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Immunology

A high-efficiency cloning system for single hapten-specific B lymphocytes that is suitable for assay of putative growth and differentiation factors

(hapten-gelatin fractionation/T-cell-independent antigens/fibroblasts/enzyme-linked immunosorbent assay)

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ABSTRACT **Fluorescein (FLU)-specific murine splenic B lymphocytes from nonimmunized adult mice were prepared by the hapten-gelatin fractionation technique and cultured singly or in very small numbers in 10-μl culture wells. Growth and differentiation to antibody-secreting status were promoted by polymeric FLU-conjugated antigens with or without added T-lymphocyte-derived conditioned media or purified cytokines. In some cultures, 3T3 fibroblasts or CBA/N thymocytes provided a source of filler cells. Anti-FLU antibody formation was detected by a sensitive enzyme-linked immunosorbent assay (ELISA). With an optimal number (around 300) of 3T3 cells per well, up to 77% of the B cells could be induced to produce detectable antibody. The ELISA permitted detection of antibody formation in essentially all wells where B-cell proliferation occurred, and it was more efficient in detecting antibody-forming clones than the hemolytic plaque assay, whether filler cells were present or not. When 10 B cells rather than 1 were included per well, the ELISA, detecting absorbance in standard fashion, provided a useful method for assessment of B-cell growth- and differentiation-promoting factors (BGDF). It was found that 3T3 cells gave less background stimulation than thymus cells, permitting the detection of as little as 1/100th as much BGDF as with thymocytes, thus offering a dynamic range of up to 30 between control absorbance in the absence of factors and the optimal factor level. Use of 3T3 cells also avoids a potential lymphokine cascade. The system has confirmed that interleukin-2 acts as a BGDF, but it has failed to establish an effect of interferon-γ on B cells. It has also shown the inactivity of a variety of hemopoietic growth factors on B lymphocytes. This system thus promises to be a useful tool in the further analysis of B-lymphocyte activation.**

It has recently become apparent that the activation of B lymphocytes into cellular division and differentiation towards antibody secretion can be profoundly influenced by a variety of B-cell growth and differentiation factors (1–14). Analysis of these factors has been rendered complex by the observation that many of them exert their effects only when 5×10^4 or more B cells are present per culture well, raising the possibility that some non-B cells in the culture may be exerting unknown effects. Following initial observations by Wetzel and Kettman (15), we have attempted to circumvent this difficulty by using single, hapten-specific B lymphocytes, in the absence of any filler, feeder, or accessory cells, as the unequivocal target for factor action (1, 16, 17). While this approach has allowed useful clarification of some of the steps in B-cell growth and differentiation, the system suffers from the major defect that the cloning efficiency is relatively low, generally permitting only 5–20% of the B lymphocytes to enter the cell cycle. This cloning efficiency was improved by adding thymus filler cells to the cultures (17, 18). However, this maneuver not only complicates interpretation through the inclusion of T lymphocytes and macrophages in the filler cell mixture but also obscures visualization of the proliferating B-cell clone.

We now report two modifications that improve the assay procedure for B-cell-active factors. Small numbers of 3T3 fibroblasts have been added as filler cells instead of thymocytes, permitting observation of B-cell proliferation while providing as much support. Second, a sensitive enzyme-linked immunosorbent assay (ELISA) technique has been used for antibody detection instead of the hemolytic plaque method. Depending on the nature of the stimulus, up to 77% of B cells can be triggered into activity, and the effect of cytokines in the system appears to be a faithful reflection of their (lesser) effect on single B cells when free of fillers.

MATERIALS AND METHODS

Mice and Preparation of Fluorescein (FLU)-Specific Splenic B Cells. Specific-pathogen-free CBA/CaH/Wehi mice, bred and maintained in our own facilities, were used as spleen donors at 8–10 weeks of age. Hapten-specific B-cell populations were prepared from spleen cell suspensions by fractionation on thin layers of FLU-gelatin as described (19, 20). Adherent FLU-gelatin was removed from the recovered binding cells by collagenase. The binding population is 97% B cells, ≈70% FLU-binding and ≈200-fold enriched for *in vitro* reactivity to FLU conjugates (1, 17, 18).

Antigens. The hapten FLU was coupled onto aminoethylcarbonylmethylated Ficoll ($AECM_{53}$Ficoll), polymerized flagellin (POL), and *Escherichia coli* lipopolysaccharide (LPS) (O111:B4, Difco) as described (18, 20). FLU_{53}Ficoll was used at a final concentration of 0.1 ng/ml, FLU-POL at 50 ng/ml, and FLU-LPS at 100 ng/ml.

EL4-Thymoma-Cell-Derived BGDF and Other Cytokines. A ×10 concentrate of medium conditioned by concanavalin A-stimulated EL4-thymoma cells prepared as described (1) was used as a source of T-cell-derived B cell growth and differentiation factors (BGDF). This is termed EL-BGDF-pik according to the recently proposed convention (21). Recombinant human interleukin 2 (IL-2) (Cetus Immune, Palo Alto, CA) was prepared and used as described (22–24). Highly purified murine P-cell-stimulating factor (PSF) was prepared as described (25) and was a gift of I. Clark-Lewis and J. W. Schrader from our laboratory. A variety of other highly purified hemopoietic growth factors—namely, puri-

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Abbreviations: FLU, fluorescein; ELISA, enzyme-linked immunosorbent assay; AFC, antibody-forming cell; PFC, plaque-forming cell; BGDF, B-cell growth and differentiation factors; PSF, P-cell-stimulating factor; POL, polymerized flagellin; LPS, *Escherichia coli* lipopolysaccharide; IL-2, interleukin 2; IFN-γ, interferon-γ; CSF, colony-stimulating factor(s) (M, macrophage; GM, granulocyte–macrophage; G, granulocyte).

fied murine macrophage colony-stimulating factor (M-CSF) (26), granulocyte–macrophage CSF (GM-CSF) (27), granulocyte CSF (G-CSF) (28), and multi-CSF (29) were prepared and kindly provided by D. Metcalf, A. W. Burgess, and N. A. Nicola. Cloned murine interferon γ (IFN-γ) (38) was kindly provided by D. Goeddel of Genentech as a supernatant medium from transfected COS cells.

B-Cell Cloning Systems. FLU-specific B cells were cultured at a mean of 0.4–4 cells per well in 60-well Terasaki trays in 10 μl of RPMI 1640 medium supplemented with 5% (vol/vol) fetal calf serum and 100 mM 2-mercaptoethanol, either in the absence of any added filler cells or in the presence of 10^5 CBA/N thymus cells as described (1, 17, 18). FLU-specific B cells were also cultured in the presence of BALB/c-3T3 fibroblast cells. The 3T3 cell line was originally provided by A. McGregor (Commonwealth Serum Laboratories, Melbourne, Australia). Routinely, 300 3T3 cells were dispensed into the culture wells in 5 μl of medium prior to the addition of the B cells to allow time for adherence. In some experiments, the 3T3 cells were x-irradiated prior to use [2000 rads (20 grays)]. To avoid intertray variance, B cells were dispensed into the wells of all trays in 5 μl of medium containing twice the required concentration of antigen. A further 5 μl of medium alone, or medium containing 10^5 CBA/N thymocytes, was then added to the appropriate trays. In nonclonal studies, hapten-specific B cells were added at a mean of 10 cells per well. Factors were added in a further 1 μl of medium.

Assessment of Clonal Proliferation. After 3–4 days, culture wells were examined, using an inverted phase microscope as described (1, 17, 18), for the presence or absence of a proliferating B-cell clone. When 300 or fewer 3T3 cells acted as filler cells, proliferating B cells could usually be distinguished because of their different morphology and substantially larger size.

Assessment of Antibody Formation. In some experiments, wells were assayed for the presence or absence of directly hemolytic anti-FLU plaque-forming cell (PFC) clones, using *in situ* plaque detection methods as described (18, 30). In the majority of experiments, we assessed antibody formation by the presence of anti-FLU antibody in the culture supernatant fluid, using an ELISA procedure similar to that used for detection of antibody-forming cell (AFC) clones by radioimmunoassay (RIA) (31). The ELISA procedure as used allows detection of AFC clones with equivalent sensitivity to RIA (unpublished data). The supernatant fluid of each culture well was individually transferred into the wells of a 96-well flexible U-bottomed polyvinyl plate (Dynatech, Alexandria, VA) precoated with FLU-bovine serum albumin at 10 μg/ml and containing 50 μl of 0.3% skim milk powder (32) in phosphate-buffered saline. After a minimum of 2 hr at room temperature, the plates were washed by immersion in phosphate-buffered saline and flicking. Horseradish peroxidase-coupled sheep antibody to murine immunoglobulin was added in a volume of 80 μl. After a minimum of 4 hr at room temperature, the plates were washed and 100 μl of the substrate 2,2'-azinodi(3-ethylbenzthiazolinesulfonic acid) (ABTS; Sigma) (diluted to 1 mM in 0.1 M citric acid, pH 4.2/0.03% H_2O_2) was added. After 1 hr the absorbance of the fluid in the wells was read with a Titertek Multiscan ML spectrophotometer (Flow Laboratories) with dual wavelengths (414 nm and 492 nm). Plates containing substrate alone were used as blanks. When adequate care was taken with the washing procedure, control wells lacking antibody consistently gave an absorbance of <0.010. Backgrounds were calculated on the basis of a large number of replicates of supernatants from wells lacking B-cell input, and a well was considered positive if its absorbance exceeded the mean ± 3 SEM of the background. The frequency of clonal precursors was determined by Poisson analysis as described (17, 33, 34). Data for nonclonal cultures (10 B cells per well) are expressed as the mean ± SEM of the absorbance of 12 replicate wells.

RESULTS

Capacity of 3T3 Cells to Support AFC Clone Formation as Assessed by a PFC Assay or by ELISA. Cultures containing a mean of 0.4–4 FLU-specific B cells were set up with either 3T3 fibroblasts or thymocytes as filler cells and FLU-LPS at 100 ng/ml as the stimulus. FLU-LPS is effective on single B cells in the absence of added lymphokines (18). After 5 days, antibody production was assessed by an ELISA or a PFC assay (Fig. 1). In all cases, the logarithm of the percentage of negative wells was linearly related to the input B cell number, showing that only one cell type was limiting. Assay of accumulated antibody in the culture supernatant by ELISA revealed more than 3 times the number of AFC clones shown by the PFC assay. Similar findings have been noted when RIA rather than ELISA was used (unpublished data). The 3T3 cells were slightly but not significantly more efficient fillers than thymocytes, allowing a 77.1% cloning efficiency with the ELISA readout.

Optimization of 3T3 Cell-Supported System. Experiments were performed to establish the optimal 3T3 filler cell number and to study the effects of x-irradiation on their support

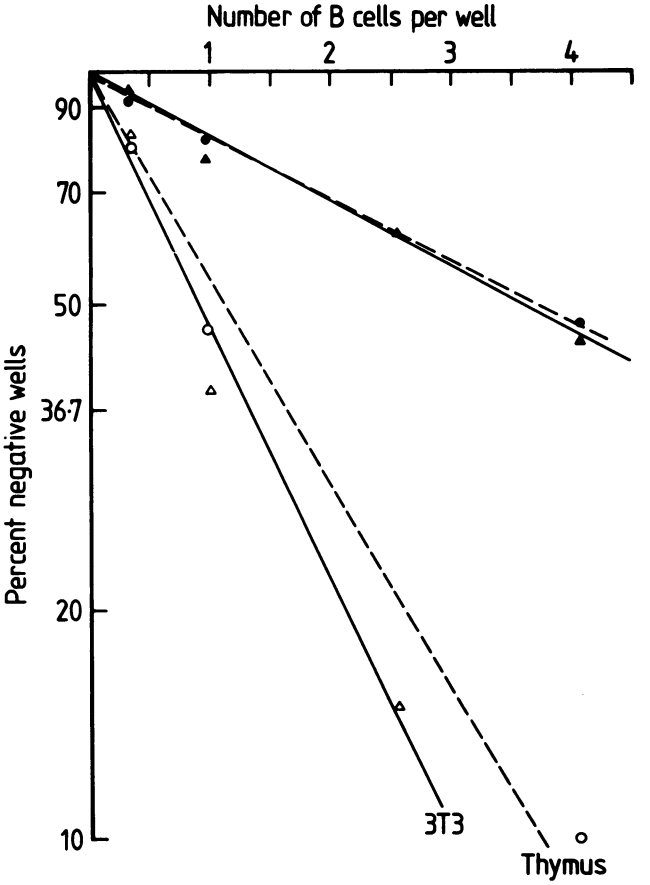

FIG. 1. Limiting dilution analysis of AFC clone development amongst FLU-specific B cells stimulated *in vitro* with FLU-LPS at 100 ng/ml in the presence of either 10^5 CBA/N thymus cells (○, ●) or 300 BALB/c-3T3 fibroblast cells (△, ▲). AFC clones were detected either by a FLU-specific hemolytic plaque method (●, ▲) or by the presence of anti-FLU antibody in the culture supernatant by an ELISA (○, △). The actual frequency values for the plaque assay were 18.9 ± 5.50% in the presence of thymus fillers and 20.0 ± 4.66% with 3T3 fibroblasts, and for the ELISA assay there were 63.5 ± 14.4% with thymus and 77.1 ± 16.7% with 3T3 cells.

Table 1. Effect of 3T3 fibroblasts on AFC clone development as detected by ELISA

3T3 cells per well	% anti-FLU AFC clones			
	Exp. I		Exp. II	
	Untreated	X-irradiated	Untreated	X-irradiated
0	8.63	8.63	8.54	8.54
30	15.5	8.78	19.8	36.0
100	25.4	15.5	41.7	31.4
300	57.1	41.6	77.4	65.7
1000	53.9	36.0	NT	NT
3000	27.9	41.6	NT	NT

Single FLU-specific B cells were stimulated with FLU-LPS in the presence of various numbers of 3T3 cells for 5 days prior to assay for AFC clones by ELISA. X-irradiated 3T3 cells received 2000 rads prior to use. NT, not tested.

capacity. The results of two such experiments are shown in Table 1. In both cases, 300 cells was found to be optimal, and nonirradiated 3T3 cells were slightly more effective. Analogous experiments using FLU-POL or FLU-Ficoll in the presence of EL-BGDF-pik as the stimuli confirmed these basic findings (data not shown).

Comparative Effects of Different Stimuli. The efficacy of 3T3 cells as fillers for responses elicited from single B cells by stimuli other than FLU-LPS was assessed. Table 2 shows the comparative data for two antigens that exhibit a requirement for BGDF for their action—namely, FLU-POL (1, 18) and a selected BGDF-dependent batch of FLU-Ficoll (24). Though the proportion of B cells forming AFC clones was not quite as high as with FLU-LPS, the 3T3 cells provided support virtually equivalent to that of thymus cells in both cases. Table 3 makes the further point that, while filler cells alone without added BGDF improved the cloning efficiency, the responses elicited by FLU-POL and FLU-Ficoll were significantly elevated by the further addition of EL-BGDF-pik. These findings are in concordance with our earlier studies using thymus filler cells and a PFC assay (17, 18).

BGDF-Dependent Antibody Formation as Assessed Under Oligoclonal Conditions. Formal clonal analysis of antibody formation is a demanding procedure, necessitating the assay of large numbers of replicate cultures. Given that our FLU-specific B-cell population contains <3% non-B cells and <0.2% T cells, and because of the high cloning efficiencies now obtainable, we thought it possible to use as many as 10 input cells per culture without running a serious risk of introducing irrelevant cells. With such oligoclonal conditions, it is possible to establish reliable mean absorbance values by using as few as 12 replicate cultures. This approach in fact provides a much more convenient means of assaying for B-cell-active factors. For example, the effect of an optimal concentration of EL-BGDF-pik on FLU-Ficoll-stimulated cultures is shown in Table 4, and Table 5 shows the effect of titration of the EL-BGDF-pik. A lower mean absorbance was obtained with FLU-Ficoll acting alone than with FLU-POL, suggesting that it is the better antigen to use in factor assays. This same difference has been noted on clonal analysis in earlier studies (24). These results also demonstrate the efficacy of the filler cells more completely than do Tables 1–3, because the absorbance reflects not only clone number but also clone size, both of which are enhanced by fillers. With 3T3 fillers the addition of as little as 0.01% of EL-BGDF-pik effectively raised the mean absorbance, whereas with thymus fillers no effect was seen because of higher background stimulation in the absence of added BGDF, which may reflect some endogenous factor production by the thymus cells *per se*.

Table 2. Comparative cloning efficiencies of FLU-specific B cells

Stimulus	% anti-FLU AFC clones		
	No fillers	3T3 cells	Thymus
FLU-LPS	7.09 ± 1.68	62.3 ± 8.74	48.6 ± 6.32
FLU-POL + EL-BGDF-pik	8.10 ± 2.24	38.8 ± 4.59	45.2 ± 10.2
FLU-Ficoll + EL-BGDF-pik	NT	37.4 ± 5.58	34.3 ± 6.99

FLU-specific B cells were stimulated as indicated, either in the absence of filler cells or in the presence of either 300 3T3 fibroblasts or 10^5 CBA/N thymocytes. Results are mean ± SEM of directly comparative experiments, four values for FLU-LPS, nine for FLU-POL + EL-BGDF-pik (10%, vol/vol), and four for FLU-Ficoll + EL-BGDF-pik. NT, not tested.

Table 3. BGDF dependence of AFC clone development in various systems

Filler cells	% anti-FLU AFC clones	
	Without BGDF	With BGDF
None	1.85 ± 0.79	12.2 ± 3.43
3T3	9.70 ± 2.02	45.2 ± 5.84
Thymus	12.3 ± 3.71	55.9 ± 13.3

FLU-specific B cells (a mean of 0.4–4 cells per well) were stimulated with FLU-POL in the presence or absence of 10% EL-BGDF-pik when cultured alone, with 300 3T3 cells, or with 10^5 CBA/N thymocytes for 5 days prior to assessment for AFC clones by ELISA. Values are from six experiments.

Assessment of Activity of Pure Growth Factors on B Cells by Using the Oligoclonal System. We have recently reported that IL-2 has significant but only moderately strong BGDF activity (24) as assessed by clonal analysis. This is confirmed by using the assay method described here (Table 6). The somewhat higher results with the thymus filler cells might have been due to IL-2 helping to initiate BGDF production by the filler cells. Table 6 also shows the results obtained using a variety of highly purified hemopoietic growth factors and lymphokines for their effects on isolated B cells. Note that multi-CSF and PSF, often termed interleukin 3 (35), were negative, as were the various factors specific for the granulocyte-macrophage lineage. The results for IFN-γ were of interest, because it has been claimed to act on B cells (10, 11). However, in these experiments no such effect was noted. These results provide a vindication of the specificity of the assay system.

DISCUSSION

The search for biologically active factors is materially facilitated by the availability of simple, reliable, and convenient bioassays. In the field of B-lymphocyte physiology, progress in defining relevant growth and differentiation factors has been slowed down by a great diversity of test systems (1–14)

Table 4. BGDF dependence of antibody formation under oligoclonal conditions

Filler cells	Absorbance × 10^3	
	Without BGDF	With BGDF
None	13 ± 6	122 ± 32
3T3	48 ± 8	807 ± 90
Thymus	214 ± 35	1179 ± 144

FLU-specific B cells at a mean of 10 per well were stimulated with FLU-Ficoll in the presence or absence of 10% EL-BGDF-pik, alone or in the presence of 3T3 or thymus cells, for 4–5 days prior to assay of culture supernatant for anti-FLU antibody by ELISA. Values are the mean ± SEM of at least 12 replicate wells in eight directly comparative experiments.

Table 5. Effect of titration of EL-BGDF on antibody formation

EL-BGDF-pik, % (vol/vol)	Absorbance × 10^3				
	FLU-POL		FLU-Ficoll		
	Filler free*	3T3 cells	Filler free*	3T3 cells	Thymus cells
0	17	126 ± 30	8	28 ± 4	217 ± 54
0.01	19	109*	0	103†	243†
0.1	33	253 ± 13	54	168 ± 29	249 ± 88
1	87	568 ± 94	72	423 ± 101	459 ± 152
10	108	1265 ± 470	120	952 ± 59	1095 ± 248
30	NT	1174†	NT	695 ± 52	918 ± 279

EL-BGDF-pik was titrated into cultures containing 10 B cells stimulated as indicated. Absorbance values were determined by ELISA 4–5 days later; results from four or five experiments are expressed as in Table 4.
*Results of a typical experiment. The response also plateaued at 10% EL-BGDF-pik in filler cell-free cultures as shown in the presence of filler cells.
†Results of two experiments.

and uncertainties as to the physiologic significance of some of these. Some systems seek to explore increased cell division rates among malignant B cells that are already dividing, or increased immunoglobulin secretion among cells that are already secreting, the relevance of which to the activation of a resting B cell could be seriously questioned. The ideal assay would have the following characteristics. (*i*) A normal resting G_0 B lymphocyte should be the unequivocal target for factor action; (*ii*) antigen, not a mitogen, should initiate activation; (*iii*) entry into G_1, S, G_2, and M phases of the growth cycle should be capable of being directly monitored, as should progressive growth of the clone; (*iv*) antibody secretion rate should be quantitated objectively; (*v*) cultures under study should not contain irrelevant cells; and (*vi*) the B cells under study should all respond uniformly.

In the present paper, we have initiated three changes that have allowed us to move closer to this ideal situation. First, a small number of 3T3 fibroblasts has replaced a large number of thymocytes as the filler cell population, permitting high efficiency B-lymphocyte cloning. Because fibroblasts adhere to and spread on the bottom of the culture well, and because 3T3 cells are substantially larger than and morphologically different from B lymphocytes, it is still possible to visualize the growing B-cell clone microscopically, though this is not as easy as in the total absence of filler cells. Furthermore, the nonlymphoid nature of the filler cell population represents an advantage for interpretation of results. Second, an ELISA has replaced the hemolytic plaque test. This permits measurement of accumulated antibody rather than current antibody-secreting status and also replaces a subjective microscopic observation with an objective spectrophotometric measurement. In the event, the ELISA also proved to be considerably more sensitive, perhaps because of its capacity to detect antibody of lower affinity. Third, as an option, the present system permits analysis of antibody formation in wells containing several B-cell clones, an approach that, while not as elegant as a full clonal analysis, provides a much more rapid and convenient screening system for potentially active factors. Oligoclonal analysis is seen not as a replacement for clonal analysis but as a useful adjunct to it.

We have not studied the nature of the effect that allows small numbers of 3T3 cells to exert their filler action. Earlier studies from our laboratory (36) had shown that normal fibroblasts and a variety of other cells are able to promote growth of B-cell colonies in agar, in line with the observations of Kurland *et al.* (37) with macrophages. It is not clear whether the cells produce some soluble growth factor that is not adequately present in fetal calf serum, or whether they exert a detoxifying influence on tissue culture media. It is evident, however, that media conditioned by filler cells cannot substitute for the cells themselves either in liquid microculture (data not shown) or in agar (36).

Our previous work using BGDF on single isolated B cells has been criticized on the grounds that the B-cell cloning efficiency in the absence of filler cells is low (1, 17, 24), raising the possibility that we may have been studying an atypical subset of B cells. Indeed, our own work has shown that the cells that yield the highest cloning efficiency under filler cell-free conditions are lymphocytes of slightly larger than median size (17), possibly activated by unknown *in vivo* events prior to initiation of culture. The fact that (depending on the stimulus) we can now provoke 40–70% of hapten-specific B cells to form AFC clones increases our confidence that factors yielding positive results are actually physiologically relevant. With any filler cell population, it remains a formal possibility that the factor is acting on the filler cell and not on the B-cell target. Even with the possibility that the 3T3 cells could synthesize factors which could influence the activation of the B cell, the use of a cloned population of

Table 6. Effects of various pure cytokines on antibody formation

Factor, units/ml*	Absorbance × 10^3													
	3T3 filler cells							Thymocyte filler cells						
	IL-2	IFN-γ	Multi-CSF	PSF	G-CSF	M-CSF	GM-CSF	IL-2	IFN-γ	Multi-CSF	PSF	G-CSF	M-CSF	GM-CSF
0	49	53	24	74	39	40	39	198	162	103	207	126	162	135
1	188	68	24	NT	NT	NT	NT	91	113	127	NT	NT	NT	NT
10	154	32	50	82	65	42	42	302	116	109	309	95	114	56
100	133	NT	34	41	79	122	57	293	NT	185	262	85	141	76
1000	208	NT	51	53	35	40	43	459	NT	173	329	70	81	37
EL-BGDF	810	686	382	934	522	753	655	1014	1103	565	1457	710	950	860

B cells at 10 per well were stimulated for 4–5 days with FLU-Ficoll in the presence of either 3T3 or thymus filler cells and the factors as indicated prior to assay for anti-FLU antibody by ELISA. Results are means for two to four experiments. NT, not tested.
*Units of activity as determined by the standard bioassay system for each factor. Titrations were performed by the providers of the factors (24–29, 38).

nonlymphoid fillers is still preferable to a heterogeneous population of lymphoid tissue-derived fillers. From this viewpoint, it should be noted that all positive results can readily be checked in the less efficient filler cell-free system.

In other studies (24) we have drawn attention to the fact that all alleged growth factors and all alleged differentiation factors that have had any effect in our single B-cell system have caused both growth and differentiation, casting doubt on the current paradigm, which sees growth and differentiation factors as separate entities. In the present high-efficiency system we have noted that essentially all wells containing proliferating B cells score positive in the ELISA. This is not to say that a "pure" growth or "pure" differentiation factor may not be defined in the future. It is evident from the literature (1–14) that at least four or five different cytokines can affect B cells. Only some of these are currently available in pure form. Working out possible synergistic effects between factors, and the exact role of each factor in the sequential events following B-cell activation, remains a major challenge for the future. Given logistic considerations, we see the oligoclonal approach in conjunction with ELISA as a promising tool in this regard.

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