DEVELOPMENT OF A HUMAN T-T CELL HYBRIDOMA SECRETING B CELL GROWTH FACTOR

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The development of human T cell hybridomas secreting immunoregulatory molecules has been an area of recent intense interest and investigation. Functional hybrids derived from both normal and malignant human T cells have been developed. These hybrids have been demonstrated to secrete interleukin 2 (IL-2)[1] (1), killer helper factor (2), and factors that help or suppress immunoglobulin production (3, 4). Application of the hybridization technique of Kohler and Milstein (5) to murine T lymphocytes has yielded an even wider variety of factors with immunoregulatory function (6–9). Factors produced by T cell hybrids have proven to be valuable as a source of purified immunoregulatory molecules for use in immunochemical as well as functional studies.

Recent studies (10–12) have demonstrated that exogenous growth factors are necessary to support proliferation of normal murine and human B cells placed in long-term culture. In the past, mitogen stimulation of human peripheral blood lymphocytes (PBL) has been used to produce supernatants with B cell growth factor (BCGF) activity (10). These conventionally generated supernatants are heterogeneous in composition, vary significantly in their ability to support B cell proliferation, and often contain growth factors for T cells as well as for B cells (13). In the present study, we report the development of a human T cell hybridoma secreting BCGF in the absence of IL-2.

Materials and Methods

Lymphocyte Preparation and Stimulation. Fresh, unfractionated PBL obtained from normal human subjects were separated by gradient centrifugation over Ficoll-Hypaque (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, NJ). T lymphocytes were separated by rosette formation with aminoethylisothiouronium bromide (AET)-treated sheep erythrocytes (14), followed by passage through a nylon wool column (15). The T cell-enriched population was suspended at 2×10^6 cells/ml in complete media (CM) consisting of RPMI 1640 plus glutamine (Flow Laboratories, Inc., Rockville, MD), 2 μg/ml amphotericin B (E. R. Squibb & Sons, Inc., Princeton, NJ), 140 μg/ml gentamicin (Schering Corp., Kenilworth, NJ), and 30% heat-inactivated fetal calf serum (FCS) (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY) screened to support the growth of T cell hybrids. T cells were stimulated with 10 μg/ml concanavalin A (Con A) (Sigma Chemical Co., St. Louis, MO) for 24 h in 24-well culture plates (3524; Costar, Data Packaging, Cambridge, MA) before hybridization.

B lymphocytes for use in the BCGF assay (described below) were prepared from normal

[1] *Abbreviations used in this paper:* AET, aminoethylisothiouronium bromide; BCGF, B cell growth factor; CM, complete media; Con A, concanavalin A; FCS, fetal calf serum; HAT, hypoxanthine/aminopterin/thymidine; IL-2, interleukin 2; PBL, peripheral blood lymphocyte.

human PBL. T cells were depleted by twice rosetting with AET-treated sheep erythrocytes, followed by treatment with anti-Leu 1 antibody (Becton, Dickinson & Co., Sunnyvale, CA) and rabbit complement. Depletion of monocytes was performed by adhering 50×10^6 T cell-depleted cells to plastic flasks (25116; Corning Glass Works, Corning, NY) containing RPMI 1640 with 1% FCS for 45 min at 37°C.

Cell Fusion Procedure. Human T cell hybridomas were produced by modification of fusion methods previously described (16–18). An azaguanine-resistant mutant of human T lymphoblastoid origin (CEM-6) was used for hybridization. This variant line, kindly provided by Dr. J. Stobo, University of California, San Francisco, CA, was grown continuously in CM containing 15% FCS and harvested at the mid-logarithmic phase of growth. 30 million Con A-activated peripheral blood T cells were mixed with 1.5×10^7 mutant CEM-6 cells and pelleted by centrifugation. The cell pellet was gently resuspended in 0.5 ml of a 50% polyethylene glycol-1500 solution. The cells were kept in suspension by gently stirring at 37°C for 5 min. The solution was then diluted to 10 ml by the addition of warm (37°C) RPMI 1640. The cells were centrifuged at 350 *g* for 5 min and resuspended in CM containing 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine (HAT; all from Sigma Chemical Co.). Cells were cultured in 96-well flat-bottomed microtiter plates (Costar 3596) at a concentration of 10^5 mutant cells/well in 0.2 ml. The cultures were kept at 37°C in a 7% CO_2 incubator, and the culture media was replaced with fresh HAT media every 3 d. Cloning of selected hybrid cells was done by the method of limiting dilution.

Assay for BCGF Activity. Assay for the effect of hybridoma supernatants on B cell proliferation was performed as previously described (19). In brief, the B cell-enriched population described above was suspended in CM containing 10% FCS and *Staphylococcus aureus*-Cowan strain I at a concentration of 5×10^{-4} vol/vol. 0.2-ml aliquots of the B cell-enriched fraction were added to the wells of a 96-well round-bottomed microtiter plate (Costar 3799) at a density of 2×10^5 cells/well. Cultures were maintained at 37°C in air with 7% CO_2 and 100% humidity. On day 3 of culture, 0.1 ml of media was removed from each well and replaced with 0.1 ml of hybridoma supernatant. Supernatant from the unfused CEM-6 line was used as a control. 48 h later, 1 μCi of [^{3}H]thymidine (New England Nuclear, Boston, MA) was added to the cultures, which were then continued for an additional 16 h. Cells were harvested using a Titertek cell harvester (Flow Laboratories, Inc.). The filter disks were placed in scintillation vials and counted in a liquid scintillation counter (LS-350; Beckman Instruments, Inc., Spinco Div., Palo Alto, CA).

Assay for IL-2 Activity. Supernatants from the T cell hybridomas were assayed for IL-2 activity in a system previously described in detail (20). Incorporation of [^{3}H]thymidine by an IL-2-dependent cytotoxic T cell line (CT6) was used as the index of IL-2 activity in the supernatants.

Gel Filtration. 200 ml of $2B_{11}$ supernatant was concentrated to 10 ml by Amicon filtration using a YM10 membrane (Amicon Corporation Scientific Division, Danvers, MA). The concentrated hybridoma supernatant was next applied to a Sephadex G-100 (Pharmacia Fine Chemicals) gel filtration column (90 × 2 cm). The column was eluted with phosphate-buffered saline (HEM Research, Inc., Rockville, MD) at a flow rate of 12 ml/h. 2.5-ml fractions were collected, and BCGF activity was assayed. Absorbance was recorded at 280 nm.

Results

Development and Cloning of Hybridomas. On the day of fusion, 768 wells were seeded with cells (Table I). The first hybrid cells were seen 18 d after hybridization. On initial screening, supernatants from three wells were found to have BCGF activity. The cells from the positive wells were expanded and subsequently cloned at 10 cells/well. Cloning efficiency was low (6%) at this cell density. One well with rapid growth characteristics ($2B_{11}$) showed marked enhancement of B cell proliferation and was chosen for further cloning. Cloning efficiency increased to 55% when plates containing 0.5 cells/well were assayed for BCGF activity.

BCGF Production by Hybridomas. To investigate the effect of human T cell hybridoma

TABLE I

Development and Screening of Human T Cell Hybridomas

Days after fusion	Number of wells cultured	Number of wells producing BCGF	Cloning efficiency*
			(%)
0	768	—	—
18	Hybrids first identified in 12 of 768 wells	—	—
41	184 Wells with hybrids screened	3	—
47	3 Positive wells cloned, 10 cells/well (288 wells)	—	—
62	230 wells screened	17	6
69	Well $2B_{11}$ recloned, 0.5 cells/well (120 wells)	—	—
88	37 wells screened	26	55

* Calculated as number of positive wells per number of wells calculated to have ≧1 cells/well.

supernatants on B cell proliferation, normal human peripheral blood B cells were activated in culture with *S. aureus*-Cowan strain I. On day 3 of culture, hybridoma supernatants were added, and the incorporation of tritiated thymidine was measured on day 6. As shown in Fig. 1, two separate T cell hybridoma supernatants had variable effects on the proliferative response of human B cells. Supernatant from clone $2B_{11}$ significantly enhanced B cell proliferation as compared with the response seen with conventional BCGF derived from phytohemagglutinin-stimulated human PBL. Intermediate BCGF activity was demonstrated in the supernatant from clone $2F_6$. The magnitude of enhancement of the proliferative response varied among experiments; however, supernatants from clone $2B_{11}$ significantly enhanced proliferation in every experiment. This variability suggests that the results seen in these experiments were greatly influenced by the responsiveness of the B cell populations used. Hybrid cells from clone $2B_{11}$ were stimulated with Con A to investigate the effect of mitogen stimulation on the hybrid cell's ability to secrete a supernatant that enhanced B cell proliferation. Mitogenic stimulation of the hybrid cells produced no significant change in the levels of proliferation measured (data not shown).

The maximum B cell proliferative response in the assay system was dependent on the concentration of T cell hybridoma supernatant added to the culture wells (Fig. 2, panel A). At an eightfold dilution, BCGF activity in the supernatants from clone $2B_{11}$ remained high. Thereafter, activity declined with subsequent dilutions, and no enhancement of B cell proliferation was demonstrable at a 1:128 dilution. In contrast, the activity of conventionally prepared BCGF diminished at a much more rapid rate so that no BCGF activity was evident at a 1:16 dilution. A question that emerged from these data was whether the T-T hybridoma supernatant could maintain growth in long-term cultures of human B cells. Simultaneous cultures of normal human peripheral blood B cells were prepared and maintained by either conventionally derived BCGF or supernatant from clone $2B_{11}$. Although those B cells supplemented with conventional growth factor showed slow inconsistent rates of proliferation, the B cell cultures supplemented with clone $2B_{11}$ supernatant have been maintained in culture for >6 wk. Some of these long-term culture wells were tested for their dependence on the $2B_{11}$ supernatant by being fed with conventional BCGF or media containing no growth factors. Shortly after $2B_{11}$ was withdrawn, proliferation of B

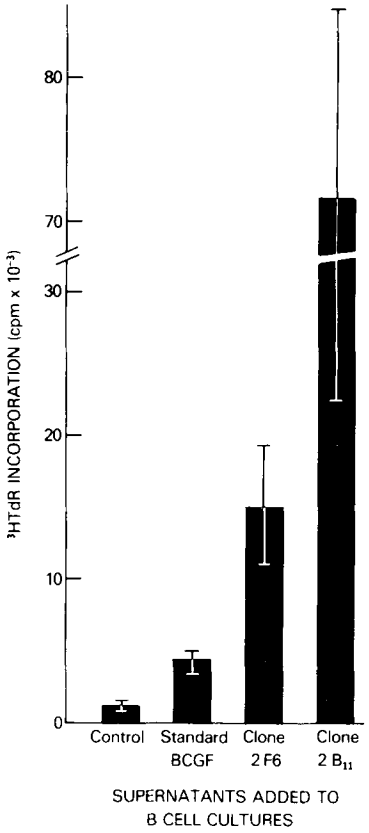

FIG. 1. Enhancement of human B cell proliferation by human T-T hybridoma supernatants. B cells (2×10^5 cells/well) that had been activated by *S. aureus*-Cowan strain I for 3 d were then cultured with 0.1 ml of supernatant derived from the CEM-6 cell line (control), phytohemagglutinin-stimulated human PBL (standard BCGF), or human T-T hybridomas. Proliferation was measured after an additional 3 d in culture. Data represent the mean (± SEM) of triplicate experiments.

cells in the culture wells fed with conventional BCGF declined significantly. B cells maintained in media containing no growth factors failed to proliferate and subsequently died. Of note is the fact that the clone $2B_{11}$ has remained stable in culture for >3 mo with no diminution of BCGF activity in the supernatant. Karyotypic analysis of the hybridoma cells revealed a chromosomal number of 48–50. In contrast, the normal human T cells as well as the CEM-6 leukemic cells were found to have a diploid number of chromosomes.

Supernatants from clone $2B_{11}$ were tested for IL-2 activity in an assay system using an IL-2-dependent T cell line (CT6). As shown in Fig. 2, panel B, serial dilutions of $2B_{11}$ supernatant failed to support proliferation of the CT6 cells. On the other hand, significant incorporation of [^{3}H]thymidine by the cells was produced by the addition of a standard IL-2 preparation. These results demonstrate no detectable contamination of BCGF with IL-2 activity. Clone $2B_{11}$ was also tested for its ability to induce B cell differentiation and antibody production in addition to proliferation. In an assay system using an Epstein-Barr virus-transformed B cell line (CESS), no evidence of differentiation was demonstrated (data not shown).

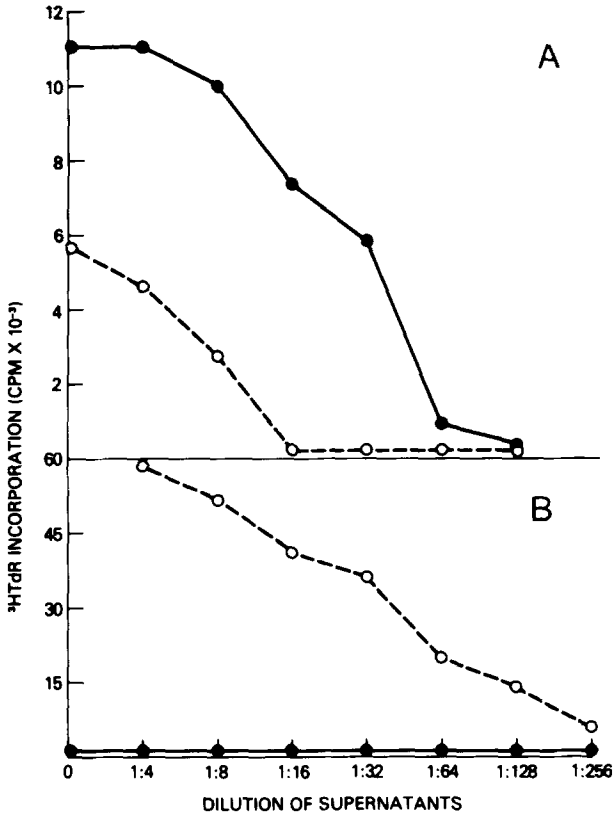

FIG. 2. (A) Relationship between the dilution of T-T hybridoma supernatant or conventional growth factor and the degree of B cell proliferation. Culture conditions in this experiment were identical to those described in Fig. 1. Normal human B cells were cultured with various dilutions of $2B_{11}$ supernatant or conventional growth factor. Proliferation, as measured by tritiated thymidine incorporation, was assayed 3 d after addition of the supernatants. ●, T-T hybrid $2B_{11}$ supernatant; ○, conventional growth factor. (B) Comparison of the effects of $2B_{11}$ supernatant and conventional IL-2 on proliferation of cytotoxic T cells. CT6 T cells (1×10^4 cells/well in 0.2 ml volume in flat-bottomed microtiter plates) were cultured for 24 h in the presence of various dilutions of hybridoma supernatant or supernatant from phytohemagglutin-stimulated PBL. Cells were pulsed with 1 μCi of tritiated thymidine for 16 h before harvest. ●, T-T hybrid $2B_{11}$ supernatant; ○, IL-2 standard.

To determine the molecular weight of the BCGF, concentrated supernatant from hybrid $2B_{11}$ was applied to a gel filtration column (G-100 Sephadex). The column was eluted, and 2.5-ml fractions were collected and assayed for the capacity to support the proliferation of normal human B cells (19). As shown in Fig. 3, the majority of protein eluted before fraction 55. Another peak of optical density was evident in fractions 58–64. BCGF activity was confined to this peak that had an apparent 18–20,000 mol wt.

Discussion

The present study reports the successful development of a human T-T cell hybridoma continuously secreting BCGF. Enhancement of human B cell proliferation was significantly increased by supernatant from clone $2B_{11}$ as compared with convention-

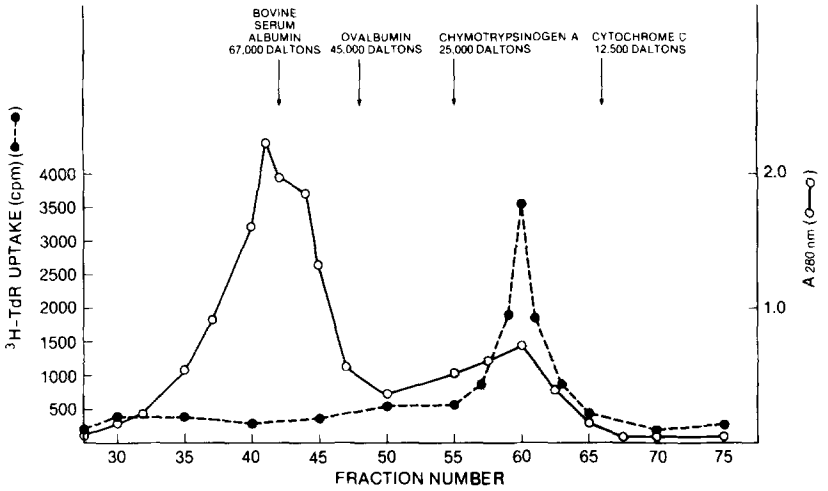

FIG. 3. Gel chromatography of $2B_{11}$ supernatant. Hybridoma supernatant was concentrated by Amicon filtration and fractionated on a Sephadex G-100 column. 2.5-ml fractions were eluted from the column and tested for their ability to support B cell proliferation with the results expressed as cpm of tritiated thymidine incorporation (●). Arrows indicate the elution profile of the proteins used for column calibration. Protein concentration of applied supernatant is represented by the absorbance of 280 nm (○).

ally prepared BCGF. In addition, the hybridoma supernatant was far superior to conventionally prepared BCGF in maintaining B cell proliferation in long-term cultures.

To optimize the conditions for cell hybridization, we attempted to obtain highly purified normal human peripheral blood T lymphocytes. An efficient enrichment procedure was found in the two-step method of rosetting with AET-treated sheep erythrocytes, followed by passage through a nylon wool column. Earlier experiments had shown a significant increase in fusion efficiency when enriched populations of human T cells were activated by mitogen before fusion (J. L. Butler and A. S. Fauci, unpublished observations). Therefore, T cell blasts induced by Con A were used in the fusion experiments. Hybrid growth and subsequent cloning were enhanced by the use of media containing 30% FCS, as reported in a previous study (21).

When the wells from the initial cloning were screened, BCGF activity was identified in 17 wells. One well was chosen for recloning because of its rapid growth characteristics, while cells in the remaining wells were kept in culture with no further cloning. On subsequent screening 4 wk later, supernatants from only three of the wells continued to enhance B cell proliferation. This loss of hybrid function could be the result of loss of genes coding for the production and secretion of BCGF. Another potential explanation of this phenomenon is overgrowth of the cultures by nonfunctional hybrids that are better adapted to tissue culture. Thus, it is possible that early, aggressive cloning is an important step in preserving function of T cell hybridomas, as has been emphasized in a recent study (3).

Although the activities of murine BCGF and IL-2 have been separated on an immunochemical basis (22), it has not been possible to achieve this separation of human BCGF and IL-2 (A. Muraguchi and A. S. Fauci, unpublished observations).

Therefore, it was conceivable that IL-2 was also present in the hybridoma supernatant and could stimulate a few residual T cells that might have been present in the responding B cell populations used in our BCGF assay system. These activated T cells could then support B cell proliferation by the elaboration of soluble growth factors. To evaluate this possible mechanism, $2B_{11}$ supernatant was assayed for IL-2 activity using an IL-2-dependent T cell line. No IL-2 activity was found in the supernatant, which indicated that the BCGF in the hybridoma supernatant was separate and distinct from IL-2. Furthermore, the hybridoma supernatant did not induce the differentiation of B cells to secrete immunoglobulin, an activity that has been identified with T cell-replacing factor (23–25).

The availability of purified BCGF in virtually unlimited quantities, which supports the proliferation of normal human B cells and which is devoid of IL-2 or T cell-replacing activity, should prove of extreme value in the precise biochemical characterization of human BCGF as well as in studies aimed at the long-term growth and cloning of normal human B cells. Finally, future development of panels of human T cell hybridomas with a range of distinct functional capabilities will make available important sources of purified factors with which to explore the mechanisms of human immunoregulation.

Summary

The success of long-term culture of normal human and murine B cells has been hampered by the limited availability of soluble factors capable of maintaining proliferation of activated B lymphocytes. Previous experiments using various culture-derived supernatants in a human system were unable to separate the activities of B cell growth factor (BCGF) and interleukin 2 (IL-2) by immunochemical means. Thus, purified factors with BCGF activity in the absence of IL-2 activity have not been available for study. In the present study, normal human peripheral blood T cells were fused with the hypoxanthine/aminopterin/thymidine-sensitive human T-leukemic cell line, CEM-6. Supernatants from the resulting hybrid cells were tested for the ability to maintain proliferation of normal human B cells in a recently described assay system for human BCGF. Hybrids demonstrating BCGF activity were cloned by limiting dilution. One hybrid clone, $2B_{11}$, continued to support proliferation of B cells in both long-term cultures and 6-d assays at a level significantly above that seen with conventionally produced growth factors. No IL-2 activity was found in the supernatant from hybrid $2B_{11}$. The hybridoma supernatant was fractionated by gel filtration, and maximum proliferation of B cells was supported by the 18–20,000 mol wt protein fraction. Thus, a human T-T cell hybridoma that has BCGF activity in the absence of any demonstrable IL-2 activity has been developed. Human T-T cell hybridomas secreting discrete immunoregulatory factors should prove to be powerful tools in dissecting the mechanisms of immunoregulation of human lymphocyte function.

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