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# Differentiation of Murine Bone Marrow Stem Cells in vitro: Long-Term Growth Promoted by a Lymphocyte-Derived Mediator

Abstract. *In attempts to induce differentiation of lymphoid cells from hematopoietic stem cells in vitro, the effects of allogeneic effect factor on the growth of murine bone marrow cultures were studied. Allogeneic effect factor is a soluble mediator derived from mixed secondary murine leukocyte cultures. For several weeks it supported the growth of bone marrow cultures, as indicated by the maintenance of stem cell activity, cellular proliferation, and heterogeneity. Another lymphokine, T cell growth factor, did not. Pre-T lymphocytes could be detected in these cultures for several weeks.*

Bone marrow is a source of pluripotent hematopoietic stem cells that can give 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 been greatly aided by the development of a tissue culture system that supports the growth and differentiation of the pluripotent stem cells of mice (*2*) and tree shrews (*3*) for many weeks. This system has made it possible to induce myeloid cells, megakaryocytes, and erythrocytes to differentiate from their precursors in vitro (*4*). However, differentiation of lymphoid cells was not observed in these cultures until very recently, although cells derived from long-term cultures of murine bone marrow were found to be capable of reconstituting the lymphoid tissues of irradiated recipients and differentiating into immunocompetent lymphocytes in vivo (*5*). While it has not been possible to maintain stem cell activity in long-term cultures of human bone marrow (*3*), sustained growth of thymus-derived (T) lymphocytes from human bone marrow was accomplished by using conditioned medium of mitogen-stimulated T lymphocyte cultures containing the lymphokine T cell growth factor (TCGF) (*6*). The development of techniques for inducing immunocompetent lymphoid cells to differentiate from pluripotent stem cells in vitro would greatly facilitate studies of the generation of the immune system and its diversity during ontogenesis.

It was recently reported that Thy-1-bearing T lymphocytes can be generated in murine bone marrow cultures with or without a stimulus by TCGF (*7*). Such cells were generated in conventional Dexter-type cultures, which require that an adherent layer of bone marrow cells be established first, followed 2 to 3 weeks later by a second inoculum of fresh bone marrow cells, in order to obtain long-term maintenance of stem cells (*2*).

For several years our laboratory has studied an immunoregulatory soluble mediator termed allogeneic effect factor (AEF), which is a product of a secondary mixed lymphocyte culture of T cells previously activated to alloantigens in vivo (*8*). Allogeneic effect factor is a glycoprotein composed of two subunits (40,000 and 12,000 daltons) and possesses Ia antigen, which is determined by the I region of the murine *H-2* major histocompatibility complex, and $\beta_2$-microglobulin determinants (*9*). Biologically, AEF induces differentiation of bone marrow-derived (B) lymphocytes to mature antibody-secreting cells (*7, 8*) and also exerts proliferative and differentiating influences on T lymphocytes. It stimulates normal T cells to develop, in the absence of exogenous antigens, into cytotoxic T lymphocytes that preferentially lyse *H-2*-identical target cells and into effector cells capable of responding to irradiated syngeneic target cells in secondary mixed lymphocyte reactions (*10*). This is in contrast to TCGF, which acts on antigen- or mitogen-activated T cells but not on normal unstimulated T cells, and thus acts only as a second signal for such cells (*11*).

These unique effects of AEF on T lymphocytes (*10*) suggest that AEF-containing supernatants may affect the differentiation of murine hematopoietic stem cells in vitro, particularly along the lymphoid line. This report describes initial investigations of this possibility. (Hereafter, the term AEF refers to AEF-containing culture supernatants that also contain a heterogeneous collection of other molecules.)

Bone marrow cultures were established in Eagle's minimum essential medium (Dulbecco's modification) supplemented with 20 percent horse serum. To test the effects of AEF on the growth of bone marrow cells, we deliberately selected conditions that resulted in the rapid decline and loss of cell function in control cultures not supplemented with AEF. These suboptimal conditions in-

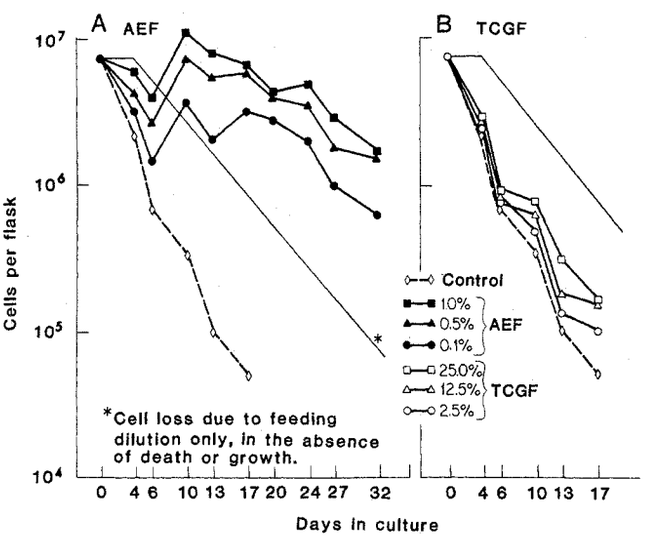


Fig. 1. Growth of $CAF_1$ bone marrow cells in the presence of AEF (A) and TCGF (B). Cultures were established with one inoculum of $7.5 \times 10^6$ fresh cells per flask with or without AEF or TCGF in the indicated concentrations. The AEF was prepared in serum-free conditions as described (*7*). The TCGF was derived from cultures of rat spleen cells ($2 \times 10^6$ cells per milliliter) stimulated for 2 days with 5 $\mu$g of concanavalin A in RPMI-1640 plus 5 percent fetal calf serum. Several lower concentrations of TCGF had no significant effect on the cultures (data not shown). Shown are the actual numbers of nonadherent cells recovered from the cultures on days of feeding.


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cluded the use of only one inoculum of bone marrow cells and of a deficient lot of horse serum that poorly supported stem cell activity in conventional Dexter cultures. Crude AEF or TCGF (derived from supernatants of concanavalin A-stimulated cultures of rat spleen cells) was added to these cultures at different concentrations when they were initiated and during subsequent feedings, which were performed twice weekly by removing half of the culture medium (including the nonadherent cells) and replacing it with fresh medium. At the time of each feeding, the recovered nonadherent cells were counted and assayed for (i) proliferation, assessed by the uptake of tritiated thymidine (*12*); (ii) stem cell activity, measured in vivo by the colony-forming unit spleen (CFUS) assay (*13*); and (iii) granulocyte-macrophage progenitors, measured in vitro by the colony-forming unit culture (CFUC) assay (*14*).

Figure 1 illustrates the effects of AEF and of TCGF on the growth of the murine bone marrow cells. The number of cells in the control and TCGF-supplemented cultures declined at a rate much higher than could be accounted for by simple dilution of the culture due to removal of half of the nonadherent cells at each feeding. This indicates that the cells stopped growing or died. In contrast, the AEF-supplemented cultures grew continuously for 32 days, at which time this experiment was terminated. Even then the harvested cells constituted 8 to 20 percent of the number of cells in the original inoculum. Since the cultures were diluted twofold at each of nine feedings, for a total dilution factor of 512, it is clear that AEF supported the continuous growth and proliferation of cells in these cultures.

The ability of AEF to support cell growth in bone marrow cultures established under suboptimal conditions is also reflected by the remarkably strong mitogenic effect of AEF on bone marrow cells. When added to cultures of fresh bone marrow cells in microtiter wells, AEF is highly mitogenic, as indicated by the dose-dependent increase in uptake of tritiated thymidine. In contrast, TCGF-containing culture supernatants were only weakly mitogenic, and even this was probably due to the presence of concanavalin A, the mitogen used to stimulate TCGF production; purified TCGF, devoid of the inducing mitogen, does not have any mitogenic effect on fresh peripheral blood lymphocytes (*15*). Serum from mice injected with endotoxin, a source of colony-stimulating activity (*16*), was moderately mitogenic.

Supplementing the bone marrow cultures with AEF not only increased cell numbers and proliferative activity (Figs. 1 and 2), it also promoted stem cell activity. The AEF-supplemented cultures had 120 units of CFUS-assayed stem cell activity and 262 units of CFUC-assayed activity per flask on days 17 and 24, respectively (*12*). In contrast, the control and TCGF-supplemented cultures were devoid of stem cell activity after 10 days.

In addition to studying the general effects of AEF on the growth and stem cell activity of bone marrow cells in vitro, we wished to ascertain whether any lymphoid stem cells or precursors are present in these cultures. We therefore determined whether cultured bone marrow cells can act synergistically with suboptimal numbers of mature syngeneic peripheral T cells (lymph node) in response to the T cell mitogen concanavalin A. Bone marrow cells respond poorly or not at all to concanavalin A stimulation while low numbers of peripheral T cells, which have the potential to respond to the mitogen, also do not do so—presumably because of limited quantities of an essential accessory cell. Cohen and Fairchild (*17*) demonstrated that, in this assay, pre-T cells in bone marrow can act as synergists with mature T cells, which then respond to concanavalin A by proliferating.

Figure 3 shows that AEF-supplemented cultures contain cells that are quite capable of such synergistic activity. Cultured separately, lymph node cells and fresh bone marrow cells responded only minimally to stimulation by concanavalin A, whereas combining the two populations resulted in a clear synergistic effect, in agreement with Cohen and Fairchild (*17*). Bone marrow cells cultured in the presence of AEF likewise possessed synergistic activity; cultures lacking AEF did not. It is ex-

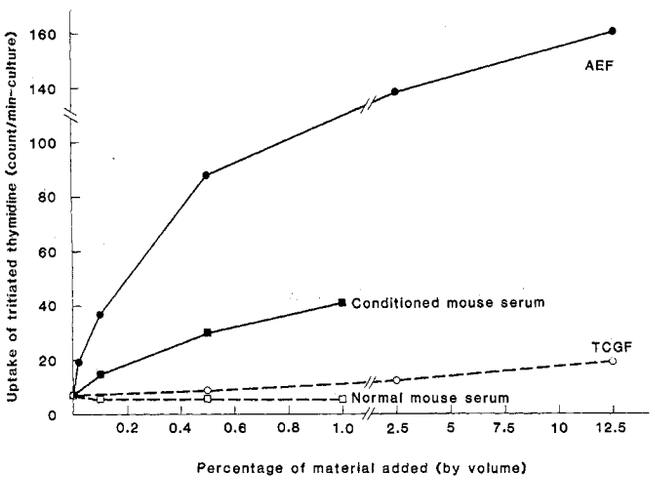


Fig. 2. Mitogenic effect of AEF on bone marrow cells. Fresh cells ($2 \times 10^5$) were cultured in triplicate in flat-bottom microtiter wells with various concentrations of AEF, TCGF, or endotoxin - conditioned mouse serum. The cultures were maintained for 6 days at 33°C in an incubator containing 10 percent $CO_2$, exposed to 1 $\mu$Ci of tritiated thymidine per well for the final 6 hours, and harvested on a Skatron cell harvester.

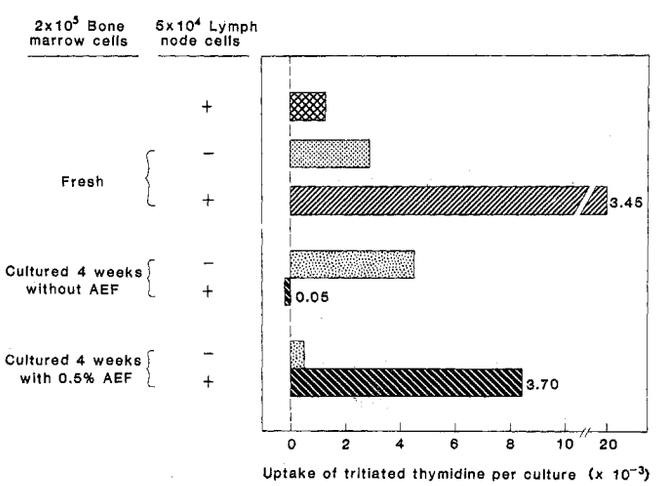


Fig. 3. Synergism between bone marrow and lymph node cells in the induction of mitogenic responses to concanavalin A. Fresh or cultured bone marrow cells ($2 \times 10^5$) were cultured alone or with $5 \times 10^4$ fresh syngeneic lymph node cells in the absence or presence of 2.5 $\mu$g of concanavalin A per milliliter. Uptake of tritiated thymidine was determined after a 6-hour exposure at the end of a 48-hour culture period. The bars represent net uptake after subtracting the levels of uptake (≤ 2100 count/min in the different groups) by unstimulated cultures. Differential cell counts on the day of the assay indicated that 100 percent of the cells in the control culture were macrophages and that the AEF culture comprised 67 percent macrophages, 26 percent granulocytes, and 7 percent undifferentiated immature cells.

tremely unlikely that the synergistic activity of the AEF-supplemented cultures is due to a carry-over of residual AEF bound to the bone marrow cells (*12*). It is noteworthy that Cohen and Fairchild reported that bone marrow cells usually lost their synergistic activity after being cultured for 2 days, the period during which residual bone marrow lymphocytes usually die in such cultures. Thus, pre-T cells were present in AEF-supplemented, long-term cultures of murine bone marrow and absent in cultures not supplemented with AEF.

An adherent cell layer, which provides an essential microenvironment for the maintenance of stem cell activity (*2*), was established much faster in the presence of AEF. This could indicate that AEF mediates its effects, in part at least, by inducing rapid development of the adherent layer. Such a layer could then provide the microenvironment necessary for the self-renewal and differentiation of residual stem cells. However, we have evidence to suggest that AEF has additional effects in these cultures. Thus, even when cultures are established under optimal conditions with two consecutive inocula of bone marrow cells (*2*), the addition of AEF together with the second inoculum (at a time when an optimal adherent layer has already been established from the first inoculum) has a cumulative effect, manifested by markedly higher cell numbers and increased proliferative and CFUC-assayed stem cell activities, compared to conventional Dexter cultures not supplemented with AEF (*12*).

Our studies are related to other recent studies in which the potential for lymphoid stem cell differentiation in vitro was investigated. It was shown that cells derived from long-term Dexter cultures can reconstitute the lymphoid system of lethally irradiated recipients (*5*) and that such cultures contain cells possessing the enzyme terminal deoxynucleotidyltransferase (*18*), considered to be a marker of primitive lymphocytes or prothymocytes (*19*). While our studies were in progress, it was reported that Thy-1-bearing cells could be found in the bone marrow cultures themselves or after exposure to TCGF (*7*).

As mentioned above, AEF-containing supernatants have some unique biological effects on T lymphocytes (*10*), which are associated with the presence on AEF molecules of Ia antigenic determinants encoded by the I region of the murine major histocompatibility complex. The Ia molecules are involved in cell-cell recognition and stimulation phenomena in the immune system (*20*). Progenitors of myeloid, erythroid, and megakaryocytic cells in human bone marrow bear the human equivalent of the murine Ia antigens (*21, 22*); it is possible, therefore, that interactions between Ia-containing molecules and/or cells are also involved in the biological effects of AEF on the bone marrow cultures.

The results presented here, together with information concerning the effects of AEF on mature lymphocytes (*8–10*), strongly suggest that AEF may prove useful for inducing lymphoid (and other) cells to differentiate from progenitors in the bone marrow. This could provide a system for analyzing the events that occur during the differentiation of immunocompetent lymphocytes from early stem cell stages and perhaps a more efficient means of culturing bone marrow for transplantation.

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## Dye Coupling and Possible Electrotonic Coupling in the Guinea Pig Neocortical Slice

Abstract. *Iontophoretic injection of the fluorescent dye Lucifer Yellow CH into single neurons of guinea pig neocortical slices resulted in the staining of more than one cell. Dye-coupled neuronal aggregates were found only in the superficial cortical layers and were often organized in vertical columns. Antidromic stimuli evoked all-or-none, subthreshold depolarizations in some superficial cells. These potentials were not eliminated by manganese and did not collide with spikes originating in the soma, suggesting that they arose from electrotonic interaction between superficial cortical neurons.*

Although interactions between neocortical neurons are believed to be mediated mainly by chemical synapses, there has been speculation that electrotonic coupling might also be present (*1*). Electrotonic transmission has been demonstrated in several mammalian subcortical structures (*2*), but there is little direct evidence for its occurrence in the cortex. However, gap junctions, the morphological substrates of electrotonic coupling, have been demonstrated in the motor cortex of primates (*3*). The fluorescent dye Lucifer Yellow CH crosses gap junc-


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