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Interleukin 4 (B-cell growth factor II/eosinophil differentiation factor) is a mitogen and differentiation factor for preactivated murine B lymphocytes

(B-cell activation/lymphokines/anti-Ig)

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ABSTRACT **Recently we described a murine T-cell hybrid that produces activities that (*i*) promote the differentiation of eosinophils (eosinophil differentiation factor) and (*ii*) cause proliferation of the BCL_1 B-cell lymphoma (B-cell growth factor II activity). Both activities appear to be associated with the same molecule, which has therefore been termed interleukin 4. The hybrid does not produce any other known lymphokines. We now find that purified interleukin 4 has no effects on small resting B cells but induces naturally occurring large B cells (which have presumably been preactivated *in vivo*) to synthesize DNA and to secrete IgM and low levels of IgG. B cells activated by anti-Ig antibodies apparently only become responsive to the factor once they have reached late G_1 stage. All bioactivities of interleukin 4 are associated with a protein of M_r 44,000 (by $NaDodSO_4$/PAGE). Therefore these results demonstrate that this lymphokine alone is sufficient to induce clonal expansion and maturation of activated B cells.**

There is considerable evidence that T-cell-derived lymphokines are involved in the control of B-cell proliferation and differentiation (1–3). However, the interrelationships of the numerous factors that have been proposed to act on B cells are still uncertain. A major source of the confusion surrounding this field is the difficulty in obtaining pure factors, since most lymphokine-producing T cells secrete a complex mixture of activities. Thus, at least two types of T-cell-derived B-cell growth factor (BCGF) have been described. Murine B-cell-stimulating factor type 1 (BSF-1) acts on resting B cells and drives them to a transitional activated state characterized by the expression of high levels of Ia antigens (4, 5). In combination with antibodies to surface Ig receptors (anti-Ig), it induces B cells to synthesize DNA (3, 6–8).

The second factor [BCGF type II (BCGF II)] was originally detected by its capacity to induce proliferation of the murine B-cell lymphoma BCL_1 *in vitro* (9, 10). Its effects on normal B cells have not been well-defined, since it is difficult to separate BCGF II from other potentially relevant factors. We have recently described a novel source of BCGF II, which is free of any other lymphokine believed to act on B cells. This activity is produced by a murine T-cell hybrid NIMP-TH1, which was originally selected for its capacity to secrete an eosinophil differentiation factor (EDF; refs. 11 and 12). The BCGF II and EDF produced by these cells copurify in every fractionation procedure employed: both activities are associated with protein(s) with an approximate M_r of 44,000 (ref. 12) and a pI of 5.0. These findings, together with earlier evidence that BCGF II and EDF are coordinately produced by a large panel of T-cell clones (13), strongly suggested that the two activities are due to the same molecule, which has therefore been named interleukin 4 (IL-4; ref. 12). We have now tested the effects of partially (and highly) purified IL-4 on normal murine B cells. The results indicate that this lymphokine does not affect resting B cells but induces DNA synthesis and Ig secretion in naturally occurring large B cells. The factor also causes DNA synthesis in B cells stimulated by anti-Ig for >36 hr, thus suggesting that it acts late in the G_1 phase of the cell cycle.

MATERIALS AND METHODS

Mice. Male BALB/c, CBA/Ca, and (CBA/Ca × C57BL/6)F_1 mice were bred under specific pathogen-free conditions at the National Institute for Medical Research and were used when 3–4 months old.

Culture Medium. RPMI 1640 medium was supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, nonessential amino acids, 0.05 mM 2-mercaptoethanol, penicillin, streptomycin, and 5% fetal calf serum.

Reagents. The preparation of affinity-purified $F(ab')_2$ fragments of rabbit anti-mouse Fab antibodies (henceforth called anti-Ig) and of fluorescein isothiocyanate-coupled monoclonal anti-I-A^k antibody have been described (14). Lipopolysaccharide (LPS; *Escherichia coli* 055:B5W) was from Difco, and [^{3}H]thymidine (specific activity, 5 Ci/mmol; 1 Ci = 37 GBq) was from Amersham International.

Preparation of B Cells. These were prepared from spleens of (CBA × C57BL)F_1 mice (unless otherwise specified) as described (14, 15). Small dense B cells were taken from the 75%/85% interface of a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient (16), and large B cells were from the 65%/50% interface. These B-cell preparations contained 83–90% surface Ig-bearing cells and no Thy-1-positive cells; the remainder were undefined null cells. In some experiments, the small dense B cells were additionally filtered through two consecutive columns of Sephadex G-10 to remove adherent cells (14).

Lymphokine Preparations. Two lymphokine sources were used: (*i*) IL-4 from NIMP-TH1 cells incubated at 5×10^5 per ml with 10 ng of phorbol 12-myristate 13-acetate (Sigma) per ml for 48 hr (ref. 11) and (*ii*) conditioned medium (CM-T2) from an alloreactive T-cell clone NIMP-T2 (ref. 17), which produces high titers of BSF-1 and IL-4 and very low levels of IL-2 and IL-3.

Purification of IL-4. NIMP-TH1 does not produce detectable amounts of IL-1, IL-2, IL-3, interferon-γ (11), or BSF-1 (unpublished results). Nevertheless, for most experiments

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Abbreviations: IL, interleukin; BCGF II, B-cell growth factor type II; BSF-1, B-cell-stimulating factor type 1; EDF, eosinophil differentiation factor; anti-Ig, anti-immunoglobulin antibodies; CM-T2, conditioned medium from the T-cell clone NIMP-T2; LPS, lipopolysaccharide; pfc, plaque-forming cell(s).

IL-4 was purified (12) from supernatants by fractional $(NH_4)_2SO_4$ precipitation, followed by affinity chromatography on lentil lectin-Sepharose and gel filtration through AcA 54-Sepharose (LKB, Croydon, Surrey) in phosphate-buffered saline (PBS) containing 50 μg of polyethylene glycol per ml. This preparation is called partially purified IL-4.

This was further purified for some experiments by reverse-phase high-pressure liquid chromatography (HPLC), using a μBondapak C_{18} column (Waters Associates), eluted with a gradient of 25–60% acetonitrile in 0.1% trifluoroacetic acid. Active fractions were pooled and run on a 10% nonreducing polyacrylamide gel in the presence of $NaDodSO_4$ (ref. 18). One portion of the gel was reserved for silver staining (19), and the remainder was sliced into 1-mm slices that were eluted with 0.5 ml of PBS containing 5% fetal calf serum and tested for lymphokine activities. IL-4 was monitored throughout the purification by assaying BCGF II activity using the *in vivo* passaged BCL_1 lymphoma line (9) and for EDF activity by the production of eosinophils in bone marrow cultures (17). A unit of BCGF II is defined as the concentration of factor that gives half-maximal proliferation of BCL_1 cells.

B-Cell Culture Systems. Assays for B-cell activation were performed as described (14): analyses of Ia antigen levels by flow microfluorimetry and entry into cell cycle by means of two-stage priming cultures. In the latter, cultures primed for 24–48 hr were washed, and 5×10^4 viable cells were recultured with further stimuli as indicated. DNA synthesis was determined by measuring [^{3}H]thymidine (0.5 μCi per well) incorporation after a 4-hr labeling period.

For assays of Ig secretion, large B cells, obtained from the 65%/50% interface of a five-step Percoll gradient, were cultured at 10^5 cells per well in 200 μl of supplemented RPMI 1640 medium containing 15% fetal calf serum, with LPS (10 μg/ml), lymphokine preparations, or medium alone. On days 4–7, cultures were assayed for total IgM- and IgG-secreting cells by the reverse plaque-forming cell (pfc) assay (20, 21).

RESULTS

IL-4 Induces Large but not Small Dense B Cells to Synthesize DNA. We first tested the capacity of semipurified IL-4 to induce proliferation in B cells of different densities, obtained by Percoll density-gradient centrifugation (Fig. 1). The factor did not induce DNA synthesis in small dense B cells, although these cells did respond to costimulation with anti-Ig plus CM-T2, which contains BSF-1 and IL-4 (numbers in parentheses). However, IL-4 stimulated marked DNA synthesis in large (low density) B cells, obtained from the 65%/50% interface of the gradient, inducing a response that was comparable to that elicited by CM-T2 plus anti-Ig.

IL-4 Does not Activate Resting B Cells. We next studied whether IL-4 could induce small dense B cells to leave G_0 stage. It is now clear that BSF-1 alone markedly increases the levels of Ia antigens on murine B cells, which is an early indicator of B-cell activation (4, 5). Table 1 shows that culturing small dense B cells for 24 hr with IL-4 had no effect on their levels of Ia antigen. However, CM-T2, presumably because it contains BSF-1, induced a marked response, which was comparable to that provoked by an optimal dose of anti-Ig.

Another way to demonstrate that resting B cells have entered the cycle is the two-stage priming culture system (14): cells are activated (primed) for 24–48 hr with various stimuli, washed, and then recultured with a mitogen in "readout" cultures. Cells that have entered the cycle will commence DNA synthesis earlier in response to restimulation. Fig. 2 shows that IL-4 did not prime small dense B cells to respond to a mitogenic concentration of anti-Ig. In contrast, and as expected, anti-Ig or CM-T2 (presumably because the latter contains BSF-1; see ref. 23) induced significant activation. Priming with CM-T2 in combination with a nonmitogenic concentration of anti-Ig produced a synergistic effect (data not shown; ref. 22). Therefore, these results indicate that resting B cells can be activated by BSF-1 (with or without anti-Ig) but not by IL-4.

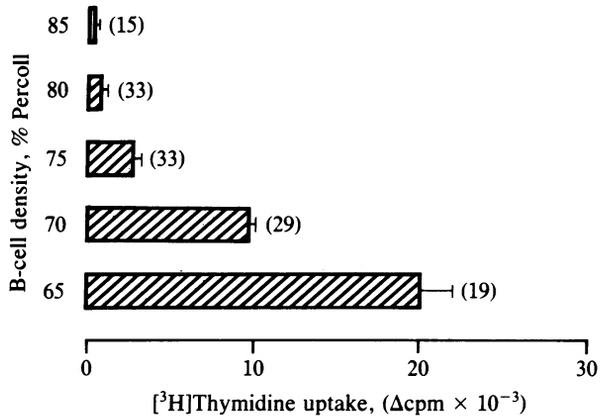

FIG. 1. IL-4 induces large but not small dense B cells to synthesize DNA. B cells were fractionated on discontinuous gradients of 85%, 80%, 75%, 70%, and 65% Percoll (approximate densities: 1.105, 1.099, 1.093, 1.087, and 1.082 g/ml). These fractions (83–88% Ig-positive) were cultured (at 5×10^4 cells per 200 μl) with or without partially purified IL-4 (2 units/ml). [^{3}H]Thymidine uptakes (mean ± SEM, $n = 3$) were measured on day 3 and are presented with backgrounds (210–3600 cpm) subtracted. Numbers in parentheses are responses of identical cultures stimulated with 5% CM-T2 plus anti-Ig at 1 μg/ml.

Small Dense B Cells Activated with Anti-Ig Become Responsive to IL-4. We then tested the proliferative responses of B cells activated by anti-Ig to restimulation with IL-4 (Fig. 3). B cells primed with 2 or 10 μg of anti-Ig per ml for 24 hr did not respond to IL-4 in the readout cultures but responded well to anti-Ig. However, if the cells were activated for 40 hr with 10 μg of anti-Ig per ml, they then responded to IL-4. In contrast, cells primed with 2 μg of anti-Ig per ml for 40 hr did not respond to the factor. Comparable results were obtained with B cells depleted of adherent cells by two cycles of Sephadex G-10 gel filtration (data not shown).

IL-4 Induces Large B Cells to Secrete IgM and IgG. In the light of the above findings it became important to establish if preactivated B cells could also be induced to secrete antibody

Table 1. Effects of BCGFs on Ia antigen levels on B cells

Addition	Median fluorescence intensity
None	57
IL-4	
5 units/ml	49 (−8)
2 units/ml	51 (−6)
0.5 unit/ml	55 (−2)
0.1 unit/ml	59 (2)
CM-T2	
5%	112 (55)
1%	111 (54)
Anti-Ig, 10 μg/ml	95 (38)

Small dense B cells obtained from CBA/Ca mice were cultured for 24 hr with the indicated concentrations of partially purified IL-4, CM-T2, or anti-Ig. Cells were then stained with a fluoresceinated monoclonal anti-I-A^k antibody and analyzed by flow microfluorometry (14). Median fluorescence intensities were determined from computer-generated histograms and are expressed as channel numbers, from 0 to 256: 51 channels are equivalent to a difference of 1 logarithm in fluorescence intensity. Numbers in parentheses represent differences (Δ) between controls and experimental cultures.

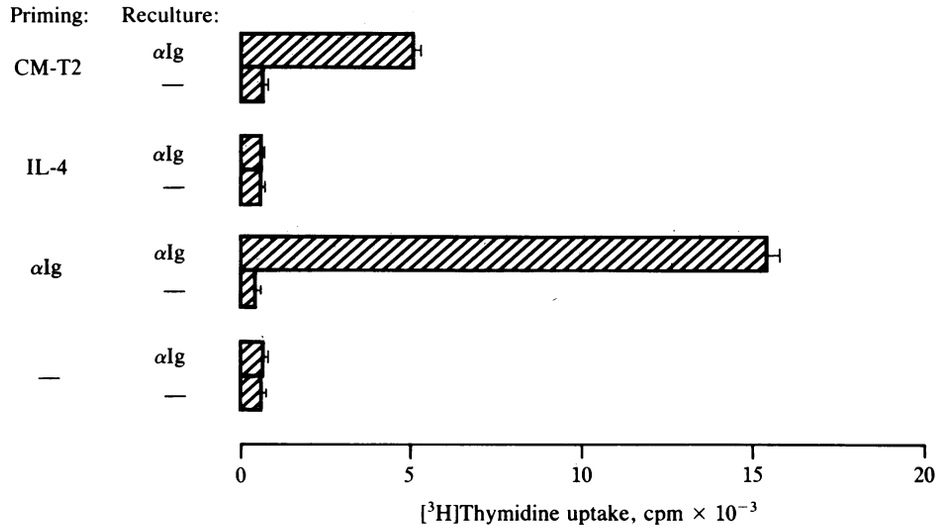

FIG. 2. Activation of B cells with growth factors for responsiveness to anti-Ig. Small dense B cells, obtained as in Table 1, were cultured (primed) for 24 hr with partially purified IL-4 (2 units/ml), CM-T2 [5% (vol/vol)], anti-Ig (αIg) (10 μg/ml), or medium alone. Cells were then washed, counted, and recultured at 5×10^4 per 200 μl with medium alone or with anti-Ig (50 μg/ml). [^{3}H]Thymidine incorporation in these second cultures was assayed after 24 hr. Results are expressed as mean ± SEM of triplicate cultures.

by IL-4. Large (low density) B cells were therefore cultured with IL-4, CM-T2, or LPS, and the cultures were then assayed for IgM- and IgG-secreting cells. Such cultures contained small numbers of IgM pfc on day 0, which increased markedly after culture with LPS or factors, and reached a plateau on days 5–7 (unpublished data). After 7 days of culture with IL-4, CM-T2, or LPS, the cultures produced large numbers of IgM pfc and significant levels of IgG pfc (Table 2). In contrast, small dense B cells responded to LPS but not to IL-4 (not shown).

Proliferation and Differentiation Activities of IL-4 Copurify. In an effort to establish if the growth-promoting and differentiation activities of IL-4 are due to the same molecule, partially purified IL-4 was further fractionated by reverse-phase HPLC, followed by NaDodSO$_4$/PAGE. The eluates from the gel slices were tested for EDF (data not shown) and BCGF II and also for their capacity to cause large B cells to synthesize DNA or to secrete Ig (Fig. 4). All of these activities comigrated in the same fractions, corresponding to a major band of protein with an approximate M_r of 44,000, thus strongly suggesting that they are all properties of a single molecule.

DISCUSSION

Murine BCGF II was originally defined by its capacity to induce proliferation of *in vivo* passaged BCL_1 lymphoma cells (9). It also synergizes with the polyclonal activator dextran sulfate to promote DNA synthesis in normal B cells (10). It was, however, not established if BCGF II behaves as an activating factor (like BSF-1) or as a late-acting growth factor in this latter system. Our results unequivocally demonstrate that IL-4, which we have used as our source of BCGF II, does not affect resting B cells (Fig. 2, Table 1) but induces DNA synthesis and Ig secretion in naturally occurring large (presumably preactivated) B cells (Fig. 1, Table 2) and stimulates

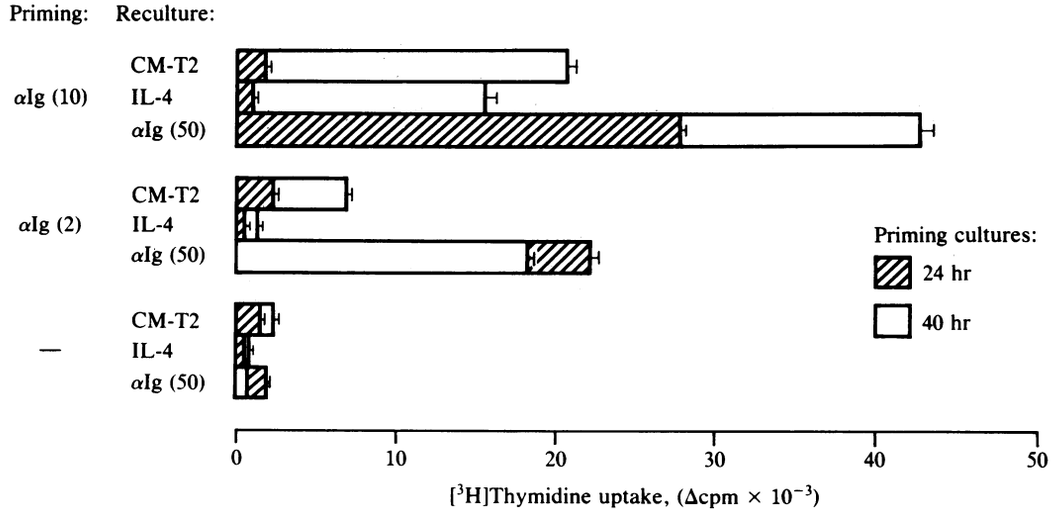

FIG. 3. Activation of B cells by anti-Ig for responsiveness to IL-4. Small dense B cells were cultured for 24 hr (hatched bars) or 40 hr (open bars) with 10 or 2 μg of anti-Ig (αIg) per ml or medium alone. Cells were then washed, counted, and recultured with medium alone, CM-T2 (5%), partially purified IL-4 (1 unit/ml), or 50 μg of anti-Ig per ml. (Numbers in parentheses represent μg of anti-Ig per ml.) [^{3}H]Thymidine incorporation in these readout cultures was assessed after 24 hr. Responses are given as Δcpm—i.e., with backgrounds (500–1500 cpm) subtracted.

Table 2. IL-4 induces large B cells to secrete IgM and IgG

Addition	pfc per culture			
	Experiment 1		Experiment 2	
	IgM	IgG	IgM	IgG
None	1,134 ± 39	0	549 ± 63	4 ± 0.8
IL-4, 2 units/ml	10,835 ± 374	635 ± 55	6,530 ± 534	710 ± 72
CM-T2, 5%	25,333 ± 785	2323 ± 4	50,250 ± 4442	9283 ± 785
LPS, 10 μg/ml	26,900 ± 393	2040 ± 52	59,750 ± 3816	3210 ± 248

Large B cells (from the 65%/50% interface of Percoll gradients) were cultured at 10^5 cells per 0.2 ml with the indicated concentrations of partially purified IL-4, CM-T2, LPS, or medium. Cultures were fed with 100 μl of fresh medium containing factors on day 4. IgM and IgG pfc were assayed on day 7. Results are expressed as mean ± SEM of triplicate cultures.

DNA synthesis in B cells appropriately activated by anti-Ig (Fig. 3). Furthermore, all of the bioactivities of IL-4 are associated with a protein band on $NaDodSO_4$/PAGE with a M_r of 44,000 (Fig. 4).

Recently, Muller *et al.* (24) found that a factor of 40–50 kDa produced by the EL4 cell line induced proliferation and IgM secretion in B cells activated for 48 hr by anti-Ig. This appeared to be distinct from IL-2 and BSF-1 but has not been extensively purified. They did not test its effects on naturally occurring large B cells. However, Layton *et al.* (25) have reported that crude supernatants from various T-cell sources stimulated DNA synthesis and Ig secretion in such B cells. In our experience, complete separation of BCGF II and BSF-1 (from EL4 cells or CM-T2) by physicochemical means is extremely difficult. The NIMP-TH1 cell line does not produce any other lymphokine believed to act on B cells apart from IL-4, which therefore obviates this problem. Furthermore, highly purified IL-4 retains all of the activities of the crude material (Fig. 4), thereby effectively eliminating any potential contribution of residual phorbol ester to the observed effects.

We further conclude that IL-4 acts on B cells late in the G_1 phase of the cell cycle. This is based on the following argument. It is known that anti-Ig causes dose-dependent cell-cycle progression of B cells (16, 26). In our hands, B cells need to be exposed to a mitogenic concentration (*ca.* 50 μg/ml) of anti-Ig for at least 36 hr before becoming committed to DNA synthesis. Ten micrograms of anti-Ig per ml stimulates a substantial proportion of B cells to progress through G_1 stage, whereas 1–2 μg/ml induces the cells to leave G_0 stage but not to progress substantially through G_1 stage (16). Therefore, the observation that B cells become responsive to

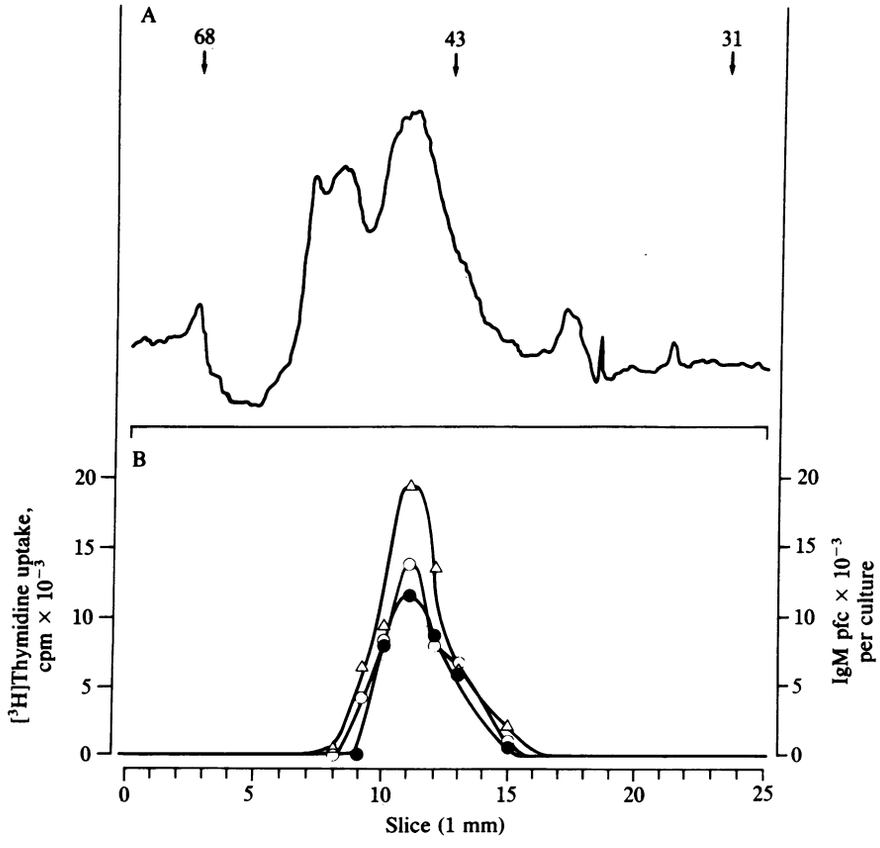

FIG. 4. PAGE of IL-4. Pooled active fractions from reverse-phase HPLC were separated by $NaDodSO_4$/PAGE under nonreducing conditions. (*A*) Densitometric scan of a silver-stained portion of the gel, with positions of molecular weight markers ($M_r \times 10^{-3}$) indicated. (*B*) Eluted material from each gel slice was assayed (at a final dilution of 1:40) for BCGF II, using the BCL_1 assay (●), and for its capacity to induce DNA synthesis (○) or IgM pfc (△) in large B cells isolated as in Fig. 1.

IL-4 after >30 hr of priming with anti-Ig at 10 μg/ml (and not 2 μg/ml) (Fig. 3) strongly suggests that IL-4 acts as a mitogen on cells in late G_1 stage. This, however, needs to be confirmed by cell-cycle analyses.

It obviously now remains to be established if the molecule responsible for the IL-4 activity produced by NIMP-TH1 is the same as those with BCGF II activity described by others (9, 24, 27). The interrelationships of BSF-1 and BCGF II in the stimulation of B cells also remain to be elucidated. Since BSF-1 with anti-Ig (presumably mimicking the effects of antigen) is sufficient to cause B cells to synthesize DNA (3, 6–8), the role of BCGF II is puzzling. Earlier studies had suggested that proliferation and maturation to antibody secretion in B lymphocytes are controlled by different lymphokines (9). Recent evidence, however, indicates that the proliferative and differentiation activities of BCGF II cannot be separated by a variety of biochemical techniques (24, 27). In agreement with this, IL-4 not only stimulates proliferation of large B cells recovered from Percoll gradients (Fig. 1) but also evokes the maturation of IgM-secreting and lesser numbers of IgG-secreting cells (Table 2). Both BCGF and differentiation activities of the lymphokine are not separable by a variety of biochemical techniques, including reverse-phase HPLC and $NaDodSO_4$/PAGE (Fig. 4). We have recently shown that IL-4 also acts as a T-cell-replacing factor, enabling T-depleted B cells to produce an antibody response to sheep erythrocytes (to be reported elsewhere).

In conclusion, it also remains to be shown if this factor exerts distinct signals for growth and differentiation in B cells, since it is possible that it merely prolongs proliferation sufficiently to allow the cells to secrete Ig. Furthermore, although it seems likely that IL-4 acts directly on preactivated B cells, we cannot at present exclude the possibility that it may synergize with other factors produced by residual accessory cells contaminating our culture system. Clearly, much further work is required to establish the precise physiological role of this lymphokine in B-cell growth and maturation.

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