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Immunology

Development of a human T-cell hybridoma secreting separate B-cell growth and differentiation factors

(T-cell hybrid/B-cell proliferation/terminal differentiation/costimulation)

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ABSTRACT **A cloned human T-cell hybridoma ($7D_5$) secreting B-cell growth factor (BCGF) and B-cell differentiation factor (BCDF) was established. Supernatant from this hybrid was capable of maintaining proliferation in anti-IgM-activated normal human B cells. In addition, the hybridoma supernatant induced differentiation and antibody secretion in *Staphylococcus aureus* Cowan I-stimulated B cells. No interleukin 2 was present in supernatant from this hybridoma. Molecular size of the hybridoma-derived BCGF and BCDF was determined by gel filtration chromatography. BCGF activity was present in the 20-kDa fractions, and BCDF activity eluted in the 30- to 35-kDa fractions. The isoelectric points of the factors, determined by chromatofocusing, were 6.6 for BCGF and 5.9 for BCDF. Finally, absorption experiments were performed using specific target cells. Phytohemagglutinin-stimulated T-cell blasts did not remove either BCGF or BCDF activity. Anti-IgM-activated B cells absorbed BCGF but not BCDF. In contrast, CESS cells removed BCDF but not BCGF. Thus, a human T-cell hybridoma secreting two distinct B-cell lymphokines was developed. Further immunochemical and functional studies of these immunoregulatory molecules should greatly enhance our understanding of the regulation of human B-cell function in normal and disease states.**

The production and characterization of immunoregulatory factors acting on B lymphocytes has been an area of recent interest and investigation (1–6). The technology of cloning and hybridization of human and murine T cells has provided renewable sources of monoclonal B-cell-specific lymphokines (7–10). These factors have proven to be powerful tools in delineating the complex mechanisms involved in the regulation of human immune responses.

In a previous report, we described the development of a human T-cell hybridoma ($2B_{11}$) secreting monoclonal B-cell growth factor (BCGF) (11). The hybridoma supernatant was devoid of both interleukin 2 (IL-2) and B-cell differentiation factor (BCDF) activities. The hybridoma-derived BCGF was capable of maintaining proliferation in normal and human B cells that had been activated by *Staphylococcus aureus* Cowan strain I (SAC) or anti-Ig (12). However, this factor was not able to induce differentiation in preactivated B cells. This finding correlated with earlier studies by ourselves and others that showed Ig production by B cells to be dependent on a sequential presentation of an activation signal, growth factor, and differentiation factor (13–15).

In the present study, we report the development of a human T-cell hybridoma secreting BCGF and BCDF in the absence of IL-2. Supernatant from this hybridoma is shown to be capable of providing both growth and differentiation signals to activated peripheral blood B cells. The B-cell factors secreted by this hybridoma have separate and distinct molecular sizes and isoelectric points, as determined by gel filtration chromatography and chromatofocusing. Finally, absorption studies show that BCGF and BCDF activities are removed by different target cells, supporting the fact that they are separate molecules acting on B cells at different stages of the cell cycle.

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MATERIALS AND METHODS

Cell Suspensions. Peripheral blood lymphocytes from normal human subjects were separated by centrifugation over Ficoll-Hypaque gradients (Pharmacia). T lymphocytes were separated by rosette formation with aminoethylisothiouronium bromide-treated sheep erythrocytes (16). The T-cell-enriched population was suspended in complete medium, consisting of RPMI 1640/glutamine (2 mM) (Flow Laboratories, Rockville, MD)/gentamicin (80 μg/ml) (Schering)/10% heat-inactivated fetal calf serum (GIBCO). T cells were stimulated with phytohemagglutinin (10 μg/ml) (Sigma) for 48 hr in 24-well culture plates (Costar 3524, Data Packaging, Cambridge, MA) before hybridization. Normal B cells were isolated from peripheral blood lymphocytes by a double aminoethylisothiouronium bromide rosette procedure with sheep erythrocytes. Monocytes were removed by adherence to glass beads.

Hybridization. T-cell hybridomas were established as described (11). Normal T cells were obtained from human peripheral blood lymphocytes and stimulated with mitogen as described above. These activated T cells were fused with CEM-6 cells (an azaguanine-resistant mutant of human T-lymphoblastoid origin) in a 2:1 ratio in the presence of polyethylene glycol and were suspended in 96-well, flat-bottomed microtiter plates (Costar 3596). Hybrids were selected in hypoxanthine, aminopterin, thymidine medium and cloned by the method of limiting dilution. Cell-free supernatants were generated from 48-hr cultures of 1×10^6 hybrid cells in complete medium.

T-Cell Factors. Sources of factors used as positive controls in the lymphokine assays were as follows: BCGF secreted by a human T-cell hybridoma ($2B_{11}$); IL-2-containing supernatant from the MLA 144 gibbon line; BCDF-containing supernatant generated from a 48-hr mixed lymphocyte reaction coculture of two unrelated donors.

BCGF Assay. Assay of T-cell hybridoma supernatants for BCGF activity was performed in an anti-μ assay system (12). Briefly, the B-cell-enriched fraction was suspended in complete medium and cultured with $F(ab')_2$ fragment goat anti-μ-specific antibody (15 μg/ml) (Cappel Laboratories, Coch-

Abbreviations: BCDF, B-cell differentiation factor; BCGF, B-cell growth factor; IL-2, interleukin 2; SAC, *Staphylococcus aureus* Cowan strain I.

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ranville, PA), 25% (vol/vol) BCGF, or anti-μ antibody and BCGF. B cells (5×10^4) in a volume of 0.2 ml were added to each well of flat-bottomed microtiter plates (Costar 3596). Cultures were incubated for 72 hr at 37°C in 7% CO_2/95% air with 100% humidity, and 1 μCi of [^{3}H]thymidine (1 Ci = 37 GBq) was added over the last 16–20 hr of culture. Cells were harvested using a Titertek cell harvester (Flow Laboratories). Standard liquid scintillation counting techniques were used to measure incorporation of [^{3}H]thymidine.

BCDF Assay. BCDF activity was assayed as described (17). The B-cell-enriched population, purified as described above, was cultured for 72 hr with 0.001% SAC (vol/vol) in complete medium in flat-bottomed microtiter plates. At the end of the culture period, these activated B cells were washed and resuspended at 3×10^4 cells per well in 0.2 ml of complete medium. Supernatants containing BCDF were added (15%, vol/vol) to the cultures, and the number of plaque-forming cells per well was determined by a reverse hemolytic plaque assay at the end of an additional 72 hr of culture.

IL-2 Assay. Cell-free supernatants from human T-cell hybridomas were assayed for IL-2 activity as described (18). Proliferation of IL-2-dependent HT-2 cells was measured by [^{3}H]thymidine incorporation and used as the index of IL-2 activity in the supernatants.

Gel Filtration Chromatography. One hundred milliliters of $7D_5$ hybridoma supernatant was concentrated to 10 ml using an Amicon YM10 membrane. This concentrated supernatant was applied onto a Sephadex G-100 column (1.6×90 cm) and eluted with phosphate-buffered saline (pH 7.4). Fractions were assayed for BCGF and BCDF activities. Protein concentration was measured by absorbance at 280 nm.

Chromatofocusing. Analysis of the isoelectric point of BCGF and BCDF was done by chromatofocusing (19). Briefly, the active fractions of $7D_5$ hybridoma supernatant were dialyzed against 0.025 M Tris acetate buffer (pH 8.3) and applied onto a PBE-94 column (1.0×38 cm) (Pharmacia). The column was eluted with 400 ml of solution composed of 7% (vol/vol) Polybuffer 74 and 3% (vol/vol) Polybuffer 96 (Pharmacia), adjusted to pH 5.0 at a flow rate of 25 ml/hr.

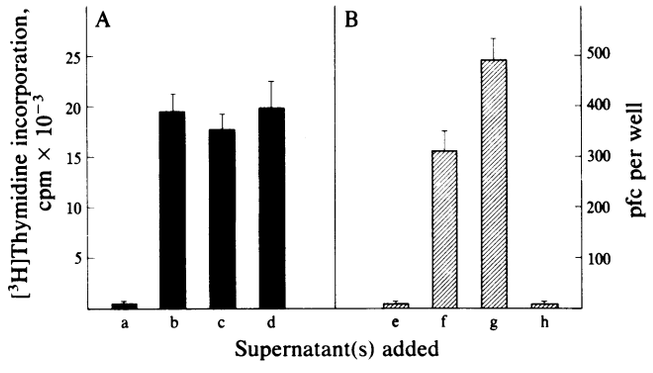

FIG. 1. (*A*) BCGF activity in the supernatant from hybrid clone $7D_5$. Anti-μ-stimulated B cells (5×10^4 cells per well) were cultured with supernatants (25%, vol/vol) derived from the CEM-6 cell line $2B_{11}$, BCGF-producing human T-cell hybridoma, or $7D_5$ human T-cell hybridoma. Cultures were maintained for 72 hr before proliferation was measured by [^{3}H]thymidine incorporation. (*B*) BCDF activity in the supernatant from hybrid clone $7D_5$. B cells were activated by 0.001% SAC (vol/vol) for 72 hr, washed, and resuspended (3×10^4 cells per well). The cells were cultured with supernatants (15%, vol/vol) from the CEM-6 cell line, a BCDF-containing mixed lymphocyte reaction supernatant, BCGF-containing supernatant from hybridoma clone $2B_{11}$, or the $7D_5$ hybridoma clone. The number of plaque-forming cells (pfc) per well was determined after an additional 3 days of culture. Data represent the mean (±SEM) of triplicate experiments. Bars are labeled as follows: a and e, CEM-6; b and h, $2B_{11}$; c and g, $7D_5$; d, $7D_5$ and $2B_{11}$; f, MLR.

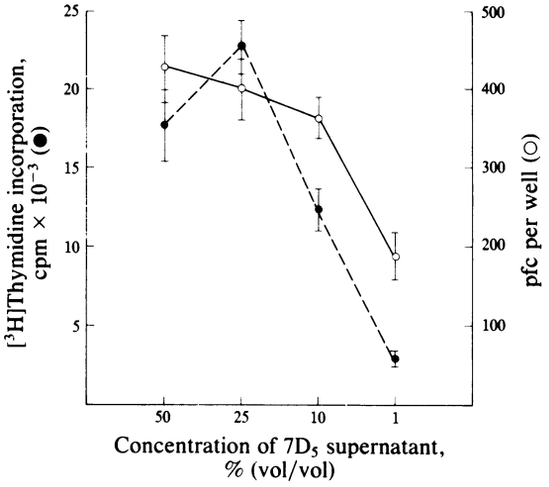

FIG. 2. Effect of dilution of $7D_5$ hybridoma supernatant on BCGF and BCDF activities. Assay systems used in this experiment were as described in Fig. 1. Activated human B cells were cultured with $7D_5$ supernatant at various concentrations for 72 hr. Proliferation and differentiation of the B cells were then measured. Background response was 475 ± 53 cpm for proliferation and 22 ± 6 plaque-forming cells (pfc) for differentiation. Data represent the mean (±SEM) of triplicate experiments.

Two-milliliter-fraction samples were collected as chromatofocusing was carried out over a pH range of 8 to 5. These fractions were assayed for BCGF and BCDF activities.

RESULTS

Screening of T-Cell Hybridomas. Three T-cell hybridization experiments were performed, and 56 wells from an initial number of 600 grew to confluence after 6 wk. Cell-free supernatants from these hybridoma cultures were assayed for BCGF, BCDF, IL-2, and γ-interferon activities. One hybrid ($7D_5$), whose supernatant contained both BCGF and BCDF activities, was successfully cloned but has required frequent recloning to maintain its activity. As shown in Fig. 1, BCGF activity in $7D_5$ supernatant was compared to the BCGF-secreting human T-cell hybrid $2B_{11}$. The BCGF activities in the supernatants of the two hybridomas were comparable and not synergistic when 12.5% (vol/vol) final concen-

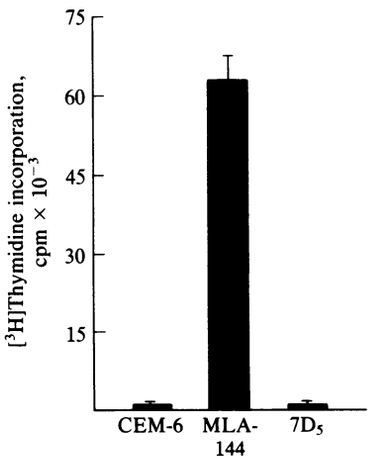

FIG. 3. No IL-2 activity was detected in $7D_5$ hybridoma supernatant. IL-2-dependent HT-2 cells (4×10^3 cells per well) were cultured with supernatants (10%, vol/vol) from the CEM-6 cell line, MLA-144 IL-2-producing gibbon cell line, or the $7D_5$ hybrid clone. Cultures were maintained 24 hr, and proliferation of the HT-2 cells was measured by [^{3}H]thymidine incorporation. Data represent the mean (±SEM) of triplicate experiments.

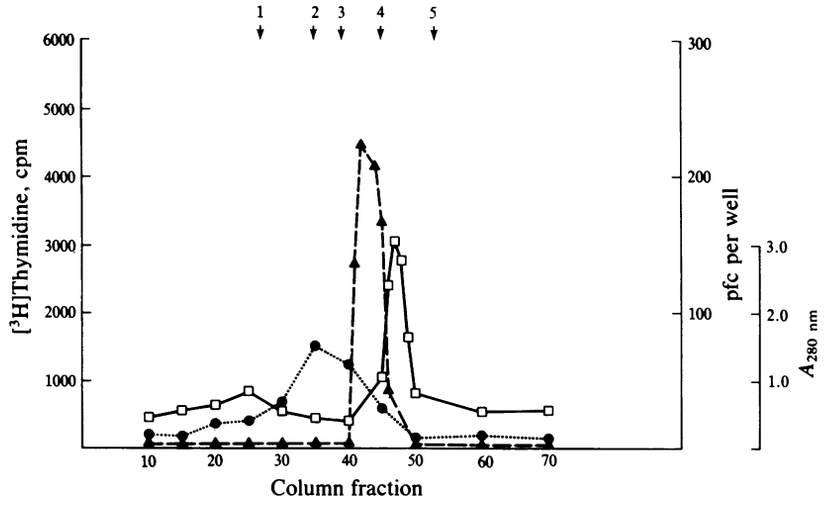

FIG. 4. Sephadex G-100 gel filtration chromatography of $7D_5$ supernatant. Concentrated hybridoma supernatant was applied onto a Sephadex G-100 column (1.6 × 90 cm). The column was eluted with phosphate-buffered saline (pH 7.4), and 1.5-ml fractions were collected. Fractions were assayed for BCGF and BCDF activity. The results are expressed as cpm of [^{3}H]thymidine incorporation (□) and as the number of plaque-forming cells (pfc) per culture well (▲), respectively. Protein concentration of the applied supernatant was measured by absorbance at 280 nm (●). The elution profile of the proteins used for column calibration is represented by numbered arrows. 1, Ferritin (540 kDa); 2, bovine serum albumin (67 kDa); 3, ovalbumin (45 kDa); 4, chymotrypsinogen (25 kDa); 5, cytochrome *c* (12.5 kDa).

tration of each supernatant was used. No BCGF activity was evident in the CEM-6 supernatant. In addition, supernatant from $7D_5$ was capable of inducing differentiation in SAC-stimulated B cells. When compared to a mixed lymphocyte culture supernatant known to contain BCDF activity, $7D_5$ supernatant was found to be more potent. As shown in Fig. 2, the BCGF and BCDF activities were proportional to the concentration of the hybridoma supernatant. Compared to IL-2-containing MLA 144 supernatant, supernatant from hybrid $7D_5$ failed to support the proliferation of the IL-2-dependent T-cell line HT-2 (Fig. 3). These data confirm the absence of IL-2 in the hybridoma supernatant. In addition, no γ-interferon activity was present in $7D_5$ supernatant, as measured in a commercial viral inhibition assay.

Biochemical Characterization. The molecular sizes of BCGF and BCDF in the $7D_5$ supernatant were determined by gel filtration chromatography. Hybridoma supernatant was concentrated 10-fold and applied onto a Sephadex G-100 column. Fractions were collected and assayed for BCGF and BCDF activities (Fig. 4). BCGF activity eluted in the 200-kDa fraction, consistent with our earlier report (11). BCDF activity was confined to fractions of 30–35 kDa.

After gel filtration, active fractions were applied to a chromatofocusing column (Fig. 5). BCDF activity was eluted in fractions with a pI of 5.9. In contrast, BCGF activity was present in separate fractions with a pI of 6.6. The results of gel filtration chromatography and chromatofocusing indicate that $7D_5$ supernatant contains two biochemically distinct molecules with different functional activities.

Absorption Studies. Absorption experiments were carried out to confirm that separate factors were responsible for BCGF and BCDF activity in $7D_5$ supernatant. As shown in Fig. 6, 1×10^7 phytohemagglutinin-stimulated T-cell blasts did not remove BCGF or BCDF activity from the hybridoma supernatant. Incubation of supernatant with 1×10^7 anti-μ-activated B cells removed BCGF activity but did not decrease BCDF activity. In contrast, 1×10^7 CESS cells removed all BCDF, but these cells did not absorb BCGF activity. These data demonstrate selective absorption of either BCGF or BCDF by specific target cells.

DISCUSSION

The present study reports the successful establishment of a human T-cell hybridoma secreting both growth and differentiation factors capable of acting on normal human B cells. Biochemical and absorption data show that the functional activities in the hybridoma supernatant reside in separate molecules.

Earlier studies have shown that activated human B cells require specific and discrete signals for proliferation and subsequent differentiation to occur (14, 17). These signals are provided by BCGF and BCDF, respectively. We recently reported the development of a human T-cell hybrid secreting BCGF (11) and described the effect of this factor on B cells in various states of activation (12, 20). However, our efforts to investigate B-cell differentiation were hampered by the lack of availability of BCDF. Previous studies had reported T-cell hybridomas secreting BCDF (10). Therefore, we developed a number of human T-cell hybridomas and screened them for BCDF activity. One hybridoma, $7D_5$, was

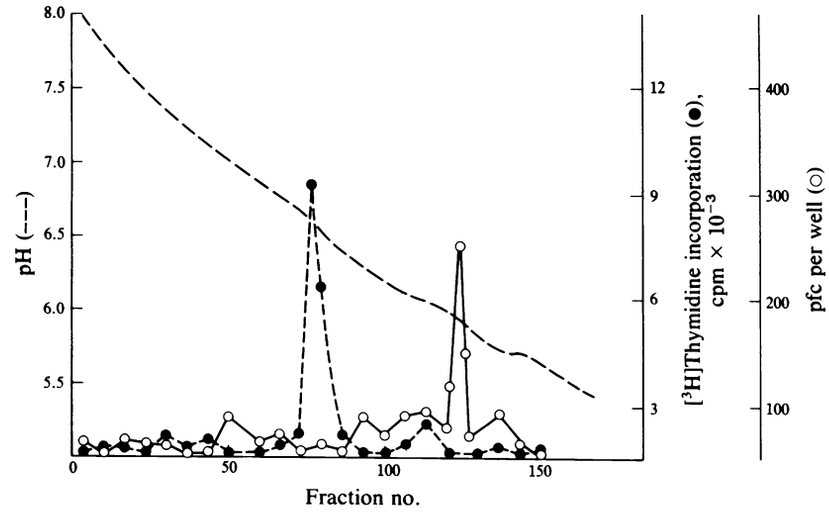

FIG. 5. pI determination of $7D_5$, BCGF, and BCDF by chromatofocusing. Fractions of $7D_5$ supernatant containing BCGF or BCDF activity were pooled and dialyzed against 0.025 M Tris acetate buffer (pH 8.3). These pooled fractions were separately applied onto a PBE-94 column (1.0 × 38 cm) and eluted with 400 ml of buffer solution [7% (vol/vol) Polybuffer 74 and 3% (vol/vol) Polybuffer 96] adjusted to pH 5.0. Fractions were collected and dialyzed against phosphate-buffered saline before use in lymphokine assays. Each fraction was assayed for BCGF (●) and BCDF (○) activities as described. pfc, Plaque-forming cells.

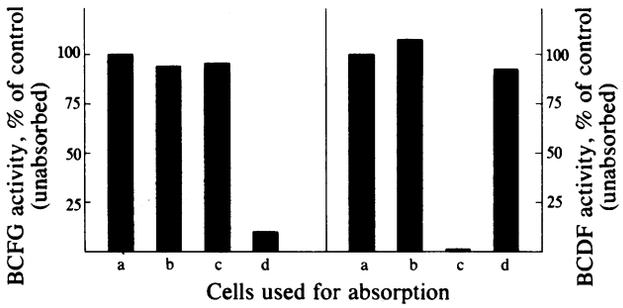

FIG. 6. Selective absorption of BCGF and BCDF from $7D_5$ hybridoma supernatant. Aliquots (0.5 ml) of cell-free supernatants, generated from 48-hr cultures of 1×10^6 $7D_5$ hybridoma cells, were absorbed with 1×10^7 of the following target cells: phytohemagglutinin-stimulated T-cell blasts (b), anti-μ-stimulated B-cell blasts (d), or CESS cells (c). Bars labeled "a" represent unabsorbed cells. Absorptions were carried out at 4°C for 4 hr. After absorption, aliquots of supernatant were diluted (15%, vol/vol) and assayed for BCGF activity on anti-μ-stimulated B cells. BCDF activity in diluted (15%, vol/vol) samples was determined using SAC-stimulated B cells. Data are presented as percentage of control values (15,888 ± 473 cpm for proliferation and 463 ± 58 plaque-forming cells for differentiation). Background levels in the assays were 652 ± 66 cpm and 32 ± 11 plaque-forming cells for proliferation and differentiation, respectively.

identified whose supernatant induced differentiation in preactivated B cells. Supernatant from $7D_5$ was also screened for other lymphokine activities. Assay for IL-2 was negative; however, the supernatant was found to have BCGF activity, as measured in an anti-μ assay system.

The question that emerged from these data was whether the BCGF and BCDF activities represented separate immunoregulatory molecules secreted by the cloned hybridoma cells. Previous studies had shown that cloned human T cells were capable of secreting multiple lymphokines (9, 19). Similar findings have also been reported in murine T-cell clones and hybridomas (21, 22). To investigate this possibility, $7D_5$ supernatant was subjected to molecular size and isoelectric point analysis. The molecular size of $7D_5$ BCGF, as determined by Sephadex G-100 gel filtration, was ≈20 kDa. A similar molecular size was reported in previous studies (11, 19). On the other hand, BCDF activity was confined to a separate peak with a molecular size of 30–35 kDa. The isoelectric points of the active column fractions were determined by chromatofocusing and were found to be distinct. BCGF activity was present in fractions with a pI of 6.6. In contrast, the pI of BCDF was determined to be 5.9. These data strongly suggest that two separate immunoregulatory molecules were secreted by the hybridoma.

Absorption experiments were carried out to determine specificity between the factors and target cells. As expected, phytohemagglutinin-activated T-cell blasts failed to absorb either BCGF or BCDF activity. Incubation of $7D_5$ supernatant with anti-μ-activated B cells abrogated BCGF activity but not BCDF activity. On the other hand, CESS cells, which are known to respond to differentiation but not growth factors (23), absorbed the majority of BCDF activity but not BCGF activity. Data from these experiments provide additional evidence that the BCGF and BCDF secreted by hybrid $7D_5$ are separate factors and are specific for their target-cell receptors.

In conclusion, we have described the establishment of a human T-cell hybridoma secreting BCGF and BCDF. Biochemical and absorption studies indicate that the activities reside in separate molecules. Finally, the use of monoclonal lymphokines derived from this and future T-cell hybridomas should allow more precise investigation of the mechanisms involved in growth and differentiation of human B cells.

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