cultures, respectively, and read after 8 days. Data reflect CFU-S and CFU-C activities per $1 \times 10^5$ cultured cells.

Taking into account the fact that the cultures were diluted 9 times (by a factor of 2 at each feeding), thus representing a total dilution factor of 512, these results clearly indicate that AEF supported the rapid growth and proliferation of cells in these cultures. This conclusion is supported by the results presented in Figure 1 depicting the levels of $^3$HTdR uptake in the cultures. It is clear that levels of $^3$HTdR uptake in the AEF-supplemented cultures were higher by far than in the control or TCGF-supplemented cultures at each given time. In that respect, there was a clear dose-response effect of AEF, with 1% being more effective than 0.5% or 0.1% AEF. Adherent cell layers became established much more rapidly in the AEF-supplemented cultures, and confluent layers were present after $\approx$2 weeks in culture.

### The Effects of AEF on Stem Cell Activity

In addition to determining the general effects of AEF on the growth of bone marrow cells in vitro, we wished to ascertain whether this accelerated growth is accompanied by stem cell activity. This was done by measuring CFU-C and CFU-S activity in AEF-supplemented cultures, according to standard procedures (Fig. 2). CFU-C and CFU-S activity in control and in TCGF-supplemented (not shown) cultures declined very rapidly and was absent in the cultures 9 days after initiation. In contrast CFU-C and CFU-S activities were existent in the AEF-supplemented cultures for 24 and 17 days, respectively. It is not surprising that stem cell activity was present for a limited period of 2–3 weeks only since, as was stated above, suboptimal culture conditions were deliberately used. The lowest concentration of AEF (0.1%) supported better maintenance of CFU-C and CFU-S than the two higher con-

centrations, in direct contrast to the effects of AEF concentrations on $^{3}$HTdR uptake (Fig. 1). It is possible that with the stronger mitogenic (and differentiation-inducing?) activity of AEF, stem cell activity is lost earlier due to the enhanced terminal differentiation of CFU-S and CFU-C in the culture. However, it should be stressed that granulocytes and blast forms were identified in the AEF-supplemented cultures for several weeks, whereas the control cultures contained only macrophages in the non-adherent fraction after 2–3 weeks of culture (not shown).

### Comparison of AEF-Supplemented Cultures With Conventional Dexter Cultures

It was mentioned above that AEF induced an accelerated growth of the adherent cell layer which provides an essential microenvironmental influence in conventional Dexter cultures. Thus, it is possible that AEF acts merely by inducing a rapid formation of the adherent cell layer, to such an extent that an optimal microenvironment is established before pluripotent stem cell activity is lost from the culture. Once such a microenvironment is established, the residual CFU-S can then proliferate and differentiate. Although such an effect of AEF is interesting in itself, we have preliminary data indicating that AEF has additional effect(s) in the cultures (Tables II and III). In the experiment presented in Tables II and III, cell numbers, $^{3}$HTdR uptake, and CFU-S and CFU-C activities were analyzed separately in the non-adherent and adherent cell fractions of cultures established under various conditions. One set of cultures was established according to Dexter et al [2] with two inocula of bone marrow (group 2) and another with just one (the second) inoculum of cells supplemented with AEF (group 3). Group 4 is a combination of groups 2 and 3 with a pre-established adherent cell layer plus AEF supplementation at the time of the second bone marrow inoculation into culture. The control group (group 1) lacked both a pre-established adherent cell layer and AEF. Despite the fact that group 3 had fewer adherent cells recovered after one week of culture than did group 2 (Table II), it contained almost three times as many non-adherent cells, which exhibited markedly higher levels of $^{3}$HTdR uptake per flask (Table II), as well as CFU-S and CFU-C activities (Table III). The combination of a pre-established adherent cell layer and AEF resulted in general in the highest levels of activity for all the parameters tested except the CFU-S activity (group 4 in Tables II and III). These results clearly demonstrate that AEF does not act by merely substituting for an optimal microenvironment, normally provided by a pre-established adherent cell, but rather has additional, still unknown, effects on these cultures.

### Cells From AEF-Supplemented Cultures Synergize With Suboptimal Numbers of Mature T Cells in Con A-Induced Mitogenic Responses

Cohen and Fairchild reported that cells in fresh bone marrow suspensions are capable of synergizing with very low numbers of peripherally derived mature T cells in the induction of strong mitogenic responses by con A, under conditions where neither the bone marrow cells nor the low numbers of mature T cells alone respond significantly to the mitogen [13, 14]. Their studies provided evidence that pre-T cells in the bone marrow are the synergizing cells in this assay, whereas the mature T cells are the ones that actually respond to con A stimulation by proliferation [13]. This conclusion was based mainly on the effects of thymosin on the synergistic activity of bone marrow cells and the direct correlation between relative levels of pre-T cells and synergistic activity in spleen vs bone marrow of athymic nude mice [13, 14]. Furthermore, bone marrow cells usually lost their synergistic activity after being cultured for 2 days [13].

**TABLE II. The Effects of AEF and Pre-Established Adherent Cell Layers on Growth and Proliferation of Bone Marrow Cells In Vitro***

| | 2.5% AEF | Pre-established adherent layer | Cells | Day 7 Cells/flask ($\times 10^{-5}$) | Day 7 $^3$HTdR uptake (cpm $\times 10^{-3}$/flask) | Day 14 Cells/flask ($\times 10^{-5}$) | Day 14 $^3$HTdR uptake (cpm $\times 10^{-3}$/flask) |
|---|---|---|---|---|---|---|---|
| 1. | – | – | Non-adherent | 5.4 | 54.7 | 1.3 | 9.8 |
| | | | Adherent | NT[a] | NT | NT | NT |
| 2. | – | + | Non-adherent | 9.6 | 134.9 | 9.4 | 90.4 |
| | | | Adherent | 15.0 | 32.5 | NT | NT |
| 3. | + | – | Non-adherent | 26.0 | 328.6 | 46.0 | 484.3 |
| | | | Adherent | 8.5 | 17.3 | NT | NT |
| 4. | + | + | Non-adherent | 38.0 | 470.0 | 64.0 | 433.9 |
| | | | Adherent | 28.0 | 81.3 | NT | NT |

*$5 \times 10^6$ $B6D2F_1$ bone marrow cells were inoculated on day 0 into empty flasks or flasks containing an adherent cell layer established 10 days earlier. AEF ($DBA/2 \rightarrow C3D2F_1$) was added to groups 3 and 4. $^3$HTdR uptake by $5 \times 10^6$ fresh bone marrow cells on day 0 was 320,200 cpm.
[a]Not tested; too few cells were available for analysis.

**TABLE III. The Effects of AEF and Pre-Established Adherent Cell Layers on CFU-S and CFU-C Activities of Cultured B6D2F$_1$ Bone Marrow***

| | 2.5% AEF | Pre-established adherent layer | Cells | CFU-S/flask | CFU-C/flask |
|---|---|---|---|---|---|
| 1. | – | – | Non-adherent | 90 | 270 |
| | | | Adherent | NT[a] | NT |
| 2. | – | + | Non-adherent | 106 | 211 |
| | | | Adherent | 71 | 210 |
| 3. | + | – | Non-adherent | 351 | 702 |
| | | | Adherent | NT | 119 |
| 4. | + | + | Non-adherent | 266 | 1216 |
| | | | Adherent | 56 | 336 |

*Cultures were established as in Table II. CFU-S and CFU-C activities of fresh bone marrow cells on day 0 were 625 and 4,250 per flask ($5 \times 10^6$ cells), respectively. Activities of cultured cells were assayed after 7 days in culture.
[a]Not tested.

**TABLE IV. Synergism Between Fresh or Cultured CAF$_1$ Bone Marrow Cells and Syngeneic Lymph Node Cells in Con A-Induced Mitogenic Responses***

| | $5 \times 10^4$ LNC | $2 \times 10^5$ Bone marrow cells | Net $^3$HTdR uptake (Δ cpm/culture) |
|---|---|---|---|
| 1. | + | None | 1,260 |
| 2. | – | Fresh | 2,872 |
| 3. | + | Fresh | 20,115 |
| 4. | – | 28-Day culture without AEF | 4,067[a] |
| 5. | + | 28-Day culture without AEF | –210 |
| 6. | – | 28-Day culture with 0.5% AEF | 521 |
| 7. | + | 28-Day culture with 0.5% AEF | 8,424 |

*$^3$HTdR uptake by unstimulated cells was <2,100 cpm in the different groups.
[a]A differential count indicated 100% macrophages in this culture. In contrast, the cells cultured with AEF (group 6) consisted of 67% macrophages, 26% granulocytes, and 7% undifferential blasts.

Thus, we wished to ascertain whether AEF-supplemented cultures contain cells capable of synergizing in the assay described above. The results of such an experiment are presented in Table IV. It can be seen that $5 \times 10^4$ lymph node cells (group 1) or $2 \times 10^5$ fresh bone marrow cells (group 2) cultured separately responded only minimally to con A stimulation, whereas combination of the two cell populations (group 3) resulted in a clear synergistic effect manifested by the highly significant con A-induced mitogenic response. This finding is in agreement with those of Cohen and Fairchild [13, 14]. When cultured bone marrow cells were tested for their synergistic activity, it was clearly demonstrated the AEF-supplemented cultures (group 7), but not the control cultures (group 5), maintained their

synergistic potential. The results of the experiment presented in Figure 3 indicate the unlikelihood that residual AEF, bound to the cultured cells themselves, mediates the synergistic effect. Thus, cultures that were not supplemented with fresh AEF for the last 6 days of culture before the assay fully maintained levels of synergistic activity comparable to those of cultures that were continuously fed with AEF. Furthermore, additional data (not shown) indicate that synergistic activity is not due to the presence of AEF in the bone marrow cultures per se. Thus, AEF-supplemented cultures maintained under suboptimal conditions for 6 weeks (ie, in the absence of a pre-established adherent layer and in the presence of deficient HS), and consisting only of macrophages, were not capable of synergizing (despite the continuous presence of AEF). We observed a correlation between the presence of blasts and the ability to synergize in bone marrow cultures. Thus, inasmuch as the synergistic activity described above reflects the presence of pre-T cells in bone marrow populations, these results indicate that pre-T cells were present in AEF-supplemented, long-term murine bone marrow cultures.

## DISCUSSION

The results presented here demonstrate that AEF, a soluble mediator generated during the course of a short-term MLC of in vivo alloantigen-primed T cells, has profound effects on cultures of murine bone marrow cells. The effects of AEF were tested under suboptimal culture conditions, which excluded supportive effects of a pre-established adherent cell layer or HS, which constitute an essential feature of the long-term murine bone marrow culture system established by Dexter [2]. This resulted in a very rapid decline in cell growth and stem cell activity in the control cultures. Under such conditions, AEF had a dramatic effect on the cultures, manifested by its ability to support cell growth and proliferation (Tables I, II and Fig. 1) and stem cell activity (Table III and Fig. 2) for prolonged periods. By contrast, TCGF was devoid of any comparable activity on such cultures. AEF probably acted not just by enhancing the development of a supportive adherent layer but also by additional effects. Thus, AEF-supplemented cultures established with one inoculum of bone marrow cells contained higher numbers of cells and manifested higher levels of proliferative CFU-S and CFU-C activities than did conventional Dexter cultures established with 2 inocula of bone marrow cells. Furthermore, the addition of AEF to conventional Dexter cultures at the time of the second inoculation of bone marrow cells into culture (when an optimal adherent layer has already been established) resulted in general in an additive effect in terms of cellular growth and proliferation and CFU-C activity (Tables II and III). This indicates that AEF probably acts via different mechanisms than the microenvironment provided by the pre-established adherent layer, although part of its effect may be related to its ability to enhance the formation of such a layer.

Since we have found that AEF contains substantial levels of CSA, one has to consider the possibility that the effects of AEF in culture are, in fact, mediated by the contaminating CSA. However, several lines of evidence make this possibility unlikely. First, while AEF maintains cellular heterogeneity in the bone marrow cultures, including the presence of undifferentiated blasts, the addition of a conventional CSA source in the form of endotoxin-induced mouse serum [17] resulted in a rapid decline of the proliferative activity and terminal differentiation to macrophages (not shown). Second, when added to cultures of fresh bone marrow cells in microtiter wells, AEF had an extremely high mitogenic activity, as tested after 6 days in culture. Endotoxin-induced CSA, at comparable CSA titers to AEF (determined in a CFU-C assay), was by far less mitogenic [Altman et al, in preparation]

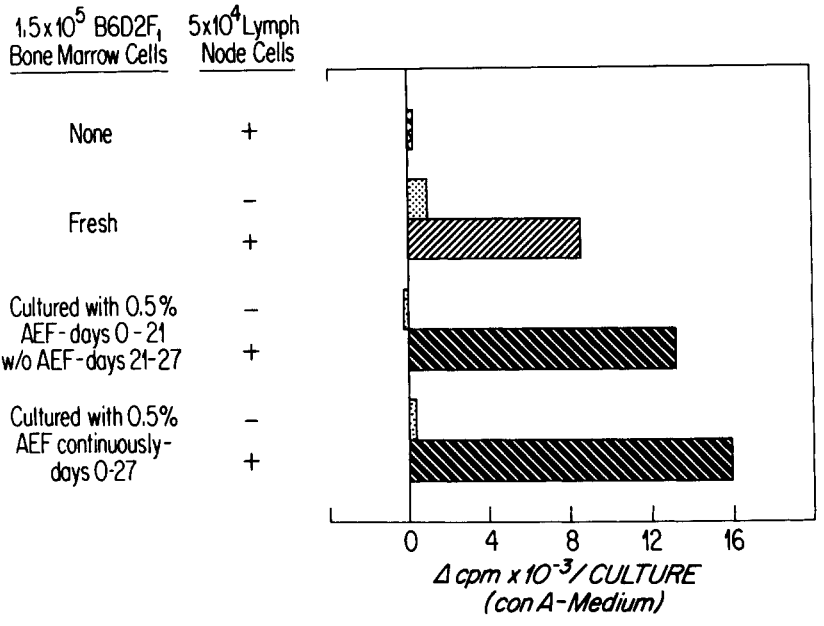


Fig. 3. Fresh or cultured bone marrow cells, as well as fresh lymph node cells, were cultured separately or mixed together, with or without 2.5 μg con A/ml. Cells from the AEF-supplemented cultures were washed twice to remove any free AEF. Levels of $^{3}$HTdR uptake in unstimulated cultures (<4117 cpm) were subtracted.

and, to the best of our knowledge, other conventional sources of CSA are not so highly mitogenic for bone marrow cells. Thus, although CSA may contribute in some way to the effects mediated by AEF, it is highly unlikely that CSA is exclusively responsible for the effects observed. We have recently obtained, by the use of several purification procedures, a semi-purified AEF preparation that is devoid of CSA activity, and we are now in the process of studying its effects in murine bone marrow cultures.

One of the most interesting aspects of this study is the possibility that AEF promotes the differentiation of lymphoid stem cells in this system. Indeed, we found that cells capable of synergizing with suboptimal numbers of mature T cells (derived from lymph nodes) in con A-induced mitogenic responses could be detected in the AEF-supplemented cultures for several weeks. Inasmuch as such cells were shown to be pre-T cells [13, 14], these results indicate that pre-T cells exist in the AEF-supplemented, but not in the control, cultures (Table IV and Fig. 3). This synergistic activity was not due to the maintenance of mature T cells in the bone marrow cultures since mature lymphocytes disappeared from the cultures within 7–10 days. Furthermore, anti-Thy-1.2 + C-treated bone marrow cells, cultured subsequently with AEF, retained their synergistic activity in this assay (not shown). In addition, cells derived from AEF-supplemented bone marrow cultures, and subsequently cultured with TCGF, gave rise to blasts with the typical appearance of T lymphoblasts, and we are now in the process of characterizing these cells.

It has been shown that cells derived from long-term Dexter cultures can reconstitute the lymphoid system of lethally irradiated recipients [7, 8] and, more recently, that such cultures contain cells possessing the enzyme terminal deoxynucleotidyl transferase (TdT

[18]), which is considered to be a marker of primitive lymphocytes or prothymocytes [19]. More recently, during the course of our own studies, it was reported that Thy-1-bearing cells could be found in the bone marrow cultures themselves [10] or following stimulation with TCGF [9, 11].

We reported recently that AEF has some unique biological effects on T lymphocytes, not mediated by other lymphokines such as TCGF. Thus, AEF is capable of inducing normal T lymphocytes, in the absence of exogenous antigens, to differentiate into activated T cells capable of lysing syngeneic target cells and responding in a secondary syngeneic mixed lymphocyte reaction [20, 21]. We postulated [20, 21] that these unique activities were associated with the presence of highly stimulatory Ia determinants on AEF molecules [22]. It has been found recently that progenitors of myeloid, erythroid, and megakaryocytic cells in the bone marrow bear Ia determinants [23; M.A.S. Moore, personal communication], and it is possible, therefore, that interactions between Ia determinants, known to be extremely important in the control and differentiation of immunological functions [24], are also involved in the biological effects of AEF on the bone marrow cultures.

Much additional work is necessary in order to analyze and understand the effects of AEF on the differentiation of bone marrow cells in culture. However, the results presented here, together with information concerning the effects of AEF on mature lymphocytes [20, 21], strongly suggest that AEF may prove to be a useful tool for the induction of lymphoid (and other) cells from progenitors in the bone marrow.

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