BOTH INTERLEUKIN 2 AND A SECOND T CELL–DERIVED FACTOR IN EL-4 SUPERNATANT HAVE ACTIVITY AS DIFFERENTIATION FACTORS IN IgM SYNTHESIS

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Resting B lymphocytes can be stimulated to proliferate and differentiate into immunoglobulin (Ig)-synthesizing cells by anti-IgM antibodies together with a series of T cell–derived growth and differentiation factors (1–7). The proliferation of anti-IgM–activated B cells can be stimulated by B cell stimulatory factor p1 (BSF-p1)[1] (formerly designated B cell growth factor) (1, 5–7). Differentiation to high rate Ig synthesis depends upon two additional T cell–derived factors (4). One (B15-TRF), which is required early in the course of the response, is found in the supernatant of the B151K12 T cell hybridoma line (4, 8), and has been partially purified by Hamaoka et al. (9). The second factor (EL-TRF), can be added relatively late in the course of the culture (4), and is found in supernatants of EL-4 thymoma cells that have been stimulated with phorbol esters. The observation that EL-TRF activity is found in the pH 4.5 fraction of an isoelectric focusing separation, which is close to the pI of interleukin 2 (IL-2) (4, 10), raises the question as to whether IL-2 is also a B cell differentiation factor. In previous work (4), we tested affinity-purified human IL-2 at a concentration of 50 U/ml and found that it did not display detectable EL-TRF activity. 1 U of IL-2 activity is defined as the reciprocal of the dilution required to cause half-maximal proliferation of the IL-2-dependent T cell line HT-2. The studies reported here indicate that molecularly cloned IL-2, at concentrations of 100 U/ml and above, does display definite EL-TRF activity and that IL-2 in EL-4 supernatants accounts for a portion of the EL-TRF activity of such material. Furthermore, we show that stimulated B cells bear receptors for IL-2 through which they may respond to this lymphokine. A second component in EL-4 supernatant, with a molecular weight of 32,000 and lacking any IL-2 activity, also displays strong EL-TRF activity.

[1] *Abbreviations used in this paper:* BSF-p1, B cell stimulatory factor p1; IL-2, interleukin 2; FCS, fetal calf serum; PMA, phorbol myristate acetate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; ELISA, enzyme-linked immunoadsorbent assay; HBSS, Hanks' balanced salt solution; NP-40, Nonidet P-40; FITC, fluorescein isothiocyanate; [^{3}H]IL-2, [^{3}H]leu,lys-IL-2.

Materials and Methods

Mice. BALB/c mice were obtained from The Jackson Laboratory, Bar Harbor, ME and used at 8–12 wk of age.

Reagents. Affinity-purified goat anti-mouse IgM antibody was prepared as previously described (11). Two rat monoclonal antibodies specific for the murine IL-2 receptor were used. 3C7 is a rat IgG antibody that can compete with IL-2 for binding to its receptor (12), and 7D4 is a rat IgM antibody that does not block the binding of IL-2 to its receptor but does inhibit IL-2-dependent T cell proliferation (13). Sequential immunoprecipitation studies demonstrated that these two antibodies were reactive with the same molecular species and precipitated identical components from the surface of T cells (12). Two rat monoclonal antibodies, kindly provided to us by J. Davie (Washington University School of Medicine, St. Louis, MO) were used as class-matched controls. 50C1C1 (50C1) is a rat IgG anti-DNP antibody and 49C2D6R5 (49C2) is a rat IgM anti-trinitrophenyl antibody. MAR 18.5, a mouse monoclonal antibody to rat kappa chain (14), was used in indirect fluorescent staining.

Culture Medium. RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 10% fetal calf serum (FCS), penicillin (50 μg/ml), streptomycin (50 μg/ml), gentamycin (100 μg/ml), L-glutamine (2 mM), and 2-mercaptoethanol (5×10^{-5} M) was used as culture medium in these experiments. In the preparation of EL-4 supernatants, RPMI 1640 medium containing 1% FCS, with supplements described above, was used.

Factor Preparation. EL-4 supernatant was prepared by culturing a cloned subline of EL-4 thymoma cells (2×10^6/ml) with 10 ng/ml of phorbol myristate acetate (PMA) for 48 h (15). Cell-free supernatant was collected and PMA was removed as described (1). Partially purified BSF-p1 was obtained from this cell-free supernatant by phenyl-Sepharose column chromatography (10). B151K12 supernatant was collected from the cloned T cell hybridoma of this designation (8). Molecularly cloned human IL-2, purified by high pressure liquid chromatography, was kindly provided to us by Dr. J. Farrar, Hoffman-La Roche, Inc., Nutley, NJ. We also received molecularly cloned human IL-2 from Dr. J. Koths, Cetus Corp., Emeryville, CA. The latter preparation was reported to contain 0.67 fg of endotoxin per unit of IL-2, as defined by assay in our laboratory.

Gel Filtration. Cell-free supernatant from induced EL-4 cells was concentrated by an Amicon concentrator using a YM10 membrane (Amicon Corp., Lexington, MA), followed by sequential precipitation with 40 and 90% ammonium sulfate. The 90% ammonium sulfate precipitate was dissolved in a small volume of Dulbecco's phosphate-buffered saline (PBS), dialyzed extensively against the same buffer, and then fractionated by gel filtration on a Sephadex G150 column that had been equilibrated with Dulbecco's PBS. Each fraction was examined for its BSF-p1, IL-2, and EL-TRF activities. BSF-p1 activity was tested in a B cell costimulator assay described elsewhere (1). IL-2 activity was measured by the stimulation of uptake of [^{3}H]thymidine by an IL-2-dependent cell line (HT-2) (16). 1 U of IL-2 is defined as the amount required to cause half-maximal uptake of [^{3}H]-thymidine by 4×10^3 HT-2 cells in the last 4 h of a 28 h culture period. EL-TRF activity was determined as described below.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). Samples (fractions 52–54) from Sephadex G150 column chromatography containing peak EL-TRF activity were further fractionated by slab gel SDS-PAGE (17). The samples were dialyzed for 16 h against sample buffer that contained Tris-HCl (pH 6.8), 1% SDS, and 10% glycerol. A running buffer system of Tris-glycine, containing 0.1% SDS, pH 8.5 was used. The stacking gel contained Tris-HCl (pH 6.8) and 4% polyacrylamide. The separation gel contained Tris-HCl (pH 8.8) and 12.5% polyacrylamide. After electrophoresis at room temperature, the gel was sliced into 2.0-mm-wide fractions. The gel slices were electro-eluted, in the presence of 1% bovine serum albumin. Samples were dialyzed at room temperature against PBS followed by dialysis against 4.0 M urea to remove the SDS. They were then dialyzed at 4°C against PBS and finally against RPMI 1640 medium containing 1% FCS. Each fraction was made sterile by filtration and examined for its BSF-p1, IL-2, and EL-TRF activities.

B Cell Preparation. We prepared B cells by pretreatment of donor mice with antithy-

mocyte serum followed by passage of spleen cells over a Sephadex G10 column and two rounds of complement-mediated lysis with monoclonal anti-Thy-1.2 and anti-Lyt-1.2 (18). In most experiments, positive selection, by adherence to and elution from plastic dishes coated with anti-IgM antibodies (19), was coupled to this negative selection procedure. Flow microfluorometric analysis on a fluorescence-activated cell sorter (FACS II; B-D FACS Systems, Sunnyvale, CA) showed that the cell populations obtained by these sequential purification procedures were ~99% surface IgM positive.

Assay for IgM-Synthesizing Cells. 2×10^5 purified B cells were cultured in flat-bottom, 96-well microtiter plates (No. 3596; Costar, Cambridge, MA) in 0.2 ml of medium containing 5 μg/ml of goat anti-IgM and various combinations of factors. The assay for EL-TRF activity was performed by culturing B cells with anti-IgM, BSF-p1, B15-TRF, and fractions to be examined for their EL-TRF activity. After 4–5 d of culture, a quantitative immunoassay for cytoplasmic IgM content was performed by using an enzyme-linked immunoadsorbent assay (ELISA) method (20). Cells in culture plates were washed four times with Hanks' balanced salt solution (HBSS). Nonidet P-40 (NP-40) was added to each well at a concentration of 0.25% to make cell lysates that were used for the detection of cytoplasmic IgM. Two rat monoclonal anti-mouse μ heavy chain antibodies (Bet-1 and Bet-2) (21) were used. Bet-1 was used for coating the microtiter plates and biotinylated Bet-2 was used as a second antibody to detect IgM bound to the Bet-1-coated plate. After extensive washing of the plates, a horseradish peroxidase–avidin conjugate was added to each well, followed by incubation with substrate. In some cases, the presence of cytoplasmic IgM was determined by immunofluoresence as described previously (4).

RNA Preparation from Resting B Cells and Cultured B Cells. After 4 d of culture, multiple wells from each group were combined and the viable cell number was determined by using the trypan blue dye exclusion method. Cell number was adjusted to 3×10^6 per group. Total RNA was obtained by lysis with 0.5% NP-40 of 3×10^6 resting B cells that had been cultured for 48 h and of 3×10^6 B cells stimulated for 4 d with anti-IgM and T cell–derived factors. After cell lysis, total RNA was extracted with cold phenol (twice) and chroloform/isoamylalcohol(24:1) (once) sequentially, and then ethanol precipitated. RNA was dotted onto nitrocellulose paper, fixed by baking, and then hybridized with a ^{32}P-labeled cDNA clone specific for the secretory terminal of the μ chain and the 3′ untranslated region immediately following the terminus. This cDNA was subcloned from pμ12; pμ12 was kindly provided to us by R. Wall (22). The specificity of the subcloned probe is described elsewhere (22a). After washing twice with 0.3 M NaCl/0.03 M trisodium citrate (2× SSC) and twice with 0.2× SSC, the filter was exposed to Kodak XAR-5 x-ray film at −70°C using an intensifying screen.

Fluorescence Staining of B Cells Stimulated with Anti-IgM Plus T Cell–Derived Factors. Direct fluorescent staining was performed by incubating the cells (1×10^6) for 30 min at 4°C with 1–2 μg of fluorescein-isothiocyanate-conjugated monoclonal antibody. After incubation, cells were washed three times with HBSS containing 0.1% NaN_3 and 3% FCS, and then analyzed on a FACS. The capacity of unconjugated 3C7 and 7D4 to compete in this binding was also assessed on the FACS; 2×10^5 cells were first incubated with the supernatants containing the indicated monoclonal antibodies followed by an additional incubation with 1–2 μg of FITC-conjugated 3C7 or FITC-conjugated 7D4. The samples were then washed and analyzed on the FACS. 10,000–20,000 cells were analyzed for each determination. Dead cells, identified by staining with propidium iodide, were excluded from analysis. Indirect fluorescent staining was performed as previously described (13) by incubating the cells (1×10^6) with an excess of culture supernatant fluids containing the indicated monoclonal antibodies. After washing and subsequent staining with FITC-conjugated MAR 18.5, the samples were analyzed on a FACS.

Radiolabeled IL-2-binding Assay. Biosynthetically radiolabeled [^{3}H]leu,lys-IL-2 ([^{3}H]IL-2) was prepared as described previously (23). The purification of human IL-2 was performed by using affinity chromatography. The eluted material was comprised of a single polypeptide (15,500 mol wt) when analyzed by SDS-PAGE, reverse phase liquid chromatography, and amino-terminal amino acid sequence analysis (24). Radiolabeled IL-2 binding to whole cells was performed as described in detail previously (23).

Results

A Portion of the EL-TRF Activity in EL-4 Supernatants Co-migrates with IL-2. High rate IgM synthesis by rigorously purified B cells stimulated with anti-IgM antibodies depends upon the presence of three T cell–derived factors: BSF-p1, B15-TRF, and EL-TRF. Omission of any one of these factors leads to a meager response but when all three factors are present, 10–30% of the resulting B cells contain substantial amounts of cytoplasmic IgM (4). In the experiment illustrated in Fig. 1, the total concentration of cytoplasmic IgM in lysates of cells stimulated with all three factors was 2.35 μg/ml, whereas stimulation of cell with any mixture of two factors led to concentrations of cytoplasmic IgM ranging from 0.15 to 0.81 μg/ml.

In an effort to characterize EL-TRF, we added graded concentrations of ammonium sulfate to supernatants of EL-4 cells and took the material that was soluble at 40% ammonium sulfate but precipitated at 90% ammonium sulfate. This fraction contained the bulk of the EL-TRF activity. After dialysis against PBS, this material was applied to a Sephadex G-150 column. Samples were tested for EL-TRF, BSF-p1, and IL-2 activity (Fig. 2). EL-TRF activity was found in two portions of the chromatogram, one with an apparent molecular weight of 32,000 and the other, 16,000. IL-2 activity was equally distributed between

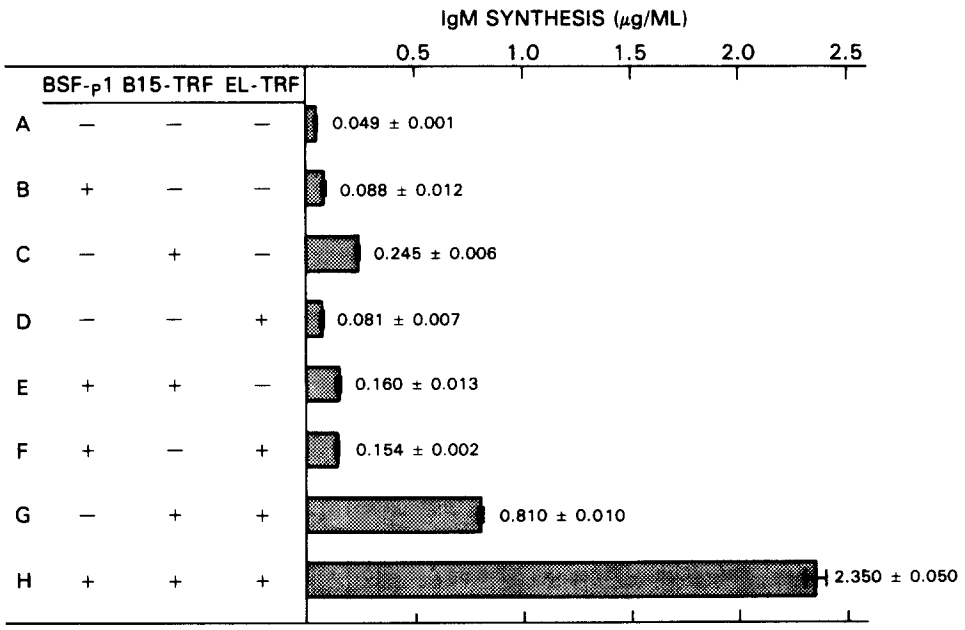

FIGURE 1. Cytoplasmic IgM in stimulated B Cells. B cells prepared by the Leibson technique followed by panning were cultured at 2×10^5 cells/well in the presence of 5 μg/ml of anti-IgM. Such cells were cultured alone, or with BSF-p1 (a 1:10 dilution of phenyl-Sepharose–purified material from EL-4 supernatant, containing 42 U of BSF-p1/ml), B15-TRF (equivalent to 125% of supernatant from B151K12 hybridoma cells), and EL-TRF (5% of an IL-2-rich phenyl-Sepharose fraction of EL-4 supernatant, containing 65 U of IL-2/ml), either individually or in various combinations. Concentrations of cytoplasmic IgM in triplicate cultures was measured on the 4th d of culture by an ELISA.

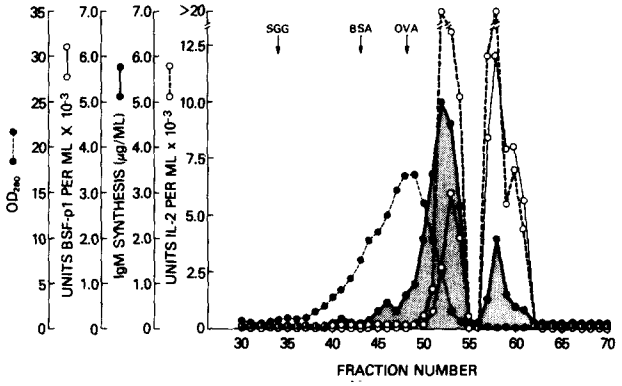

FIGURE 2. Gel filtration of EL-TRF. Induced EL-4 supernatant, after ammonium sulfate precipitation (see Materials and Methods), was applied to a Sephadex G-150 column. Individual fractions (2 ml) were tested for OD_{280}, IL-2, BSF-p1, and EL-TRF activity. The column had previously been calibrated with sheep gamma globulin (*SGG*; mol wt 155,000), bovine serum albumin (*BSA*; mol wt 68,000), and ovalbumin (*OVA*; mol wt 43,000).

these two fractions, while the bulk of the BSF-p1 activity was found in the lower molecular weight fraction.

Since the higher molecular weight peak had considerably more EL-TRF activity than the lower molecular weight peak, we concentrated our attention on this material. Fractions 52, 53, and 54, from the higher molecular weight peak, were pooled, concentrated, and applied to a SDS-polyacrylamide gel (12.5% acrylamide). After electrophoresis, EL-TRF, IL-2, and BSF-p1 activities in individual gel slices were measured. EL-TRF activity was found in two areas of the electropherogram, one corresponding to 32,000 (fraction 13), the other, 16,000 (fraction 22) (Fig. 3). We will designate the former material as high molecular weight EL-TRF and the latter as low molecular weight EL-TRF. IL-2 and BSF-p1 activity were found only in the lower molecular weight fraction. Thus, EL-TRF appears to exist in two forms, separable by SDS-PAGE. One of the two, low molecular weight EL-TRF, co-migrates with IL-2 and BSF-p1; the other, high molecular weight EL-TRF, is well separated from these two factors.

IL-2 Has EL-TRF Activity. To further explore the relationship between IL-2 and EL-TRF, we tested the ability of a monoclonal antibody to the murine IL-2 receptor to inhibit the differentiative activity of EL-TRF. 3C7 has been shown to inhibit the action of IL-2 on activated T cells, to block the binding of [^{3}H]IL-2 to the IL-2 receptor, and to precipitate a molecule of 48,000–62,000 mol wt in size from radioiodinated mouse T cell membranes. The latter is analogous to the characteristics of the IL-2 receptor on activated human T cells (26). Furthermore, affinity-purified human IL-2 blocks the binding of 3C7 to T cells. This led to the conclusion that 3C7 is specific for the mouse IL-2 receptor and that it competes with IL-2 for binding to that receptor (12). Although 3C7 did not show any effect on proliferation of B cells stimulated with anti-IgM and BSF-p1 (K. Nakanishi and T. Malek, unpublished observations), ascitic fluid containing 3C7 showed substantial, although incomplete, inhibition of the EL-TRF activity of low molecular weight EL-TRF from SDS-PAGE (Table I, Exp. 1). This fraction contained 70 U/ml of IL-2, as judged by the HT-2 assay. By

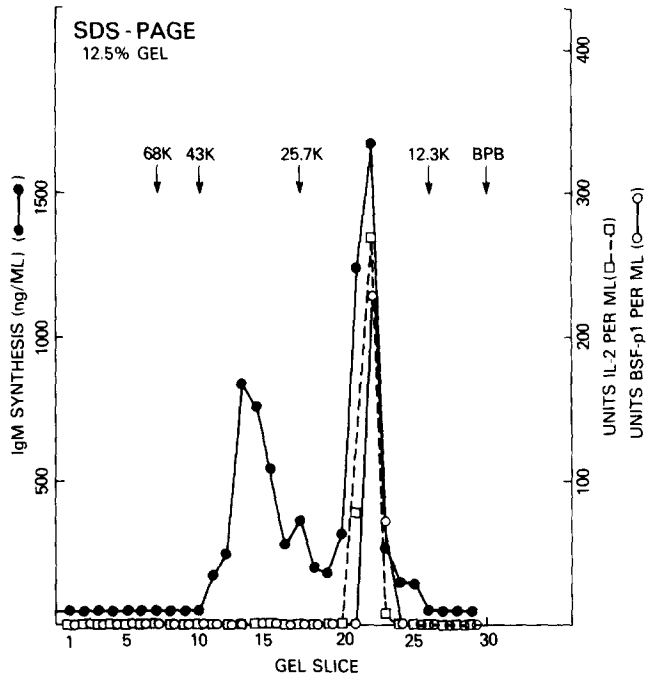

FIGURE 3. SDS-PAGE of EL-TRF. Fractions 52–54 from Sephadex G-150 gel filtration (see Fig. 2) were pooled, concentrated, and applied to a 12.5% polyacrylamide slab gel containing 1% SDS and electrophoresed for 6 h at a constant voltage of 150 V. Standards used were BSA (mol wt 68,000), OVA (mol wt 43,000), α-chymotripsinogen (mol wt 25,700), β-lactoglobulin (mol wt 18,400), and cytochrome *c* (mol wt 12,300). Fractions (2 ml) were electroeluted, dialyzed, and tested for IL-2, BSF-p1, and EL-TRF activity.

contrast, 3C7 had no inhibitory action on the EL-TRF activity of high molecular weight EL-TRF, which had no measurable IL-2 activity.

This result was confirmed in a second experiment (Table I, Exp. 2) in which molecularly cloned human IL-2 was examined for its capacity to mimic EL-TRF by causing IgM synthesis in B cells cultured with anti-IgM, BSF-p1, and B15-TRF. The differentiative activity of low molecular weight EL-TRF was diminished by 76% by 3C7, in comparison with the result obtained with 50C1, a control ascitic fluid. 3C7 caused no inhibition of stimulation of Ig synthesis by high molecular weight EL-TRF. Cloned IL-2, obtained from Hoffman-La Roche, Inc., at concentrations of 100–1,000 U/ml caused IgM synthesis in the presence of anti-IgM, BSF-p1, and B15-TRF; maximum responses required 500 U/ml. This stimulation was inhibited by 3C7; the degree of inhibition was greatest when the lowest concentrations of IL-2 were used.

In separate experiments, comparable results were obtained with molecularly cloned human IL-2 provided to us by Cetus Corporation. 1,000 U of IL-2 were required for maximal IgM synthesis with this material. This should contain 0.67 pg of endotoxin, which is far too low to induce B cell differentiation in the presence of anti-IgM (data not shown). This, together with the specific inhibitory effect of 3C7 on IL-2-induced IgM synthesis, indicates that small amounts of

TABLE I

Inhibition of IgM Synthesis by Monoclonal Anti-IL-2 Receptor Antibody (3C7)

B cells cultured with anti-IgM, BSF-p1, and B15-TRF plus:	IgM synthesis (ng/ml)			Percent suppression* of IgM response	
	Ascites used in the cultures:				
Exp. 1	None		3C7	A	
Nothing	288 ± 16		232 ± 16		
High mol wt EL-TRF (<1 U/ml)‡	1,100 ± 52		1,410 ± 50	−45.1	
Low mol wt EL-TRF (70 U/ml)	1,640 ± 8		668 ± 18	67.8	
Exp. 2	None	50C1	3C7	A	B
Nothing	180 ± 21	161 ± 13	172 ± 6		
High mol wt EL-TRF (< 1 U/ml)	920 ± 13	1,020 ± 120	1,280 ± 40	−49.7	−29.0
Low mol wt EL-TRF (140 U/ml)	1,020 ± 45	1,100 ± 34	400 ± 10	72.9	75.7
rIL-2 (100 U/ml)	584 ± 20	520 ± 35	212 ± 52	90.1	88.9
rIL-2 (200 U/ml)	1,680 ± 82	1,492 ± 100	420 ± 95	83.5	81.4
rIL-2 (500 U/ml)	2,280 ± 67	2,110 ± 50	1,100 ± 150	55.8	52.4
rIL-2 (1,000 U/ml)	2,560 ± 95	2,230 ± 150	1,320 ± 30	51.8	44.5

Culture conditions are described in Materials and Methods and in the legend to Fig. 1

* The degree of suppression of IgM synthesis was calculated using the following formula: percent suppression = [1 − (experimental group − background)/(positive control − background)] × 100. (*A*) In this comparison, the positive control was B cells stimulated with anti-IgM and factors in the absence of any ascites. (*B*) In this comparison, the positive control was B cells stimulated with anti-IgM and factors in the presence of 50C1 ascites (a rat IgG monoclonal anti-dinitrophenyl antibody). In both cases, background is IgM synthesis of B cells stimulated with anti-IgM, BSF-p1, and B15-TRF, without any source of EL-TRF.

‡ IL-2 activity in units per milliliter.

endotoxin contaminating molecularly cloned preparations of IL-2 are not responsible for their observed B cell differentiation activity.

To further test the capacity of IL-2 to act as a B cell differentiation stimulus, we examined the capacity of RNA from such cells to hybridize to a cDNA probe specific for the C terminal and the 3′ untranslated regions of the secretory form of μ heavy chain mRNA. Resting B cells cultured for 48 h and B cells stimulated for 96 h with anti-IgM and BSF-p1 with or without B15-TRF have relatively small amounts of RNA complementary to the cDNA probe (Fig. 4). The addition of cloned IL-2 to cultures containing anti-IgM, BSF-p1, and B15-TRF caused an 8–16-fold enhancement in levels of secretory μ chain mRNA. 1,000 U/ml of IL-2 caused greater enhancement than did 100 U/ml.

Differentiation Effects of Adding Cloned IL-2 and High Molecular Weight EL-TRF. The finding that two apparently distinct entities in induced EL-4 supernatant, IL-2 and a high molecular weight EL-TRF, act as differentiation factors in the induction of high rate IgM synthesis by B cells treated with anti-IgM, BSF-p1, and B15-TRF, raises the question of the effect of mixing the two factors. To study this, we mixed a low concentration (5%) of fraction 13 from SDS-PAGE, which is devoid of IL-2 activity and contains high molecular weight EL-TRF, with graded concentrations of cloned IL-2 (Fig. 5). At all concentrations of IL-2, from 31 to 8,000 U/ml, the addition of fraction 13 caused a greater than additive enhancement of the concentration of cytoplasmic IgM in the stimulated cell population. The mechanism underlying this synergistic effect of IL-2 and high molecular weight EL-TRF remains to be elucidated. However, this effect may explain why pH 4.5 fractions of isoelectric focusing (IEF) separations of EL-4 supernatant, which contained relatively small amounts of IL-2 (50 U/ml),

B CELL CULTURE CONDITION	1/DILUTION OF RNA 1 2 4 8 16 32	CYTOPLASMIC IgM (% Positive Cells)
A RESTING B CELL (0 Hr)		0.01
B RESTING B CELL (48 Hr)		n.t.
C ANTI-IgM + BSF-p1 (96 Hr)		0.3
D ANTI-IgM + BSF-p1 + B15-TRF (96 Hr)		0.6
E ANTI-IgM + BSF-p1 + B15-TRF + rIL-2 (100U/ml) (96 Hr)		5.1
F ANTI-IgM + BSF-p1 + B15-TRF + rIL-2 (1000U/ml) (96 Hr)		14.0

FIGURE 4. Expression of μ_s mRNA in B cells. Resting B cells were cultured for the times indicated with or without stimulants. RNA was extracted and "dotted" as described in Materials and Methods. Dots were probed with a ^{32}P subclone of pμ12; radioautographs were exposed for 40 h with an intensifying screen at −70°C. Percent of cells with cytoplasmic IgM was determined by indirect immunofluorescence on fixed cells from replicate cultures.

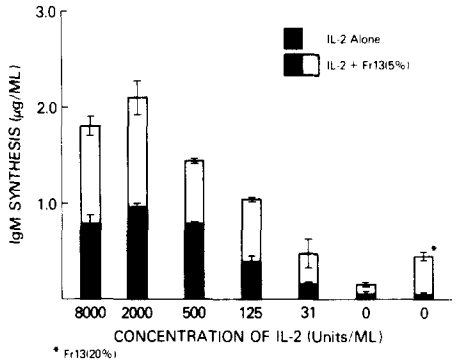

FIGURE 5. IgM synthesis in cultures containing mixtures of IL-2 and high molecular weight EL-TRF. B cells were cultured with anti-IgM, BSF-p1, and B15-TRF either with no source of EL-TRF, with molecularly cloned IL-2, with high molecular weight EL-TRF (5% of fraction 13 from SDS-PAGE), or with mixtures of IL-2 and high molecular weight EL-TRF. Concentrations of cytoplasmic IgM were measured on day 4 of culture by ELISA.

nonetheless gave very substantial EL-TRF activity in our initial studies (4). In addition, this may account for the much stronger differentiation activity of the high molecular weight fraction from Sephadex G-150. This fraction contains both high molecular weight EL-TRF, and IL-2, presumably in a dimeric form.

Expression of IL-2 Receptors on Activated B Cells. If the B cells are the direct target of IL-2, then they should express receptors for this lymphokine. We examined this in two ways. First, we tested the capacity of monoclonal antibodies to the T cell IL-2 receptor to bind to B cells; second, we tested biosynthetically radiolabeled IL-2 for binding to B cells.

B cells were stimulated with anti-IgM and BSF-p1 with or without B15-TRF

for 2 d. Cells harvested at that time consisted almost exclusively of IgM-bearing cells; no Thy-1-bearing cells were detected (Fig. 6*A*). This indicates the purity of the cell population used in these studies. B cells stimulated with anti-IgM, BSF-p1, and B15-TRF bind small but significant amounts of 3C7 (Fig. 6*B*) and somewhat greater amounts of 7D4 (Fig. 6*C*). The specificity of this binding is shown by its inhibition by preincubation with the homologous unlabeled monoclonal antibody but not by a monoclonal antibody of the same class that is not specific for the IL-2 receptor. The greater fluorescence intensity of B cells stained with FITC-7D4 may reflect the fact that FITC-7D4 has a higher fluorescein-to-protein ratio than FITC-3C7 and that it is an IgM whereas 3C7 is an IgG.

B cells incubated with anti-IgM, BSF-p1, and B15-TRF bind somewhat more FITC-3C7 and FITC-7D4 than do B cells incubated with anti-IgM and BSF-p1, only (Fig. 7). This suggests that B15-TRF may slightly enhance the expression of IL-2 receptors on activated B cells.

To compare the expression of antigens recognized by 3C7 and 7D4 on stimulated B cells and on cells from an IL-2-dependent T cell line (HT-2 cells), we carried out indirect immunofluorescence studies using FITC-MAR 18.5 to detect binding of 3C7 and 7D4. Fig. 8 illustrates results using B cells cultured with anti-IgM, BSF-p1, and B15-TRF for 3 d and HT-2 cells fed with IL-2 2 d previously. We determined the mean fluorescence intensities of HT-2 cells and of stimulated B cells stained with 3C7, 7D4, or SP2/O ascitic fluid, followed by FITC-MAR 18.5. Using these data, we estimate that HT-2 cells bound 125-fold more 3C7 and 56-fold more 7D4 than did B cells stimulated with anti-IgM, BSF-p1, and B15-TRF.

The B cell preparation used in the experiment described in Fig. 6 was also tested for its capacity to bind radiolabeled human IL-2. B cells cultured for 2 d with anti-IgM, BSF-p1, and B15-TRF were incubated with various concentrations of [^{3}H]IL-2, ranging from 0.31 to 2 nM (Fig. 9). At the highest concentration of

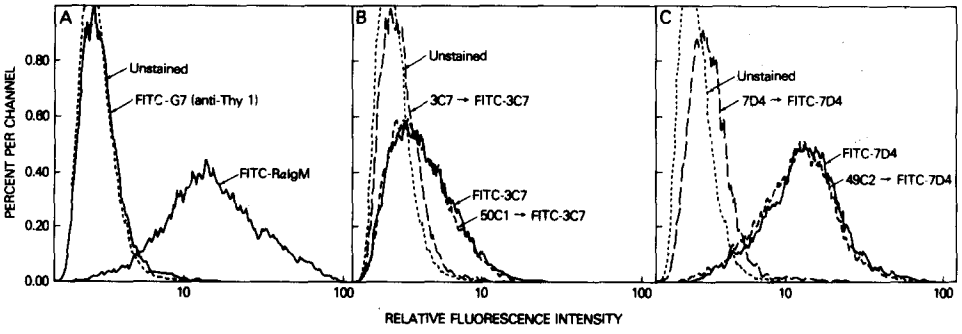

FIGURE 6. Direct immunofluorescence staining of stimulated B cells. Highly purified B cells were cultured for 2 d with anti-IgM, BSF-p1, and B15-TRF. Cells were harvested, stained, and analyzed on a FACS II. (*A*) Fluorescence histograms of cells stained with rabbit anti-mouse IgM (FITC-RαIgM) or with monoclonal rat anti-Thy-1 (G7). (*B*) Fluorescence histograms of B cells stained with FITC-3C7. Cells were preincubated with nonfluoresceinated 3C7 or 50C1. Unstained cells are included as an additional control. (*C*) Fluorescence histograms of cells stained with FITC-7D4. Cells were preincubated with nonfluoresceinated 7D4 or 49C2. Unstained cells are included as an additional control.

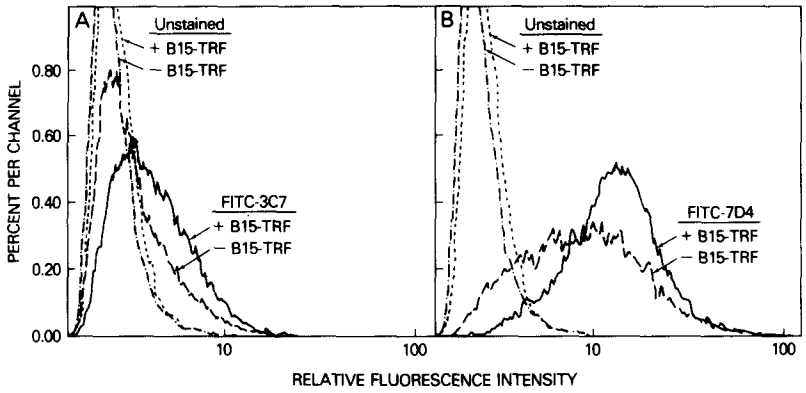

FIGURE 7. Comparisons of 3C7 an 7D4 staining of B cells stimulated with or without B15-TRF. Highly purified B cells were cultured for 2 d with anti-IgM and BSF-p1 with or without B15-TRF. The cells were harvested and stained with FITC-3C7 or 7D4. (*A*) Comparison of cells stained with 3C7. (*B*) Comparison of cells stained with 7D4.

[^{3}H]IL-2 used, we also examined binding when 200 nM cold IL-2 was present. At a total concentration of 2 nM [^{3}H]IL-2, 1,500 molecules of [^{3}H]IL-2 were bound per B cell. In the presence of 100-fold molar excess of unlabeled IL-2, this was reduced to 900 molecules per cell, suggesting that ~600 molecules per cell of [^{3}H]IL-2 were specifically bound by stimulated B cells. Similar amounts of [^{3}H]IL-2 were bound by B cells that had been cultured with anti-IgM plus BSF-p1. The binding of [^{3}H]IL-2 to HT-2 cells was also tested in these experiments. HT-2 cells expressed 59,000 receptors for IL-2 per cell; the equilibrium constant of the binding of IL-2 to these receptors was 2.1×10^{10} M^{-1} (K_d = 0.047 nM). In these experiments HT-2 cells bound ~100 times more [^{3}H]IL-2 than did B cells cultured with anti-IgM, BSF-p1, and B15-TRF. This multiple is quite consistent with that observed for the relative binding of 7D4 and 3C7 by the two cell populations. In addition, it should be noted that the binding of IL-2 to T cells was close to saturation at a free concentration of IL-2 of 0.5 nM; our results suggest that B cell binding continued to increase between 0.9 and 1.9 nM of free IL-2. However, the very small amounts of IL-2 bound per B cell make it difficult to determine whether the binding [^{3}H]IL-2 to B cells resulted from a lower affinity receptor than that detected in the binding of low concentrations of IL-2 to HT-2 cells.

Discussion

We have previously (4) demonstrated a role for two distinct differentiation factors for the induction of high rate IgM synthesis by highly purified B cells cultured with anti-IgM antibodies and BSF-p1. One of these is found in the supernatant of EL-4 cells that have been induced with phorbol esters (4). In the present studies, we found that IL-2, which is present in such supernatants at a relatively high concentration, has such EL-TRF activity. The evidence that supports this conclusion is that IL-2 and EL-TRF activity co-migrate on gel filtration and that IL-2 and low molecular weight EL-TRF activity co-electrophorese on SDS-PAGE. The activity of low molecular weight EL-TRF is inhibited

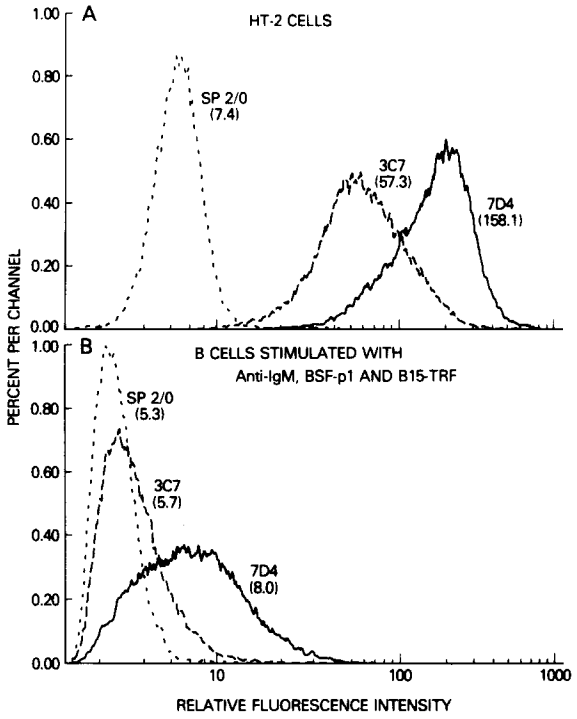

FIGURE 8. Comparison of immunofluorescence staining by 3C7 and 7D4 of stimulated B cells and HT-2 Cells. B cells were cultured for 3 d with anti-IgM, BSF-p1, and B15-TRF. HT-2 cells, a continuously growing IL-2-dependent cell line, were harvested 2 d after the last feeding. Cells were incubated with a control ascitic fluid (SP2/0) or with ascitic fluid from mice bearing 3C7 or 7D4 hybridomas. Cells were stained with FITC-MAR 18.5. Mean fluorescence intensities were calculated and are indicated next to each histogram. (*A*) Fluorescence histograms of HT-2 cells. (*B*) Fluorescence histograms of B cells stimulated with anti-IgM, BSF-p1, and B15-TRF. Relative staining was calculated according to the formula: Relative staining = (mean fluorescence of HT-2 cells stained with anti-IL-2 receptor and FITC-MAR 18.5 − mean fluorescence of cells stained with SP2/0 and FITC-MAR 18.5.)/(mean fluorescence of stimulated B cells stained with anti IL-2 receptor antibody and FITC-MAR 18.5 − mean fluorescence of stimulated B cells stained with SP2/0 and FITC-MAR 18.5). For 3C7, relative staining is 57.3 − 7.4/5.7 − 5.3 = 125; for 7D4, relative staining is 158.1 − 7.4/8.0 − 5.3 = 56.

by 3C7, a monoclonal antibody to the mouse IL-2 receptor, and molecularly cloned IL-2 exhibits EL-TRF activity which can be blocked by 3C7. Not only did IL-2 prove to have EL-TRF activity by measurement of the induction of cytoplasmic IgM, it also caused an 8–16-fold increase in levels of mRNA for the secretory form of μ heavy chains when used on B cells cocultured with anti-IgM, BSF-p1, and B15-TRF. An important observation regarding the action of IL-2 as EL-TRF is that high concentrations (100 U/ml and above) of this lymphokine are required for induction of IgM synthesis. We will discuss this point in greater detail below.

Since we used very highly purified B cells, it seemed quite likely that IL-2 was acting directly upon B cells. However, IL-2 can stimulate activated T cells to secrete factors such as BSF-p1 (27) and γ-interferon (28, 29). Thus, the possibility must be considered that IL-2 is acting indirectly, by stimulating residual T cells

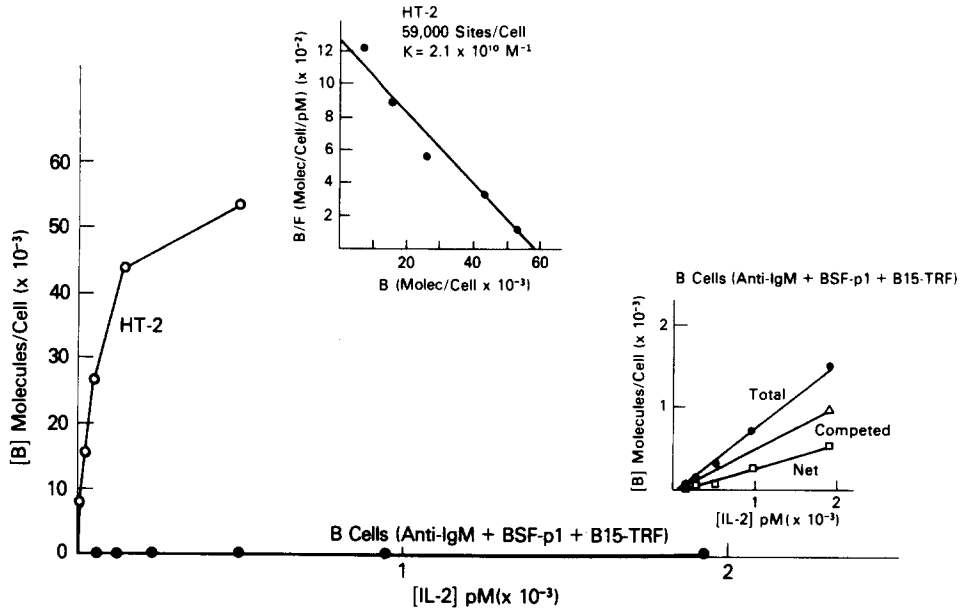

FIGURE 9. Binding of [^{3}H]IL-2 to HT-2 cells and B cells stimulated for 2 d with anti-IgM, BSF-p1, and B15-TRF. Abscissa represents the concentration of free [^{3}H]IL-2; ordinate represents the number of bound [^{3}H]IL-2 molecules per cell, obtained after correction for nonsaturable binding. Scatchard plot analysis of [^{3}H]IL-2 binding to HT-2 is also presented, where bound [^{3}H]IL-2 molecules per cell is plotted on the abscissa, and the ratio of bound [^{3}H]IL-2 molecules over free concentration of [^{3}H]IL-2 is plotted on the ordinate. The binding curve of [^{3}H]IL-2 to stimulated B cells is also presented here by using a different scale for each axis. The number of [^{3}H]IL-2 bound by B cell in the presence or absence of a 100-fold excess of unlabeled IL-2, and the net number of [^{3}H]IL-2 molecules per B cell, are presented.

to make a B cell differentiation factor. Until cloned B cell lines or single-cell assays are used, absolute proof that IL-2 acts on B cells will be lacking. However, we have shown that activated B cells express small numbers of membrane molecules that bear epitopes in common with the T cell IL-2 receptor and that activated B cells bind small numbers of IL-2 molecules. Indeed, relatively similar multiples are obtained by comparing numbers of molecules of [^{3}H]IL-2 bound by HT-2 cells and by stimulated B cells with the relative binding of 7D4 and 3C7 by these two cell populations. Both approaches suggest that HT-2 cells have 56–125-fold more IL-2 receptors than B cells stimulated with anti-IgM, BSF-p1, and B15-TRF.

The binding of 3C7 and 7D4 to B cells activated with anti-IgM and BSF-p1, with or without B15-TRF, is perhaps not surprising. Mouse B cell blasts induced with lipopolysaccharide have already been shown to bind 7D4 (13) and human hairy cell leukemias, which are B cells as judged by Ig gene rearrangement, and some human lymphoblastoid cell lines have been shown to possess the Tac antigen (30, 31), which is an epitope on the human T cell IL-2 receptor. Thus, the action of IL-2 as EL-TRF, its inhibition by 3C7, the presence of the 3C7 epitope on activated B cells, and the capacity of activated B cells to bind small amounts of [^{3}H]IL-2, all are in keeping with a direct action of IL-2 on B cells that have been cultured with anti-IgM, BSF-p1, and B15-TRF.

The high concentrations of IL-2 required for EL-TRF activity need further consideration. This requirement could be partly explained if it is postulated that B or T cell responses to IL-2 require the binding of IL-2 to a fixed number of IL-2 receptors per cell. The formation of a specific number of IL-2-receptor complexes would depend upon the free concentrations of both IL-2 and IL-2 receptors. Thus, if one cell type bore fewer IL-2 receptors than a second cell type, a greater concentration of free IL-2 would be required to cause the latter cell to achieve comparable numbers of bound receptors. However, we do not believe such an argument can completely explain the requirement for high concentrations of IL-2 to stimulate B cells. First, such an argument assumes that both B and T cell responses require that similar numbers of receptors must be occupied. No evidence exists on this point. Second, the concentrations of IL-2 required to cause B cell differentiation appear to be in excess of those necessary to saturate T cell receptors for IL-2, even when T cell IL-2 receptors are present at much higher number than B cell IL-2 receptors. We base this on calculations of the concentrations of IL-2 required for stimulating B cell activation and for saturating T cell IL-2 receptors. Cantrell and Smith (25) have reported that 1 U/ml of affinity-purified IL-2 is equivalent to 5.3×10^{-11} M.[2] Thus, 100 U/ml, the concentration of IL-2 required for effects on B cells cultured at 2×10^5/well, would be 5.3 nM. Assuming that no substantial amounts of IL-2 are consumed in these B cell cultures (consistent with bound/free ratios observed in our binding experiments), the available amount of IL-2 should be more than sufficient to saturate receptors on HT-2 cells cultured at densities similar to those at which the B cells are cultured. In the binding study presented in Fig. 9, a total concentration of ~0.8 nM of IL-2 essentially saturated the IL-2 receptors of 5×10^5 HT-2 cells. This result suggests that the affinity of the B cell receptor for IL-2 is lower than that detected on HT-2 cells. The initial binding data reported in Fig. 9 is consistent with this possibility. Such a difference is reminiscent of the affinity of binding sites for macrophage-specific colony-stimulating activity (CSA-M) when examined by competitive binding using lineage-specific CSAs. Granulocyte-specific CSA (CSA-G) competes for CSA-M binding to its receptor, but 100-fold higher CSA-G concentrations are required compared with those of CSA-M (32).

The action of IL-2 in causing B cells to differentiate to high rate IgM-synthesizing cells is dependent upon their treatment with B15-TRF. Without B15-TRF, neither enhancement in levels of mRNA for the secretory form of μ heavy chains nor the presence of cells with cytoplasmic IgM is observed. However, B cells cultured with anti-IgM and BSF-p1, without B15-TRF, do express epitopes recognized by 3C7 and 7D4 and these stimulated B cells can bind small amounts of [^{3}H]IL-2. Thus, the expression of IL-2 receptors on B cells does not appear to depend upon the presence of B15-TRF. Fluorescence histograms of binding of 3C7 and 7D4 to B cells indicate that B15-TRF-treated B cells do bind somewhat more 3C7 and 7D4 than activated B cells not cultured with this factor. Whether this modest increase in receptor expression difference accounts for the

[2] In the calculation of specific activity of [^{3}H]IL-2, it should be noted that the unit which Cantrell and Smith (25) adopted is equal to 10 of the units used in this work. Thus, their value of 1 U/ml = 5.3×10^{-10} M leads to 1 U/ml in our system being equal to 5.3×10^{-11} M.

differences in responsiveness to IL-2 or whether the action of B15-TRF as a B cell differentiation molecule is mediated through a distinct mechanism, not dependent in changes in expression of IL-2 receptors, cannot be answered at this time.

These results also support the concept that IL-2 is not limited in its function to the stimulation of proliferation. Thus, its action on B cells appears to be principally to cause Ig synthesis. We have not detected evidence that IL-2 drives B cell proliferation directly. The possibility that IL-2 causes a terminal round of B cell proliferation that may be necessary for Ig synthesis has, however, not been excluded.

Our results also indicate the existence in induced EL-4 supernatants of a high molecular weight EL-TRF. This material, which has an apparent molecular weight of 32,000 by gel filtration and SDS-PAGE, lacks both IL-2 and BSF-p1 activity. Its structure and its relation to other differentiation factors has not yet been defined. However, no interferon activity was detected in high molecular weight EL-TRF using as an assay interference of the infection of L cells by vesicular stomatitis virus (T. Hecht, personal communication). Similarly, a sample of genetically cloned mouse γ-interferon provided to us by Dr. P. Gray (Genentech, Inc., So. San Francisco, CA), did not display EL-TRF activity when added on the first day of culture.

The relative roles of high molecular weight EL-TRF and IL-2 as B cell differentiation factors in vivo will need to be carefully considered. This is particularly so because the need for very high concentrations of IL-2 to induce differentiation to high rate IgM synthesis of B cells activated with anti-IgM, BSF-p1, and B15-TRF naturally raises the question of the physiologic significance of this process. Since information regarding the local concentrations of IL-2 in the immediate vicinity of IL-2-secreting cells in vivo is not yet available, it would be premature to reach a decision on this issue. One must entertain the possibility that IL-2 is not a physiologic ligand in promoting IgM secretion, but rather, another, as yet unidentified, activity is responsible for the B cell IgM secretion mediated through B cell IL-2-binding sites.

Summary

B cells cultured with anti-IgM, BSF-p1, and B15-TRF will differentiate into high rate IgM-synthesizing cells in the presence of supernatants from EL-4 cells that have been induced with phorbol myristate acetate. These supernatants contain two molecular species (EL-TRFs) that have differentiative activity. One co-migrates with interleukin 2 (IL-2) and its activity is blocked by antibody to the IL-2 receptor. Furthermore, molecularly cloned IL-2, at concentrations of 100 U/ml or more, expresses such EL-TRF activity. The EL-TRF activity of cloned IL-2 can also be inhibited by antibody to the IL-2 receptor. The other material with EL-TRF activity has a molecular weight of ~32,000. This material lacks IL-2 activity. Antibody to the IL-2 receptor does not impair its function.

B cells stimulated with anti-IgM and BSF-p1, with or without B15-TRF, express determinants that react with two monoclonal antibodies which recognize distinct epitopes on the T cell IL-2 receptor. These determinants are present at much lower density (~100-fold) on stimulated B cells than on HT-2 cells, an IL-2-

dependent T cell line. Very small amounts of [^{3}H]IL-2 (<1,000 molecules per cell) bind to activated B cells. These results indicate that IL-2 binds to a receptor on appropriately prepared B cells and causes them to differentiate into high rate IgM-synthesizing cells. The physiologic significance of the B cell differentiative activity of IL-2 remains to be investigated.

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