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Proliferation and differentiation of single hapten-specific B lymphocytes is promoted by T-cell factor(s) distinct from T-cell growth factor

(fluorescein-polymerized flagellin conjugate/B-cell clone/T-cell hybridoma/antigen–lymphokine synergy)

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ABSTRACT **Hapten-specific B lymphocytes reactive to fluorescein were prepared from mouse spleen, placed singly in 10-μl culture wells, and stimulated with fluorescein-polymerized flagellin in the presence of conditioned media (CM) from various concanavalin A-stimulated cloned T-cell tumors or hybridomas. Antigen plus appropriate CM triggered 5–9% of the B cells into both clonal proliferation and differentiation into antibody-forming cells. Antigen alone stimulated 0.5–0.8% of B cells and CM alone stimulated <0.1%. This bioactivity was termed B-cell growth and differentiation factor(s) (BGDF). Four CM rich in T-cell growth factor (TCGF)—namely, CM from spleen and the lines EL4, T6, and 123—contained BGDF. The lines T19.1 and WEHI-3 lacked BGDF and TCGF. Four lines of evidence suggested that BGDF and TCGF were distinct molecules. First, the BGDF/TCGF ratios in the various CM varied. Second, on gel filtration, TCGF eluted as a sharp peak corresponding to a M_r of about 35,000, whereas BGDF eluted over a range corresponding to a M_r of 25,000–60,000. Third, the activity of TCGF in EL4-CM was markedly reduced by treatment with guanidine HCl while BGDF activity was not. Fourth, BGDF showed more heterogeneity than TCGF on hydrophobic chromatography. All CM or fractions active in promoting B-cell division also promoted differentiation to antibody-forming cells. These results provide unequivocal evidence that antigen and a T-cell product can synergize to directly activate a single B lymphocyte.**

The *in vitro* growth and differentiation of B lymphocytes in response to an antigenic or mitogenic stimulus can be markedly influenced by factors derived from other cell types—e.g., macrophages (1–6) or activated T lymphocytes (7–13). Despite much recent work, the number and nature of these factors remain controversial. Certain T-cell hybridomas and tumor lines can act as defined sources of factors that directly or indirectly promote B-cell growth or differentiation (7, 11, 13–15). However, the bioassay systems used for analysis of these factors suffer from a major defect. Target cell populations used contain not only B cells but variable numbers of other cell types, such as macrophages or T cells, that could exert profound effects and confuse interpretation of results.

The development of a liquid microculture system in which individual murine splenic B cells can be triggered into clonal proliferation by mitogens in the complete absence of any other cell (16) thus represented a major advance. We have recently (17) adapted this system to study the development of clones of antibody-forming cells by using specific antigen rather than mitogens as the initiating stimulus. A significant proportion of hapten-specific B cells selected by affinity fractionation responded, as single cells, to haptenated antigen, provided that medium conditioned by concanavalin A (Con A)-stimulated spleen cells (CAS) was present. We now report that certain cloned T-cell tumor lines and hybridomas secrete a factor (or factors) capable of promoting the growth and differentiation of single B cells. This activity was distinct from T-cell growth factor (TCGF).

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MATERIALS AND METHODS

Mice and Preparation of Fluorescein (FLU)-Specific Splenic B Cells. Inbred male CBA/CaHWehi mice 8–10 wk old were used. Spleen cell suspensions were fractionated on hapten/gelatin as described (18–21), FLU acting as the hapten throughout. Routine checks using the fluorescence-activated cell sorter have shown this population to be 97 ± 2% B cells.

FLU Conjugates. FLU-protein conjugates were prepared as described (19); FLU-polymerized flagellin (FLU-POL) = 0.7 mol of FLU per mol of monomeric flagellin, and FLU-gelatin = 4 mol of FLU per mol of gelatin (M_r, 100,000).

Cloned Cell Lines. The T-cell hybridoma 123 (14) resulted from fusion of lymph node cells from mice immunized with keyhole limpet hemocyanin and line BW5147. The lines T6 and T19.1 (11, 22) came from fusions of Con A-activated CBA spleen cells with the AKR thymoma TIKAUT. The EL4 line was obtained from the Salk Institute (La Jolla, CA) and has been maintained at the Hall Institute *in vitro* over many years. WEHI-3 is a cloned myelomonocytic line isolated at this Institute.

Preparation of Conditioned Media (CM). Con A-stimulated CBA spleen CM (CAS) was prepared as described (23). CM from EL4, 123, T19.1, and T6 were prepared by culture of cells at 2×10^6/ml in serum-free Dulbecco's modified Eagle's medium containing Con A at 5 μg/ml (11, 22). WEHI-3 CM was prepared by growing the cells to a density of 2×10^6/ml in medium containing 10% (vol/vol) fetal calf serum without deliberate stimulation. The CM were collected at 24 hr, filtered (Whatman GF/A glass microfiber filters), and concentrated to 1/10th vol by using an Amicon hollow fiber system (cutoff: M_r, 10,000), sterilized by Millipore filtration, and stored at −20°C. All CM were used as 10-fold concentrates.

Biochemical Methods. The gel filtration techniques used were essentially as described (22). Fractionation was carried out on a Sephadex G-75 superfine column run in phosphate-buffered saline or a Sephacryl S-300 column (each 100 × 1.5 cm) run in phosphate-buffered saline containing 6 M guanidine HCl (Gdn·HCl). EL4-CM was treated with 6 M Gdn·HCl. After 1

Abbreviations: BGDF, B-cell growth and differentiation factor(s); CM, conditioned medium (media); Con A, concanavalin A; CAS, Con A spleen cell CM; FLU, fluorescein; Gdn·HCl, guanidine hydrochloride; pfc, plaque-forming cell(s); POL, polymerized flagellin; TCGF, T-cell growth factor.

hr at 20°C, the Gdn·HCl was removed by gel filtration on a Sephadex G-25 column equilibrated in Hepes-buffered medium.

Assay for B-Cell Growth and Differentiation Factor(s) (BGDF). A cloning system that allows single B cells to proliferate and differentiate in the absence of filler cells and in the presence of antigen and added factors (17) was used to assess the BGDF activity in various CM. Microcultures were set up in 60-well 10-μl Terasaki trays using RPMI 1640 medium/5% (vol/vol) fetal calf serum/0.1 mM 2-mercaptoethanol. A mean of 1–100 FLU-enriched splenic B cells was added in 5 μl of medium containing FLU-POL at 0.2 μg/ml, the tray order was randomized, and the appropriate dilutions of the test CM or fractions were added in 5 μl. Cultures were held for 3 days in humidified 10% CO_2/90% air.

Assessment of Clonal Proliferation. After 3 days, culture wells were examined by using an inverted phase-contrast microscope at 100-fold magnification for the presence of a B-cell clone as described (17). Frequently, single B cells grew as clusters, but sometimes they grew as dispersed clones or a mixture of both. In some experiments, trays were surveyed after 18 hr of culture to detect wells containing exactly one cell, and clone formation was scored as positive if four or more cells were observed later. Where culture wells were set up to contain a small number of cells (e.g., 30) a well was scored as positive if one or more clusters of >3 blast cells was observed or if the number of cells present was clearly greater than the input number.

Assay for Anti-FLU Plaque-Forming-Cell (pfc) Clones. Cultures were assayed individually for the presence of anti-FLU pfc by transfer to 96-well flat-bottomed trays together with a mixture of haptenated sheep erythrocytes and complement (17, 24). After incubation, wells were scored for the presence of directly hemolytic IgM pfc. The frequency of FLU-specific pfc precursors was determined by Poisson analysis (18, 25).

Assay for TCGF. Assay for TCGF was performed as described (22). Two hundred T-cell blasts, prepared from Con A-stimulated CBA lymph node cells and passaged for up to 3 wk in the presence of CAS (3%), were added in 5 μl of medium to 5 μl of appropriate dilutions of the test CM. The number of viable cells was counted on day 3 by using an inverted microscope.

RESULTS

Synergy of Antigen and EL4-CM in Promoting the Clonal Proliferation of Hapten-Specific B Lymphocytes. The first aim was to show that certain T-cell tumor lines or hybridomas, when stimulated with Con A, could produce factor(s) capable of promoting the growth or differentiation or both of B cells stimulated with their specific antigen. Such activity has been demonstrated in CAS (17). Initial studies showed that CM from the T-cell line EL4 on Con A stimulation was active. Cultures of 30 FLU-specific B cells were set up in medium with FLU-POL alone, FLU-POL together with EL4-CM, EL4-CM alone, or medium alone. After 3 days, wells were scored for proliferation and assayed for anti-FLU pfc. The frequency of clone formation with FLU-POL alone (0.5%) was increased 13-fold (to 6.3%) by the addition of EL4-CM (Table 1). In medium alone or in EL4-CM alone, only 0.1–0.2% of wells contained clones. The pfc results closely resembled the proliferation results (data not shown). On purely operational grounds, we termed this synergistic capability "B cell growth and differentiation factor(s)," or BGDF. EL4-CM from unstimulated EL4 cells did not contain any significant BGDF.

Action of Antigen and EL4-CM at the Level of the Single Cell. As 97% of FLU-gelatin-binding cells are B cells, cultures of 30 cells would rarely have contained more than 1 non-B cell. To avoid any ambiguity regarding the target of action of antigen/lymphokine, a mean of 1.5–2 cells was placed in each well and, 18 hr later, wells containing exactly 1 cell were noted. This method has the advantage that Poisson statistics need not be used. As only 50–70% of dispensed B cells are detected after the initial 18 hr, this approach yields higher frequency values than that based on the imputed input cell number (16, 17). The results (Table 2) agree with those of Table 1 (based on Poisson statistics) when the early cell death is taken into account. As in our studies using CAS (17), most clones generated pfc. The question of what proportion of proliferating B cells were pfc still remains, as many clones are tight clusters of up to or >20 cells, which impedes (*i*) accurate cell counting and (*ii*) accurate pfc enumeration. Such nondispersed clusters frequently yield large plaques with an intact central cell cluster. It was, however, clear that many individual B cells were not pfc, with most clones containing some pfc and some non-pfc.

Table 1. Proliferation of FLU-specific B cells with EL4-CM and FLU-POL

	Stimulus	
	EL4-CM/ FLU-POL	FLU-POL alone
Wells, no.	120	180
Cells per well (mean)*, no.	30	30
Negative wells, %	15	86.1
Input cells proliferating†, %	6.3	0.5
95% confidence limits†	4.9–8.0	0.3–0.7

EL4-CM was used at 3% (vol/vol) (see Fig. 4) and FLU-POL was used at 0.1 μg/ml.
* Imputed cell input number at initiation of culture based on hemocytometer count, not microscopic examination of wells.
† According to the Poisson equation (25).

Screening of Other CM for BGDF Activity. CM from six different cell sources were titrated for their ability to synergize with FLU-POL to allow a significant proportion of B cells to proliferate and differentiate: murine spleen cells (CAS) and EL4 (as above); two other known sources of TCGF—i.e., the T-cell hybridomas T6 and 123 (19); a third T-cell hybridoma, T19.1, that produces a granulocyte/macrophage colony-stimulating factor but not TCGF (11); and a myelomonocytic leukemia cell line, WEHI-3, that produces factor(s) active on a range of hemopoietic progenitor and stem cells and a factor resembling lymphocyte-activating factor or interleukin 1 (26) but not TCGF. The same batches of CM were also assayed for TCGF activity. The results (Fig. 1) show that all four CM containing TCGF also contained BGDF and that, in three cases, BGDF was detectable at about 0.3% of the optimum concentration. Supraoptimal concentrations were inhibitory. Each CM showed a distinct bioactivity ratio, the T6-CM had more TCGF than BGDF and the EL4-CM had more BGDF than TCGF. Significant batch

Table 2. Proliferation and generation of pfc clones by single isolated FLU-specific B cells stimulated with FLU-POL/EL4-CM

Wells, no.	600
Input cells per well (mean), no.	1.8
Cell death in first 18 hr, %	34
Wells with exactly 1 cell, no.	
At 18 hr	217
Showing proliferation	20 (9.2%)
Showing pfc formation	18 (8.3%)

A simultaneous Poisson study of the same cell population cultured at 20 per well gave the following background frequencies (%) for proliferation and pfc clones respectively: medium alone, 0.08, 0.08; EL4-CM, 0.04, 0.17; FLU-POL, 0.76, 0.81.

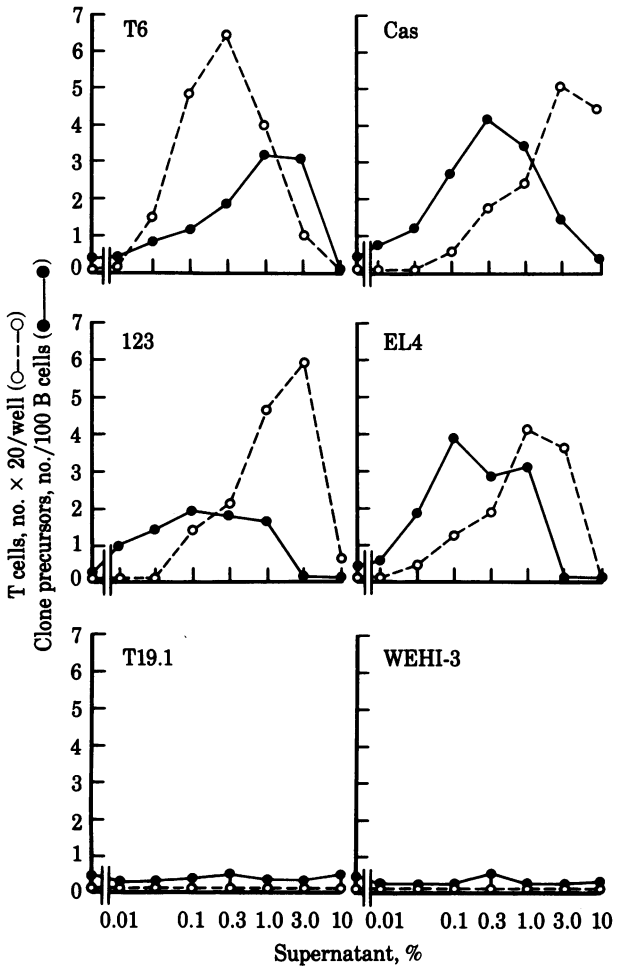

FIG. 1. Titration of BGDF (●) and TCGF (○) in CM prepared from different cell sources.

variation was noted in this respect—e.g., some batches of EL4-CM exhibited high BGDF levels but virtually lacked TCGF. The behavior of 123-CM is of particular interest as it shows strong TCGF activity but only suboptimal BGDF activity and only at low concentrations. Mixing experiments (data not shown) proved that 123-CM contains inhibitory material, effective at low concentrations, that neutralizes the BGDF activity in EL4-CM. Interestingly, this inhibitor appeared not to affect TCGF activity. Ammonium sulfate precipitation of 123-CM did not remove the inhibitory effect. CM from T19.1 and WEHI-3 showed neither BGDF nor TCGF activity (Fig. 1), and mixing experiments (data not shown) failed to demonstrate any inhibitory activity against EL4-CM or BGDF nor any detectable enhancement of growth or differentiation. CAS harvested after 5 days of culture, claimed to have no TCGF [allegedly because of adsorption by proliferating T blasts (12)] but significant B-cell stimulatory activity, in our hands, contained both BGDF and TCGF and thus was not helpful in distinguishing the two activities.

Characterization of EL4-CM BGDF by Gel Filtration. In this antigen-driven system in which CAS (17) or EL4-CM was used as the source of BGDF (Table 2), most B-cell clones formed some pfc. This suggested a strategy for the molecular characterization of BGDF. Fractions were screened in the presence of FLU-POL for BGDF using 80–100 FLU-specific B cells per well, and the mean number of pfc per well (rather than clone frequency) was determined. Although less technically demanding than carrying out a clone-frequency analysis at each concentration of each fraction, this approach may miss factors promoting division but not differentiation. Subsequent analysis of fractions at the clonal level, however, has shown the activity profiles generated to be almost identical. Fig. 2 shows the bioactivity profile of EL4-CM fractionated on Sephadex G-75. Although BGDF activity appeared to peak at about the same fractions as TCGF, corresponding to a M_r of around 35,000, BGDF activity was also detected over more than a third of the adjacent column fractions. Assay for BGDF at lower concentrations resulted in only a slight narrowing of this elution profile (data not shown).

To reduce the effect of molecular interaction between BGDF and other proteins, EL4-CM was subjected to gel filtration in the presence of Gdn·HCl. Preliminary studies showed treatment of EL4-CM with 6 M Gdn·HCl to have no effect on BGDF activity. Interestingly, the particular batch of EL4-CM used in this fractionation showed minimal TCGF activity that was completely removed by treatment with Gdn·HCl. Another batch of EL4-CM selected for good TCGF and BGDF activity was subjected to Gdn·HCl treatment. Little or no effect was demonstrable on the BGDF activity, whereas the TCGF activity was reduced by >90% (Table 3). Previous studies (22) have shown that treatment with Gdn·HCl reduced TCGF levels in 123-CM and CAS by 80–90%.

The BGDF activity profile of EL4-CM fractionated on Sephacryl S-300 in 6 M Gdn·HCl is shown in Fig. 3. No TCGF activity was demonstrable in the original sample, or in any fraction, or in fraction pools on repeated assays. To ensure that the profile obtained by the simplified method for BGDF titration was the same at the clonal level, fractions 12–31 were pooled in groups of 4, as indicated in Fig. 3, and these pools were titrated in a formal limiting-dilution assay for both frequency of proliferating clones and pfc clones (Fig. 4). Pools II and III were about as active as unfractionated EL4-CM, a result consistent with Fig. 3. Some definite BGDF activity was present in pools

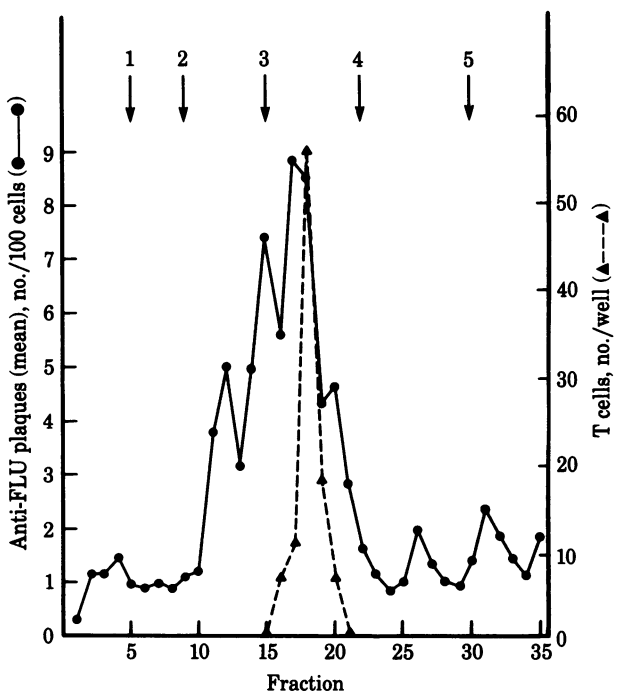

FIG. 2. Gel filtration in phosphate-buffered saline of EL4-CM on superfine Sephadex G-75. Each fraction was assayed for BGDF (●) and TCGF (▲) activity. M_r markers: 1, blue dextran (200,000); 2, bovine serum albumin (68,000); 3, ovalbumin (43,000); 4, α-chymotrypsinogen (25,000); 5, RNase (12,000).

Table 3. Effect of Gdn·HCl on BGDF and TCGF in EL4-CM

Exp.	% EL4-CM (vol/vol)	B-cell clone frequency, % Control	Treated	TCGF activity*
A	3	5.3	4.0	Undetectable
	0.3	2.8	3.1	
	0.03	1.1	1.1	
	0	0.3	0.3	
B	3	4.4	3.1	Control, 54;
	0.3	2.1	3.4	treated, <6
	0.03	1.7	0.9	
	0	0.5	0.5	

* Expressed as reciprocal of endpoint dilution.

I and IV, even at low concentrations, but no concentration produced optimal growth. The results for pfc formation were not substantially different (data not shown). Titration of pools of all other peripheral fractions showed no significant BGDF activity. No fraction favoring proliferation relative to differentiation was identified within EL4-CM.

DISCUSSION

These results provide unequivocal evidence that a T-cell-derived lymphokine can synergize with a specific antigen to cause a B lymphocyte to proliferate and produce antibody. With one exception, previous attempts to address this issue have used B-cell populations in which the possibility of some contribution from contaminating cells such as T cells and macrophages could not be formally ruled out. A culture system described by Wetzel and colleagues (7, 16, 27) having a mixture of two mitogens, *Escherichia coli* lipopolysaccharide and dextran sulfate, as the triggering stimulus allows a single B cell to be the object of study. With some batches of fetal calf serum, these mitogens, unaided by exogenous growth factors, initiate extensive proliferation in nearly every B cell, but with other batches, the addition of supernatant from a monoclonal T-cell line C.C3.11.75 (7) or peritoneal exudate cells (27) is required to achieve this high cloning frequency. The mitogen-driven system favors proliferation over differentiation, and only a proportion of the B-cell clones generate pfc (16, 17). While our present antigen-driven system resembles that of Wetzel and colleagues (7, 16, 27), it differs in some important respects. First, by using B cells chosen for their affinity for the hapten FLU, we can (*i*) use authentic antigen, not a mitogen, as a stimulus and (*ii*) measure the production of specific antibody. Second, the antigen-driven system has only a relatively low background when antigen acts alone in the absence of added lymphokines, regardless of the batch of fetal calf serum used.

The conclusion emerging (7, 12, 13) is that B-lymphocyte growth and differentiation may require the simultaneous or sequential cooperative action of two or more growth regula-

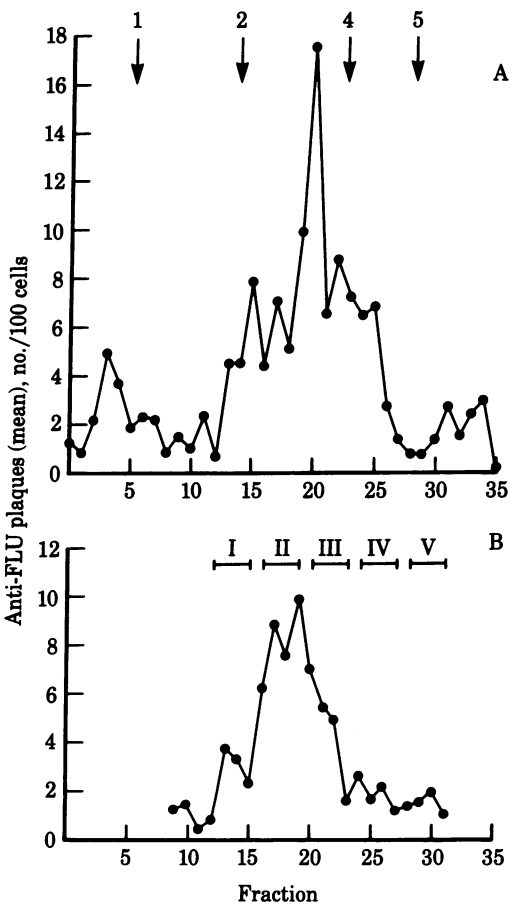

FIG. 3. Gel filtration in 6 M Gdn·HCl of EL4-CM on Sephacryl S-300. Each fraction was assayed at 3% (*A*) and 0.3% (*B*) for BGDF activity. No detectable TCGF activity was demonstrable in any fraction. M_r markers are as in Fig. 2. Pool selections are indicated: I, fractions 12–14, corresponding to M_r 67,000–94,000; II, fractions 15–19, corresponding to M_r 38,000–67,000; III, fractions 20–23, corresponding to M_r 24,000–38,000; IV, fractions 24–27, corresponding to M_r 15,000–24,000; V, fractions 28–31, corresponding to M_r 9,700–15,000.

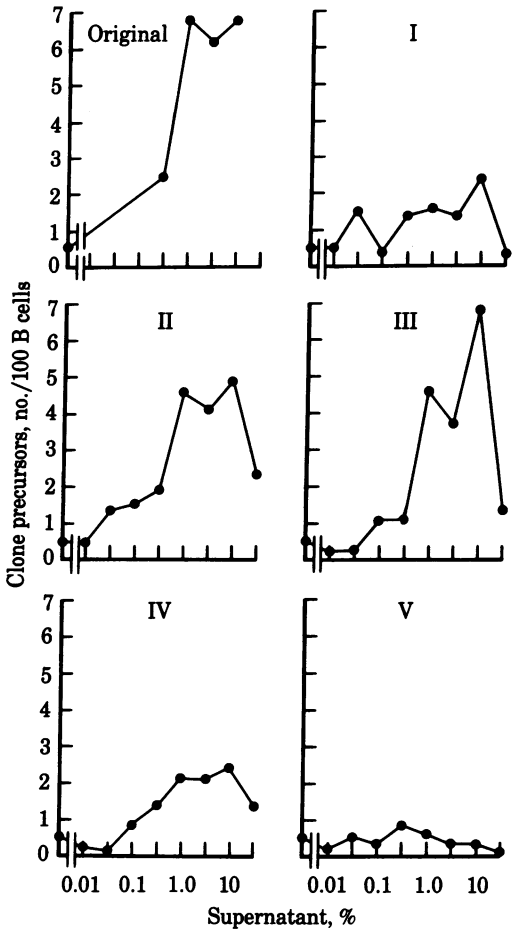

FIG. 4. BGDF activity profiles of pooled fractions (see Fig. 3) carried out at the clonal level. Each pool was titrated in the presence of FLU-POL with 30 FLU-specific B cells per culture. Data shown represent the frequency of proliferating cells.

tors—e.g., growth factors together with differentiation factors. The issue is made more complex by the possibility that different B-cell subsets respond to different sets of signals. Some aspects of the present study are consistent with a diversity of factors—e.g., the apparent heterogeneity of BGDF on gel filtration or chromatography and the observation that certain fractions may be stimulatory but reach a plateau where higher concentrations do not result in an optimal response (Fig. 4). However, the point is certainly not settled. It would be helpful to identify fractions that clearly stimulate division more than differentiation. Using other systems, other authors have claimed a major distinction between division-promoting factors and factors that aid differentiation to pfc. We have found no such distinction, as all CM or fractions promoted both division and antibody formation. As CM contain multiple factors and our attempts to characterize EL4-CM have produced such heterogeneous profiles, it is possible that separate components within the overall bioactivity termed BGDF will soon be identified. In our preliminary studies with EL4-CM fractionated by hydrophobic chromatography on phenyl-Sepharose, TCGF gave a relatively sharp peak, whereas BGDF activity showed a highly complex "saw-toothed" profile, with some activity coinciding with the TCGF peak but many TCGF-free fractions containing BGDF. Farrar *et al.* (13) report success in separating a B-cell growth factor from TCGF by this method. Howard and colleagues (10, 13), in seeking to define B-cell growth and differentiation factors, have used cultures of 10^3–10^5 cells, significantly depleted of macrophages or T cells, and anti-IgM antibodies to provide a mitogenic stimulus. The B-cell co-stimulatory activity in this system could be separated from TCGF by gel filtration (see figure 3 in ref. 10) and spread over an apparent M_r of 10,000–25,000. As we find BGDF activity in the M_r 25,000–60,000 range, with no activity at M_r 15,000 and below, the activities measured in the two systems are unlikely to be identical. Despite the incompleteness of our biochemical characterization, we believe that BGDF and TCGF are distinct. Apart from the different behaviors on fractionation discussed above, BGDF is unaffected by 6 M Gdn·HCl, whereas TCGF activity is markedly reduced. Furthermore, the widely varying relative activity levels of different sources argue in the same direction.

The search for the molecular basis of B-cell activation is just beginning and the present experimental system offers several advantages for future work. It uses authentic antigen, the physiological trigger of B-cell responses, as the initiating stimulus and an individual antigen-specific B cell as the target cell. It thus avoids the major complexities of other systems. Fetal calf serum is still required, which may contain unknown factors important in the response; however, our system is not dependent on special rare batches of fetal calf serum, with most batches tested giving comparable results. We need to define the heterogeneity in the population of target B cells and to use B cells with a higher cloning efficiency than <10%, ideally with a lower frequency of cells responding to antigen alone. If the assay could be improved in these respects, biochemical characterization of the effective lymphokine(s) could proceed rapidly.

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