B CELL GROWTH FACTORS AND B CELL DIFFERENTIATION FACTOR FROM HUMAN T HYBRIDOMAS

Two Distinct Kinds of B Cell Growth Factor and Their Synergism in B Cell Proliferation*

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Discovery and use of T cell growth factor, interleukin 2 (IL-2),[1] have made it possible to immortalize functioning T cells and have facilitated studies on the activation, function, and antigen recognition of T cells (1–3). Human and murine neoplastic T cells secreting IL-2 have been particulary useful for the isolation and molecular characterization of IL-2 (4, 5).

Recent studies with successful long-term culture of murine or human B cells (6, 7) as well as with short-term B cell culture (8) have suggested the requirement of T cell-derived growth factor(s) for maintaining the continuous proliferation of activated B cells. Our studies with human leukemic B cells also showed that anti-immunoglobulin (anti-Ig) and growth factor were required for proliferation of B cells and three signals, anti-Ig, growth factor, and differentiation factor, were required for Ig production (9). However, little is known about B cell-specific growth factor (BCGF), and the lack of homogeneous BCGF preparations without IL-2 activity has hampered the establishment of nontransformed B cell lines.

Previously, we showed the successful establishment of stable human T hybridomas secreting several monoclonal immunoregulatory molecules involved in the proliferation and differentiation of T cells, such as IL-2 or killer helper factor(s) (10). In the present study, we used a similar method for obtaining homogeneous factors involved in the proliferation or differentiation of B cells. T hybridomas secreting BCGF or B cell differentiation factor (BCDF) have been established, and the results show the presence of molecular and functional heterogeneity in BCGF and the synergistic effect of two distinct kinds of BCGF on the proliferation of B cells. The results also show that BCDF involved in the final differentiation of B cells into Ig-producing cells is different from BCGF or IL-2. By using these monoclonal factors, activation process of B cells into Ig-producing cells are able to be dissected and reconstituted in vitro.

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[1] *Abbreviations used in this paper:* BCDF, B cell differentiation factor; BCGF, B cell growth factor; CEM-AG^R, azaguanine-resistant human T leukemic cell line, CEM; IL-1, IL-2, interleukin 1 and 2; PHA, phytohemagglutinin.

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Materials and Methods

Cells. An azaguanine-resistant human T cell line, CEM-AG^R, used for the establishment of T hybridomas, was described previously (10). An Epstein-Barr virus-transformed B cell line, CESS, which was able to differentiate into IgG-producing cells in the presence of BCDF, has also been described (11). An IL-2-dependent human cytotoxic T cell line was established by mixed lymphocyte reaction and maintained with IL-2 (12). Normal B cells were isolated from human peripheral blood lymphocytes (PBL) after rosette formation with sheep erythrocytes (SRBC) twice, and adherent cells were removed by dish adherence.

Establishment of T Hybridomas. The method used has been described (10). T cells isolated from human peripheral blood were stimulated with protein A (10 μg/ml, Pharmacia Fine Chemicals Div. Of Pharmacia Inc., Piscataway, NJ) for 3 d and hybridized with CEM-AG^R cells. Hybrid cells were selected in hypoxanthine, aminopterin, thymidine (HAT) medium and cloned by a limiting dilution method. Hybrid clones were incubated at a cell density of 1×10^6/ml in RPMI 1640 with 10% fetal calf serum (FCS) (Centaurus, Santa Ana, CA) for 48 h, cell-free supernatants were recovered, and the activities of BCGF, BCDF, and IL-2 were assessed.

Preparation of Conventional T Cell Factors. 1×10^6 purified peripheral T cells were stimulated with 0.1% protein phytohemagglutinin (PHA-P) for 48 h and partially purified by gel filtration on Sephadex G-100 in 0.5 M Nacl. Fractions of 15,000–25,000 mol wt were used, and the activity from 10^6 T cells was defined as 1 U.

Measurements of BCGF, BCDF, and IL-2 Activities. BCGF activity was assessed by using anti-μ-stimulated B cells or B cells recovered from PHA-induced B cell colonies. 5×10^4 nonadherent B cells in 0.2 ml medium (RPMI 1640 plus 10% FCS) were stimulated with 10 μg/ml of affinity-purified rabbit anti-human IgM coupled to Sepharose 4B in the presence or absence of varying concentrations of culture supernatants from T hybridomas. After 4 d culture, cells were pulsed with 1 μCi of [^{3}H]TdR for 5 h and the uptake of [^{3}H]TdR was measured.

For the assessment of the activity of the supernatants to maintain the continuous growth of B cells, B cells from PHA-induced B colonies were used. The method of B cell colony formation was exactly the same as described (13). 2×10^6 B cells in 2 ml McCoy's 5A modified medium with 15% FCS and 5×10^{-5} M 2-mercaptoethanol were co-cultured with the same number of X-irradiated autologous T cells and stimulated with PHA-P (1:1,600). After 3 d liquid culture, cells were harvested and transferred to soft agar cultures with double layers as described previously (13). After 7 d culture in soft agar, colonies were picked up and cells were pooled. 92% of pooled cells were positive for surface Ig by a direct immunofluorescent staining technique with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human Ig (Behringwerke AG, Marburg, Federal Republic of Germany), and 3.5% of the cells formed SRBC rosettes. To remove T cells, the pooled cells (2×10^5) were treated with 100 μl of the culture medium from anti-Leu-1-producing hybridomas, a gift of Dr. R. Evans (Memorial Sloan-Kettering Cancer Center, New York) and complement (1:4 diluted newborn rabbit serum). 1×10^3 B cells thus obtained from B colonies were incubated with 0.2 ml of supernatant from T hybrid clones for 3 d, and 0.5 μCi [^{3}H]TdR was pulsed for 6 h. All cultures were set up in triplicate.

BCDF activity in the supernatants was detected either by using CESS cells, which could differentiate into IgG-producing cells in the presence of BCDF (11), or Cowan I-stimulated normal B cells (14). 1×10^4 CESS cells were incubated with 200 μl of the supernatants from T hybridomas for 48 h and the number of IgG-producing cells was enumerated by reverse plaque assay using protein A-coupled SRBC and anti-γ antiserum. BCDF activity for normal B cells was measured by using Cowan I-stimulated normal B cells. 1×10^6 purified B cells in 1 ml culture medium were stimulated with 0.0025% *Staphylococcus aureus* Cowan I (Calbiochem-Behring Corp., La Jolla, CA). After 3 d culture, cells were harvested and 2×10^5 cells were cultured with 0.2 ml supernatants from T hybridomas for 2 d. IgG-producing cells were enumerated by reverse plaque assay.

Activity of IL-2 in the supernatants was measured by using an IL-2-dependent human cytotoxic T cell line as described previously (12).

Results

T Hybridomas Secreting BCGF. Over 100 hybrid clones were established by hybridization of protein A-stimulated peripheral T cells with CEM-AG^R and their culture

supernatants were tested for BCGF, BCDF, and IL-2 activities. As shown in Fig. 1, culture supernatants from three hybrid clones, 77-A, 94-C, and 98-F, augmented the proliferation of anti-IgM-stimulated B cells. The activity of the supernatants was proportional to their concentrations, and the diluted (1:124) culture supernatant obtained from 10^6 of 77-A cells showed BCGF activity comparable to that from 10^6 PHA-stimulated normal T cells, demonstrating that BCGF activity from 77 A cells was >100 times higher than that from PHA-stimulated normal T cells. The culture supernatants from the three hybrid clones, 77-A, 94-C, and 98-F, however, showed no IL-2 activity when tested with an IL-2-dependent human cytotoxic T cell line. Moreover, a hybrid clone 24-A, which had been shown to secrete IL-2 in our previous study (10), did not show BCGF activity. Taken collectively, these results clearly show that the molecules distinct from IL-2 were involved in the proliferation of anti-Ig-activated B cells.

Existence of Two Distinct BCGF and Their Synergism. To study whether BCGF detected by anti-IgM-stimulated B cells could maintain the continuous growth of B cells, the following experiments with B cells obtained from PHA-induced B cell colonies were carried out. 1 $\times 10^3$ B cells obtained from PHA-induced B cell colonies were cultured with 0.2 ml of supernatants from hybrid clones and proliferation was assessed by [^{3}H]TdR uptake. As shown in Figs. 1 and 2, the culture supernatant from 77-A clone induced an increase in [^{3}H]TdR uptake. On the other hand, the culture supernatant from 94-C clone, which augmented the proliferation of anti-IgM-stimulated B cells, did not show any activity to induce the proliferation of colony-forming B cells. The result suggested the presence of two distinct kinds of BCGF, i.e., one

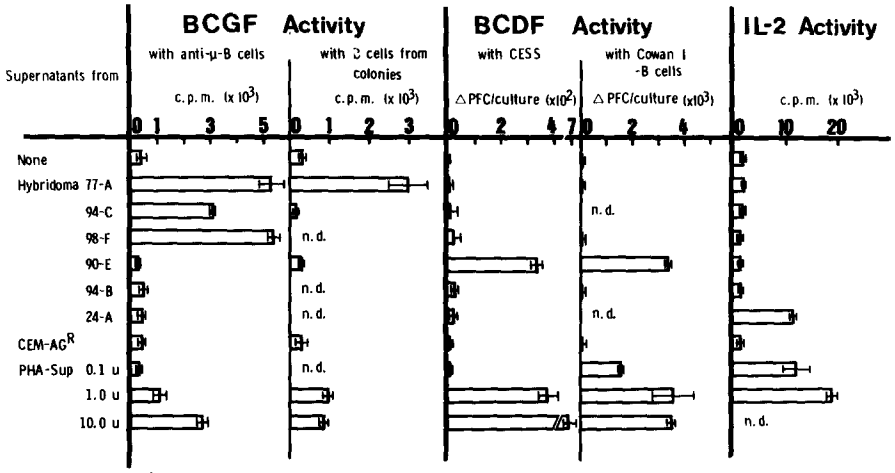

FIG. 1. BCGF, BCDF, and IL-2 activity in the culture supernatants of human T hybrid clones. BCGF activity was assessed by using anti-μ-stimulated human peripheral B cells or B cells recovered from PHA-induced B colonies. BCGF activity in the 1:4-diluted culture supernatants is shown (mean ± SE of triplicate cultures). BCDF activity in the supernatants was detected by using either CESS cells or Cowan I-stimulated normal B cells. IgG producing cells were enumerated by reverse plaque assay. Δ PFC/culture = PFC/culture (factors[s] added) − PFC/culture (no factor[s]). Mean ± SE of duplicate cultures was shown. Background PFC without factors were 217 and 1,185 in CESS cells and Cowan I-stimulated B cells, respectively. Semipurified PHA supernatant obtained from 10^6 T cells was defined as 1 U.

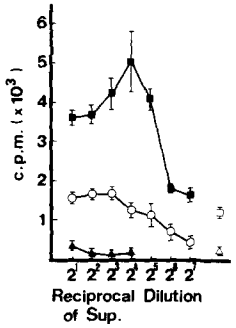

FIG. 2. Synergistic effect of two different BCGF on the continuous proliferation of B cells. Colony-forming B cells were obtained from PHA-induced B colonies as described in Materials and Methods. 1,000 B cells were suspended in 0.2 ml of varying concentrations of the supernatants from 77-A cells (○), 94-C cells (▲), or in the mixture of the varying concentrations of 77-A supernatants and twofold dulution of 94-C supernatants (■). After 3 d culture, 0.5 μCi [^{3}H]TdR was pulsed for 6 h and the uptake of [^{3}H]TdR was measured. The activity of the supernatant from PHA-stimulated normal T cells (□) or the supernatant from CEM-AG^R cells (△) was also measured. All cultures were done in triplicate and the mean ± SE is shown.

(77-A) that was effective on both anti-Ig-stimulated B cells and colony-forming B cells, and another (94-C) effective only on anti-Ig-stimulated B cells.

The most interesting finding was the synergistic effect of the supernatants from 77-A and 94-C cells on the proliferation of colony-forming B cells. Although the supernatant from 94-C did not induce any proliferation of colony-forming B cells, its addition to the supernatant from 77-A clone tremendously augmented the activity of 77-A supernatant to induce proliferation of colony-forming B cells. The maximum proliferation of colony-forming B cells was observed when 16-fold-diluted supernatant of 77-A cells was mixed with 2-fold-diluted supernatant of 94-C cells as shown in Fig. 2. The addition of the supernatant of 90-E cells, which showed BCDF activity, to that of 77-A cells did not show any augmentation of [^{3}H]TdR uptake by colony-forming B cells. The addition of the 94-C supernatant to that of 77-A cells also showed the synergistic effect on the proliferation of anti-μ-stimulated B cells. However, the effect was not as prominent as that observed in colony-forming B cells, because the supernatant of 94-C cells could itself induce the proliferation of anti-μ-stimulated B cells. The result demonstrated the presence of two different kinds of BCGF and their synergistic effect on the proliferation of a certain subset of B cells or of B cells in a certain activation stage.

Howard et al. (15) reported that IL-1 from macrophages acted on anti-Ig-activated B cells to induce proliferation in a synergistic manner with BCGF. To exclude the possibility that either clone 77-A or 94-C were hybridomas between macrophages and CEM-AG^R and secreted IL-1, surface phenotypes of the hybrid clones were analyzed using a human monocyte-specific monoclonal antibody (16) or a monoclonal anti-DR antibody (10). A macrophage cell line, U937, or a B cell line, CESS were used as a positive control. Monocyte-specific antigens or DR antigens were not detected on either 77-A or 94-C cells, suggesting that 77-A or 94-C cells were not hybridomas between macrophages and a T cell line. Moreover, the supernatant of 94-C cells did not augment the proliferation of Con A-stimulated murine thymocytes, excluding the possibility that hybridomas secreted IL-1.

BCDF from T Hybridomas. A B lymphoblastoid cell line, CESS, or Cowan I-stimulated normal B cells were used for the assessment of BCDF activity in the culture supernatants. CESS cells have been shown to differentiate directly into IgG-producing cells without any requirement of cell division when BCDF is provided (11). As shown in Fig. 1, the culture supernatant from clone 90-E can induce an increase in IgG-producing cells in CESS cells or in Cowan I-stimulated normal B cells. The activity to induce IgG in CESS cells was dose dependent, but 1:8 diluted supernatant lost the activity. When the supernatant (1:2 dilution) was added to Cowan I-stimulated normal B cells, both IgM- and IgG-producing cells were induced, i.e., 705 IgM producing cells and 3,037 IgG producing cells per 10^5 cultured cells. Thus, the effect of 90-E supernatant was not isotype specific. The result indicated that the culture supernatant from clone 90-E was able to induce final differentiation of B lymphoblastoid cells as well as of normal B cells into Ig-secreting cells. The same culture supernatant, however, showed no BCGF or IL-2 activity. The culture supernatants from 77-A, 94-C, and 98-F, which showed BCGF activity, did not induce any increase in IgG-producing cells in CESS cells or Cowan I-stimulated normal B cells. From these results, it appears that BCGF is not involved in the final differentiation of B

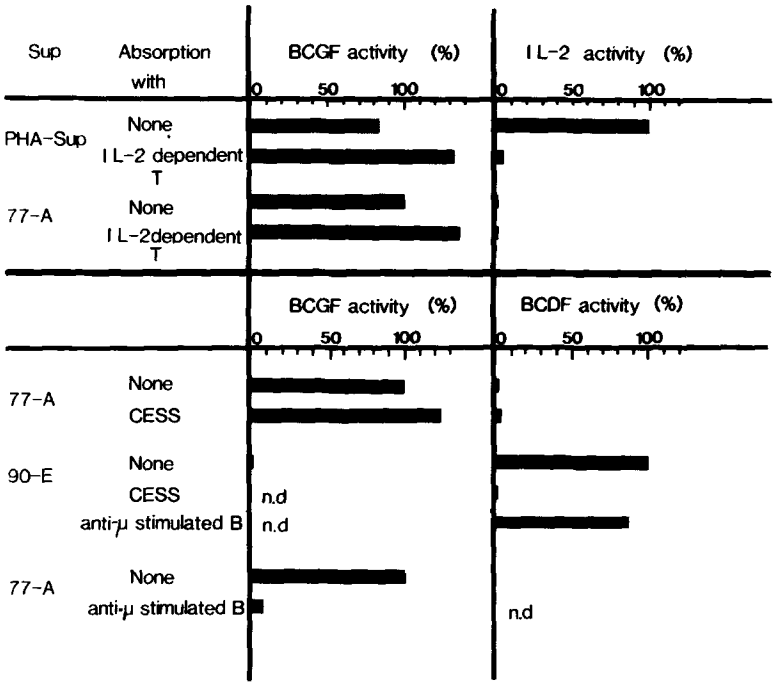

FIG. 3. Absorption of BCGF or BCDF activity with IL-2-dependent T cells, CESS cells, or anti-μ-stimulated B cells. BCGF, BCDF in 1 ml of the culture supernatant from 1×10^6 of 77-A or 90-E cells, or IL-2 including 5 U of activity in 1 ml of PHA-supernatant were absorbed twice with 1×10^7 IL-2-dependent T cells and 2×10^7 CESS cells for 2 h at 4°C, or once with 7×10^6 anti-μ-B cells for 4 h at 4°C. After absorption, activity of 1:20 diluted BCGF, 1:2 diluted BCDF, or 1:5 diluted IL-2 preparation was assessed with anti-μ-stimulated B cells, CESS cells, or IL-2-dependent T cells, respectively. BCGF activity in the 1:20 diluted nonabsorbed supernatant from 77-A cells, BCDF activity in the 1:2 diluted supernatant from 90-E cells, and 1 U of IL-2 activity were shown as 100%.

cells and that the factor responsible for the differentiation of B cells does not induce their proliferation.

Absorption of BCGF or BCDF Activity by the Target Cells. We confirmed that BCGF was a different molecule than IL-2 or BCDF using absorption experiments. As shown in Fig. 3, 1×10^7 IL-2-dependent T cells could absorb 1 U of IL-2 activity from 10^6 PHA-stimulated T cells, whereas the same number of dependent T cells did not remove any BCGF activity in PHA supernatant or 77-A supernatant. BCDF activity from 10^6 90-E cells was completely removed by the absorption with 2×10^7 CESS cells, but the same absorption procedures did not remove any BCGF activity in 77-A supernatant. On the other hand, BCGF activity from 10^6 77-A cells or 94-C cells was removed by the absorption with 0.7×10^7 of anti-μ-stimulated B cells. The same number of anti-μ-stimulated B cells, however, did not absorb BCDF activity in 90-E supernatant. The result showed the presence of acceptors specific for each factor on their target cells.

Discussion

The present study showed the successful establishment of human T hybridomas secreting monoclonal immunoregulatory molecules involved in the proliferation or differentiation of B cells. The presence of the B cell-specific growth factor distinct from IL-2 has been suggested in murine and human system (7, 8). Howard et al. (8) demonstrated that a phorbol myristate acetate (PMA)-stimulated murine thymoma, EL-4, secreted BCGF that could be separated from IL-2 by molecular sieving or by absorption experiments. In the human system, discrepancies in the induction of proliferation of T and B cells in the culture supernatants of mitogen-stimulated T cells suggested the presence of the B cell-specific growth factor (7). More recently, absorption experiments (17) or chromatographical separation (18) have indicated that BCGF and IL-2 are different molecules. However, it has not yet been demonstrated that BCGF or BCDF without IL-2 is sufficient to induce proliferation or final differentiation of B cells, since monoclonal BCGF or BCDF preparations without IL-2 or other lymphokine activity have not yet been obtained. In this case, T hybridomas secreting monoclonal immunoregulatory molecules proved powerful tools, and the results herein clearly show that BCGF distinct from IL-2 or BCDF was present and could induce B cell proliferation without any contamination of IL-2.

Moreover, the present result with 77-A and 94-C cells showed the presence of the functional heterogeneity of BCGF and their synergism in the induction of B cell proliferation. In another experiment in our laboratory (19), it was also demonstrated that an IL-2-dependent human helper T cell clone secreted BCGF with the molecular weight of 50,000, and this BCGF showed an synergistic effect, with the 20,000-mol wt-BCGF, on the proliferation of anti-μ-stimulated B cells. These results indicate the presence of at least two different kinds of BCGF. At moment, the cellular or molecular mechanism of the synergism between the two BCGF is not known. A possibility is that one BCGF can act on B cells in G_0 phase and activate them into G_1 phase, while another BCGF might push B cells in G_1 phase into S phase. If this is the case, those BCGF with distinct functions will provide a powerful strategy for the establishment of nontransforming B cell lines.

Absorption experiments shown in Fig. 3 clearly demonstrated the strict specificity between T cell-derived factors and their target cells. IL-2-dependent T cells could

absorb IL-2 activity but not BCGF activity, and BCGF activity was only absorbed with anti-μ-stimulated B cells, demonstrating the presence of the growth factor(s) specific for T or B cells. CESS cells, which may represent the final differentiation stage of B cells, could absorb BCDF but not BCGF activity, whereas anti-μ-stimulated B cells could not absorb BCDF activity, suggesting that acceptors for BCGF or BCDF may be expressed only in a certain activation stage of B cells. By the presence of such strict specificity between acceptors and factors and the temporal expression of acceptors, the specificity of the immune responses may be maintained even if they were regulated by antigen-nonspecific factors.

In the present experiments, both growth and differentiation factors for B cells were obtained from monoclonal T cell hybridomas. In our preliminary experiment, anti-Ig, BCGF, and BCDF from hybrid clones could induce Ig production in leukemic B cells, demonstrating that these three signals could induce proliferation and differentiation of a certain subset of B cells or B cells in a certain activation stage into Ig-producing cells without help from MHC-restricted T cells. As our BCGF or BCDF did not have any IL-2 activity, we can analyze the direct effect of the factors on B cells without worrying about the contamination of T cells in the B cell fraction. Thus, by using those monoclonal factors, we will be able to dissect the activation process of B cells and to analyze the signals required for each step of the activation process.

Summary

Human T hybridomas secreting B cell growth factors (BCGF) and B cell differentiation factor (BCDF) have been established. Hybrid clones 77-A, 94-C, and 98-F secreted BCGF that induced proliferation of anti-IgM-stimulated normal B cells. The culture supernatant from 77-A cells could also maintain continuous proliferation of colony-forming B cells, but the factor from 94-C could not. The addition of the supernatant from 94-C cells to that from 77-A cells, however, synergistically augmented the proliferation of colony-forming B cells, demonstrating the existence of two distinct kinds of BCGF and the synergism between them. These supernatants, however, showed no interleukin 2 (IL-2) or BCDF activity. A hybrid clone, 90-E, secreted BCDF. The culture supernatant induced Ig production in Cowan I-stimulated normal B cells or in a transformed B cell line, CESS. However, the supernatant had no BCGF or IL-2 activity. Anti-Ig-stimulated B cells, but not IL-2-dependent T cells, absorbed BCGF activity and CESS cells absorbed BCDF activity but not BCGF activity in the culture supernatants from T hybridomas. Taken collectively, the results demonstrated that IL-2, BCGF, and BCDF were different molecules and acceptors specific for the each molecule are present on the each target cell.

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