INTERLEUKIN 2 INDUCES ANTIGEN-REACTIVE T CELL LINES TO SECRETE BCGF-I

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Regulation of B cell immune responses requires an understanding of the cellular and molecular processes that govern such events. The role of T lymphocytes in B cell development has been recognized for over a decade, but recently this concept has matured to encompass a battery of antigen-nonspecific genetically unrestricted T cell–derived factors that regulate B cell function (1–16). We have identified one such factor, initially designated B cell growth factor (BCGF),[1] which appears to be required for the proliferation of a population of splenic B cells (13). This factor has recently been renamed BCGF-I to distinguish it from a second B cell proliferation cofactor, BCGF-II (17). BCGF-I synergizes with anti-IgM antibodies to cause polyclonal B cell proliferation (13). It appears to act on early G_1 phase B cells (18) and does not lead to development of Ig-secreting cells in the absence of additional factors (13, 16). It is present in induced supernatants of the cloned murine thymoma EL4 (13) and thus is presumably T cell derived.

To explore the mechanism of production of BCGF-I in a normal antigen-driven immune response, we have examined in detail the products of a continuously growing T cell line following antigenic stimulation. Antigen-reactive T cell lines can be generated by serial restimulation of antigen-primed T cells with irradiated spleen cells plus antigen, followed by antigen-free periods when the cells are maintained on irradiated spleen cells alone (19). Such lines are capable of supporting a variety of in vitro B cell responses, and thus seemed likely candidates for potential BCGF-producers. A T cell line specific for the protein antigen ovalbumin (OVA) was produced and tested over the course of 18 mo. When the line was stimulated with OVA in the context of syngeneic antigen-presenting cells (APC), it rapidly produced levels of BCGF-I comparable to those in induced EL4 supernatants. As few as 10^4 T cells produced sufficient BCGF-I to signficantly enhance proliferation of 5×10^4 purified anti-Ig activated B cells. Antigen-reactive T cells produced large amounts of BCGF-I in the absence of presented antigen if instead they were provided with purified preparations of interleukin 2 (IL-2). From these and our previously published results, we propose

[1] *Abbreviations used in this paper*: APC, antigen presenting cells; BCGF, B cell growth factor; IEF, isoelectric focusing; IL-2, interleukin 2; OVA, ovalbumin; PMA, phorbol myristate acetate.

a model for the cellular and molecular chain of events leading to B cell proliferation in an antigen-induced immune response.

Materials and Methods

Mice. BALB/c, B10.A, B10.S(9R), and CBA/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). CBA/N mice were obtained from the Division of Research Services, National Institutes of Health. All mice were used at 8–12 wk of age.

Anti-IgM. Affinity column-purified goat antibody specific for mouse μ heavy chains (anti-μ) was prepared as previously described (20).

Factor Preparations. EL4 supernatant was obtained by stimulating a cloned subline of EL4 thymoma with 10 ng/ml phorbol myristate acetate (PMA) as described elsewhere (21). For some experiments, an IL-2-rich, BCGF-I-free component of these supernatants was obtained either by isoelectric focusing (IEF) or by phenyl Sepharose chromatography according to procedures published elsewhere (22, 23). Purified human IL-2 kindly provided by Dr. Kendall A. Smith (Dartmouth Medical School, Hanover, NH) was prepared as follows. IL-2–containing supernatant was obtained from a phytohemagglutin (1.5 μg/ml, Wellcome Reagents Limited, Beckenham, England) and PMA (50 ng/ml, Consolidated Midland Corp., Brewster, NY) stimulated human leukemic T cell line (Jurkat subclone 6.8). This supernatant was concentrated and applied to an immunoaffinity column constructed with an IgG_{2a} monoclonal antibody to human IL-2. Bound IL-2 was eluted with 0.2 N acetic acid. The eluted material appeared to be a single polypeptide, 15,500 (M_r) when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and showed a single spot on two-dimensional PAGE. For all preparations of IL-2, activity was measured via proliferation of the IL-2-dependent T cell clone HT2, as detailed elsewhere (13).

Culture Medium. The culture medium used throughout was RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (Reheis, Kankakee, IL), penicillin (50 μg/ml), streptomycin (50 μg/ml), gentamicin (100 μg/ml), L-glutamine (200 mM), and 2-mercaptoethanol (5×10^{-5} M).

Preparation of Antigen-reactive T Cell Lines. A continuously growing OVA-reactive T cell line was produced and maintained according to the procedure of Kimoto and Fathman (19). Briefly, BALB/c mice were immunized in the footpad with 100 μg OVA emulsified in complete Freund's adjuvant (Difco Laboratories Inc., Detroit, MI). Draining lymph nodes were removed 7 d later, T cells were purified by passage over nylon wool columns (24), and then cultured in medium containing OVA 100 μg/ml and γ-irradiated (3,300 R) syngeneic spleen cells as a source of antigen-presenting cells (APC). A long-term T cell line was established from this initial culture by serial restimulation of Ficoll-recovered T blasts (10^5/ml) with either γ-irradiated spleen cells (5×10^6/ml) alone, or γ-irradiated spleen cells plus antigen (100 μg/ml). This line has been maintained in vitro without loss of antigen specificity for more than 18 mo. The proliferative response of the line was periodically assayed by culturing 10^4 Ficoll-purified T cells together with 10^5 irradiated (3,300 R) syngeneic spleen cells with or without OVA (100μg/ml) in microtiter wells (Costar 3596) for 2.5 days, followed by a 16-h pulse with [^{3}H]thymidine.

Several other antigen-reactive T cell lines were also developed as previously described (25). Clones from these lines were derived by limiting dilution, plating the cells at an initial average density of 0.3 cells/well. The antigen specificities and genetic haplotypes of these lines were as follows: A3 is a B10.A-derived pigeon cytochrome *c*-specific T cell line; A2.9 is a B10.A pigeon cytochrome *c*-specific T cell clone; and 9R.1 is a B10.S(9R)-derived pigeon cytochrome *c*-specific T cell clone.

BCGF-I Assay. Full details of this co-stimulator assay are given elsewhere (13). Briefly, splenic B cells were purified by the procedure of Leibson et al. (9), then cultured at a density of 5×10^4 per 200 μl medium in flat bottom 96-well microtiter plates. Some cultures contained affinity-purified goat anti-μ antibodies at 5–10 μg/ml final concentration and/or dilutions of the various growth factor preparations. Cultures were incubated at 37°C in a humidified atmosphere of air containing 7.5% CO_2 for 3 d. The proliferative

response of these cultures was determined by adding [^{3}H]thymidine for the last 16 h of culture and measuring ^{3}H-TdR incorporation. A relative unit of BCGF-I activity was defined as the amount of material required to produce 50% of the proliferation caused by a saturating amount of EL4 supernatant. Thus, the inverse of the dilution that produced 50% of the proliferation obtained with a saturating level of EL4 supernatant defined the number of relative units per milliliter (U/ml).

Biochemical Characterization of B Cell Growth Activity. The B cell co-stimulating activity in antigen-stimulated supernatants from the continuously growing OVA-reactive T cell was characterized biochemically by phenyl Sepharose chromatography and IEF. Full details of these procedures are given elsewhere (22, 23).

Lyt Phenotype Analysis. The Lyt phenotype of BCGF-I producing T cells was assessed by selective cytotoxicity. Long-term OVA-reactive T cell blasts were recovered on Ficoll gradients following a 2-wk antigen-rest period, washed twice, then incubated at 2×10^5/ml with either the rat anti-mouse Lyt-1 hybridoma (clone 53-7.3) and/or rat anti-mouse Lyt-2 hybridoma (clone 53-6.7) at final concentrations of 1:10. As these IgG_{2a} monoclonal antibodies are not directly cytotoxic, it was essential to include the complement-binding monoclonal mouse anti-rat kappa antibody (MAR 18.5) to this cocktail in order to form a cytotoxic sandwich. The anti-Lyt and anti-kappa reagents were produced and generously provided by J. Ledbetter and L. Lanier, respectively (26, 27). Following incubation for 30 min at 4°C, the cells were washed, incubated for 45 min at 37°C with rabbit complement at 1:15 (Cedarlane, Low-tox), then washed twice in culture medium. To ensure comparison of equivalent precytotoxicity numbers of T cells, the entire contents of both control and test samples were then placed into a microtiter well in 200 μl of culture medium containing 5×10^6 γ-irradiated syngeneic spleen cells and OVA (100 μg/ml). Supernatants were collected 24 h later and assayed for BCGF-I production.

Anti-IL-2 Receptor Antibody (α7D4). A monoclonal rat anti-mouse IL-2 receptor antibody was produced by immunization of rats with the IL-2-dependent T cell line HT2. Full details of this antibody and its speciticity for the IL-2 receptor are presented elsewhere (28). Briefly, this reagent reacts with a membrane antigen of 50,000–60,000 daltons expressed on activated but not resting lymphocytes (28), immunoprecipitates cell associated IL-2 (28), and blocks proliferation of IL-2-dependent HT2 and CTLL cells (28), but not the proliferation of LPS or anti-Ig activated B cells (T. Malek and M. Howard, unpublished observations).

Results

BCGF-I Activity in Antigen-stimulated T Cell Line Supernatants. OVA-primed T cells were grown in continuous cell culture according to the procedure of Kimoto and Fathman (19). Activation of this line to proliferate required specific antigen presented in the context of syngeneic irradiated spleen cells. Supernatants collected 4 d after stimulating the T cell line with OVA plus irradiated syngeneic spleen cells were tested for BCGF-I activity using the B cell co-stimulator assay developed previously (13). Such supernatants (T_{OVA} sup) did not directly induce proliferation of purified B lymphocytes, but were capable of synergizing with anti-Ig antibodies to induce proliferation levels comparable to those achieved with induced EL4 supernatants (Fig. 1). The activity of T_{OVA} sup was not H-2 restricted, as BALB/c, CBA/J, and B10.A B cells all responded in comparable fashion to the BALB/c T cell line product (Figs. 1 and 2). B cells from mice bearing the xid-determined immune defect (i.e. CBA/N mice) failed to proliferate in the presence of anti-Ig plus T_{OVA} sup (Fig. 1). By these functional criteria T_{OVA} sup could not be distinguished from PMA-induced EL4 sup (Figs. 1 and 2). Indeed, no synergistic or additive proliferation levels were obtained when anti-Ig activated B cells were cultured with T_{OVA} sup supplemented with optimum

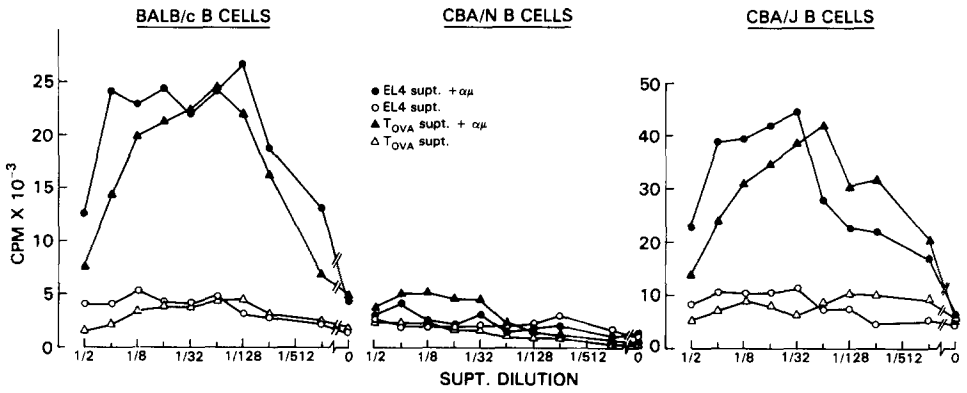

FIGURE 1. Proliferative response of purified B cells from different strains of mice to anti-IgM antibodies plus various dilutions of either a 2-d PMA-induced EL4 supernatant or a 4-d OVA-induced long-term T cell line supernatant. All groups represent the mean of duplicate cultures incubated for 3 d, with proliferation assessed via a final 16-h pulse of [^{3}H]thymidine.

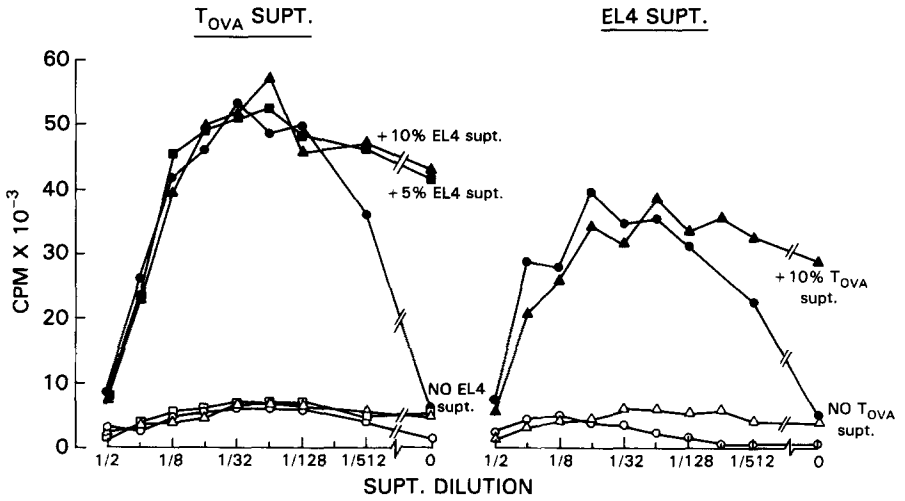

FIGURE 2. Proliferative response of purified B10.A B cells cultured with (● ■ ▲) or without (○ □ △) anti-IgM antibodies and various dilutions of either 4-d OVA-induced long-term T cell line supernatant (*left*) or 2-d PMA-induced EL4 supernatant (*right*). Some cultures were additionally supplemented with 5% (■ □) or 10% (▲ △) of either the EL4 supernatant (*left*) or the T cell line supernatant (*right*). Other details as in Fig. 1.

amounts of EL4 sup, or vice versa (Fig. 2).

The above data strongly suggest that B cell co-stimulating activities in T_{OVA} sup and EL4 are identical. To confirm this, the active component of T_{OVA} sup was characterized by IEF and phenyl Sepharose chromatography and its properties compared to those previously reported (22, 23) for BCGF-I obtained from EL4 cells. The B cell co-stimulating factor in T_{OVA} sup had isoelectric points for 6.4 and 7.4 (Fig. 3) and on phenyl Sepharose hydrophobic chromatography eluted in the 10–30% ethanediol fraction (Fig. 4). Thus, by both procedures, it was indistinguishable from the BCGF-I in induced EL4 sup (22, 23). From these biological and biochemical data we therefore conclude that antigen-stimulated

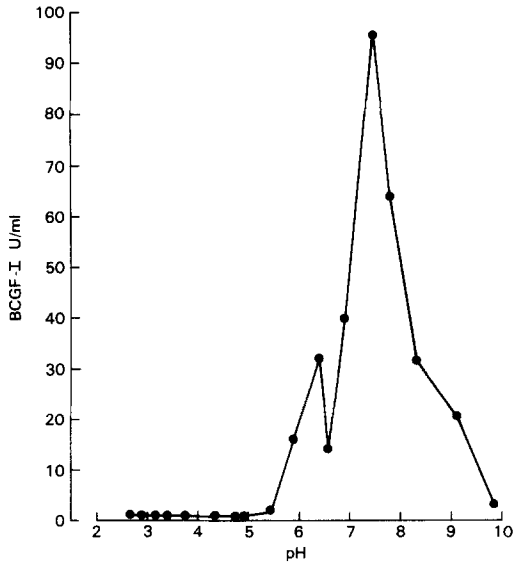

FIGURE 3. Isoelectric focusing analysis of B cell co-stimulator activity in 4-d OVA-induced long-term T cell line supernatant. Fractions collected after isoelectric focusing were dialyzed extensively, then added at six serial dilutions to duplicate cultures of purified B cells and anti-IgM for assay of co-stimulator activity. Proliferation was assessed at day 3, with units of activity calculated as described in the Materials and Methods.

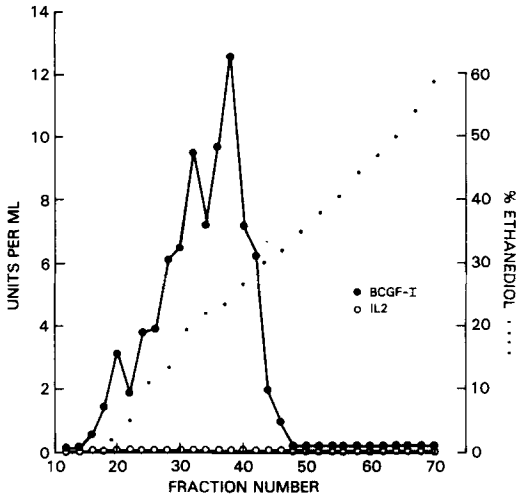

FIGURE 4. Phenyl Sepharose chromatographic analysis of B cell co-stimulator activity in 4-d OVA-induced long term T cell line supernatant. Fractions were assayed for B cell co-stimulator activity as described in Fig. 3 legend. Fractions were assayed for IL-2 activity via proliferation of the IL-2-dependent T cell clone, HT2, as described elsewhere (13).

T cell lines produce BCGF-I. Interestingly, T_{OVA} supernatant collected 4 d after antigenic stimulation ws found to lack the T cell proliferation cofactor IL-2 (Fig. 4; unpublished observations), thus further distinguishing these two lymphokines and providing a convenient IL-2–free source of BCGF-I.

Characteristics of BCGF-I Production by Antigen-stimulated T Cell Lines. To investigate the kinetics of BCGF-I production by antigen-activated T cell lines, OVA-reactive T cells were collected after they had been cultured for 2 wk in the presence of APC but in the absence of antigen. Such "rested" cells were stimulated with OVA plus irradiated syngeneic spleen cells, and cell-free supernatants were collected 1, 2, 3, 4, and 5 d thereafter. Excellent BCGF-I activity was detected in each supernatant, with essentially no quantitative difference betweeen the different time points (Fig. 5). The amount of BCGF-I produced within 24 h of stimulating 10^5 OVA-reactive T cells was comparable to that produced by 20 times as many EL4 cells stimulated for 48 h with phorbol myristate acetate (Fig. 5). No BCGF-I was detected in supernatants collected from cultures of irradiated syngeneic spleen plus OVA (Fig. 5). Small but detectable amounts of BCGF-I were found in supernatants from antigen-free cultures containing T cell plus irradiated syngeneic spleen cells (Fig. 5). As some proliferation was also seen under these latter conditions, this production might reflect either a small degree of antigen carry-over or a small degree of autologous MLR activity by cells in this line. No BCGF-I activity was found in supernatants collected from OVA-reactive T cells stimulated with antigen presented in the context of irradiated allogeneic spleen cells (data not shown), demonstrating that induction of BCGF-I production is H-2 restricted. Treatment of the OVA-reactive T cells with cytotoxic anti-Lyt reagents before antigenic stimulation revealed that the cells in this line which are involved in BCGF-I production bear the Lyt 1^+2^- phenotype (Fig. 6). As few Lyt 2^+ T cells are propagated by this method of continuous

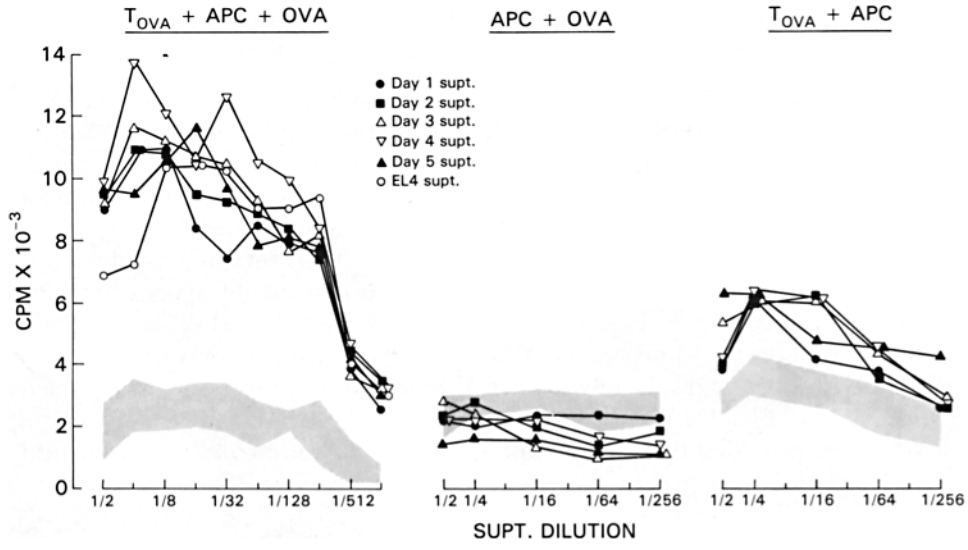

FIGURE 5. Kinetics of BCGF-I production by antigen-stimulated T cells. Cultures containing various combinations of 10^5 Ficoll-purified long-term OVA reactive T cells collected at the end of a 2-wk antigen rest period, 5×10^6 irradiated (3,300 rads) syngeneic spleen cells (APC), and OVA at 100 μg/ml were incubated for 1, 2, 3, 4, or 5 d. Supernatants collected thereafter were assayed for BCGF-I activity via their addition at various dilutions to cultures of purified B cells and anti-IgM. The range of proliferative responses observed in control cultures lacking anti-IgM is shown by the hatched regions of the figure. All groups represent duplicate cultures.

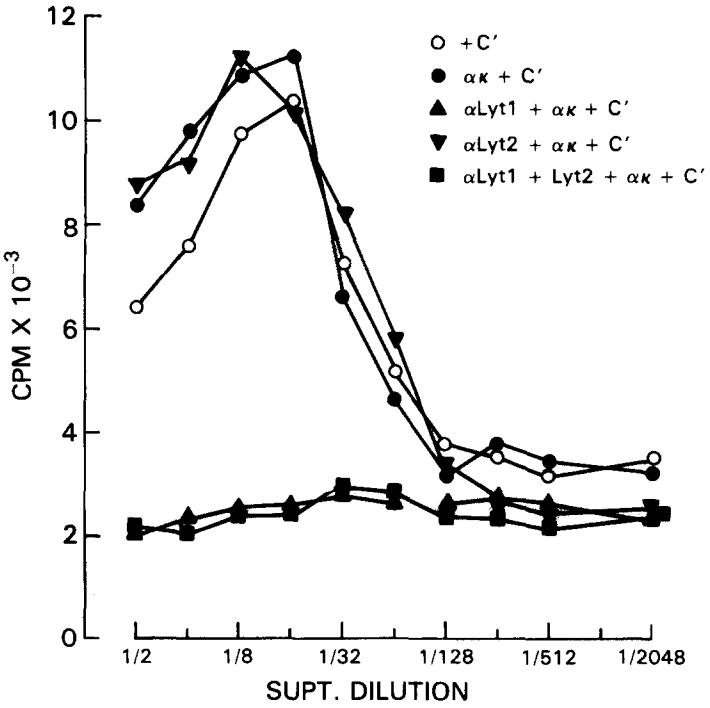

FIGURE 6. Lyt phenotype of BCGF-I producing cells in the OVA-reactive long term T cell line. T cells were treated with cytotoxic monoclonal anti-Lyt reagents plus complement before their stimulation with OVA and irradiated spleen cells. Supernatants collected 2 d later were assayed for BCGF-I activity as described in Fig. 5. All groups represent duplicate cultures.

antigen-induced T cell cultivation, it should not, however be concluded that Lyt 2^+ T cells fail to produce BCGF-I. Finally, the relationship betwen T cell density and BCGF-I production (Fig. 7) was found to be nonlinear and showed that as few as 10^4 OVA-reactive T cells produced detectable levels of BCGF-I within 24 h of stimulation with antigen plus irradiated syngeneic spleen cells.

IL-2 Induces BCGF-I Production by T Cell Lines. Current models of T cell activation propose that antigen presented in the context of syngeneic APC induces resting T cells to express IL-2 receptors, and that IL-2 causes these activated lymphocytes to proliferate (29). The above experiments demonstrate that in the case of Lyt 1^+ T cells at least, this activation process is accompanied by induction of BCGF-I release. To test whether the stimulus for lymphokine release is also provided by IL-2, OVA-specific T cells were collected at the end of a 2-wk antigen rest period and stimulated with purified BCGF-I-free preparations of IL2. Cell-free supernatants collected 2 d later were assayed for BCGF-I activity. Both phenyl Sepharose–purified IL-2 from PMA-induced EL4 supernatant and affinity-purified human IL-2 induced 10^5 OVA-specific T cells to produce substantial amounts of BCGF-I (Table I). Similar results were obtained using IEF purified IL-2 from PMA-induced EL4 supernatant (data not shown). In all experiments of this type, the level of IL-2-induced BCGF-I production corresponded to between 10 and 20% of that achieved when T cells were

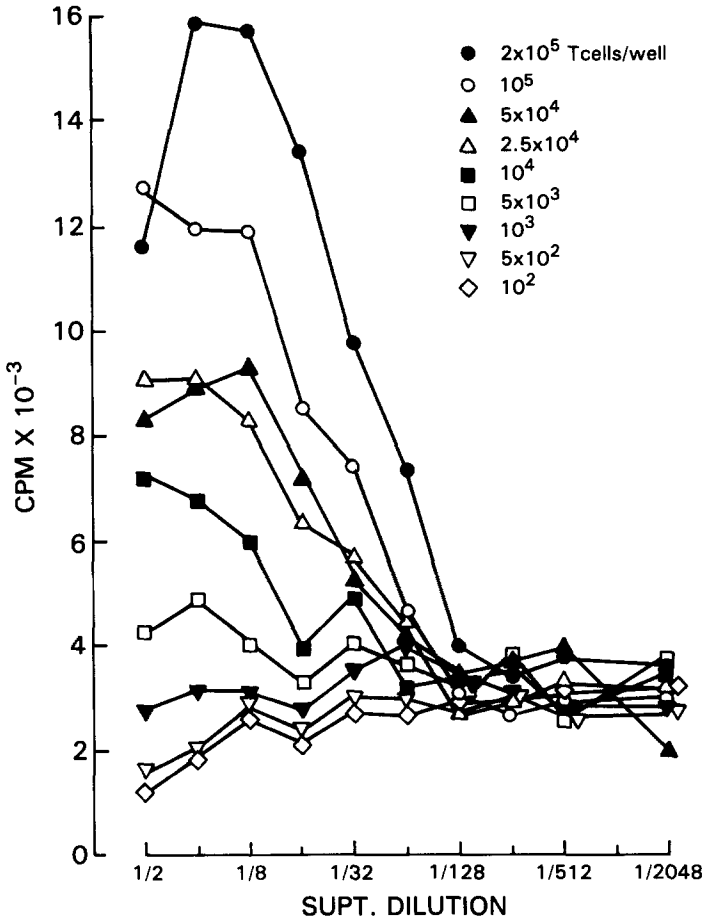

FIGURE 7. Relationship between T cell density and BCGF-I production. Various numbers of OVA-reactive T cells collected at the end of a 2-wk antigen rest period were stimulated with OVA and irradiated spleen. Supernatants collected 1 d later were assayed for BCGF-I activity as described in Fig. 5. All groups represent duplicate cultures.

stimulated with antigen in the context of syngeneic spleen cells (Table I). Titration of the amount of IL-2 required for BCGF-I production showed that as little as 10 U/ml IL-2 induced 10^5 T cells to produce optimum amounts of BCGF-I (Fig. 8). This production was totally blocked by a monoclonal rat anti-mouse IL-2 receptor antibody (designated α7D4) but not by a monoclonal rat anti-mouse Lyt-1 antibody (Fig. 8). As the anti–IL-2 receptor antibody by itself showed no intrinsic blocking effects in the BCGF-I assay (M. Howard and T. Malek, unpublished data), these data indicate that BCGF-I production in the above experiments had indeed resulted from a direct effect of IL-2 at the T cell's IL-2 receptor. While abrogation of IL-2-induced BCGF-I production in the above antibody-blocking experiments was accompanied by elimination of the T cell proliferative response (Table II), separate experiments showed that non-proliferating irradiated T cells were capable of producing optimum amounts of

TABLE I

Production of BCGF-I by an OVA-Specific T Cell Line in Response to Various Stimuli

Stimulus	BCGF-I			
	Exp. 1*		Exp. 2*	
	+ T cells	− T cells	+ T cells	− T cells
	U/ml			
0	4	<2	3	<2
Mouse IL-2‡	96	<2	272	<2
Human IL-2§	96	<2	256	<2
OVA-irradiated spleen‖	1024	<2	1792	<2

* 10^5 antigen-rested T cells were cultured with various stimuli for 2 d; supernatants were harvested and assayed for BCGF-I. Parallel cultures lacking T cells were included to control for any BCGF-I derived from the stimulant.

‡ Mouse IL-2 = phenyl Sepharose–purified IL-2 from PMA-induced EL4 supernatant added to cultures at ~10 U/ml.

§ Human IL-2 = affinity-purified IL-2 from induced Jurkat supernatant, provided by Dr. K. Smith, added to cultures at ~25 U/ml.

‖ Syngeneic spleen cells were cultured with OVA 100 μg/ml for 3 h, washed, irradiated at 3,300 rads, and added to cultures at 5×10^6/ml.

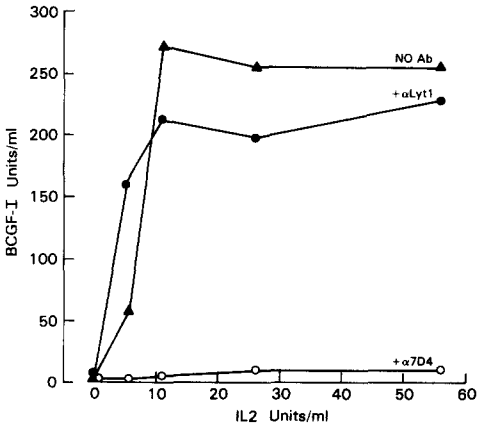

FIGURE 8. BCGF-I production by long-term OVA-reactive T cells stimulated for 2 d with various concentrations of phenyl Sepharose–purified murine IL-2. Some cultures also contained either anti–Lyt-1 hybridoma supernatant or α7D4 (anti–IL-2 receptor) hybridoma supernatant added at final concentrations of 1:10. Control experiments showed that 56 U/ml of the partially purified IL-2 contained <2 U/ml BCGF-I activity. All groups represent duplicate cultures.

BCGF-I in response to either IL-2 or antigen-pulsed APC (Table III). Thus, while proliferation and lymphokine release are apparently mediated via the same receptor-ligand interactions, they are nevertheless independent events. The results of Table III also demonstrate that Con A, particularly when presented in the context of syngeneic irradiated spleen, is capable of inducing antigen-reactive T cells to secrete BCGF-I. While this observation is readily explained by Con A–inducing T cells to secrete IL-2 which in turn induces BCGF-I production, it is nevertheless intriguing in light of the apparent difficulty of detecting BCGF-I in Con A–induced spleen cell supernatants (M. Howard, unpublished observations).

TABLE II

Effect of Anti–IL-2 Receptor Antibody (α7D4) on Antigen or IL-2 Induction of an OVA-reactive T Cell Line

Stimulus*	BCGF-I Production	Proliferation
	U/ml	*cpm*
0	4	893
IL2	256	9,809
IL2 + α7D4	12	603
IL2 + αLyt1	228	8,695
OVA-irradiated spleen	1,792	39,291
OVA-irradiated spleen + α7D4	1,536	29,292
OVA-irradiated spleen + αLyt1	2,048	31,685

* 10^5 antigen-rested T cells were cultured with the various stimuli listed. Induction of BCGF-I production was assessed by assaying the amount of BCGF-I in supernatants collected 2 d later. Induction of proliferation was assessed via the uptake of ^{3}H-TdR on day 3. For details of the mouse IL-2 and OVA pulsed irradiated spleen cell stimulants, refer to Table I. For details of the α7D4 and αLyt1 antibodies, refer legend to Fig. 8.

TABLE III

BCGF-I Production by and Proliferation of Nonirradiated and Irradiated T Cells following Stimulation

Stimulus	BCGF-I Production*			Proliferation‡	
	No T cells	Normal T cells	Irradiated T cells§	Normal T cells	Irradiated T cells§
		U/ml		*cpm*	
+0	<2	<2	<2	2,100	450
+IL2‖	<2	96	72	16,670	363
+Irradiated spleen & OVA	<2	512	512	33,019	450
+Con A¶	<2	320	ND	5,099	ND
+Con A + irradiated spleen	<2	960	ND	75,842	ND

* BCGF-I production estimated as in Table I.

‡ Proliferative response estimated by culturing 2×10^4 OVA reactive T cells per microtiter well plus the various stimuli for 3 d with a final 16-h pulse of [^{3}H]thymidine.

§ Irradiated T cells received 3,300 rads before culture.

‖ See footnote ‡ of Table I.

¶ Con A was used at 1 μg/ml.

Interestingly, the anti–IL-2 receptor antibody used in the above experiments failed to abrogate either BCGF-I production or proliferation of T cells stimulated with antigen and syngeneic APC (Table II). Possible explanations for this observation are discussed below.

Finally, to test the generality of the above findings, the same BCGF-I induction experiments were performed using other antigen-reactive T cell lines. Three long-term lines, two of which had additionally been cloned by limit dilution, were used for this purpose. Two of these three lines produced BCGF-I in response to specific antigen presented in the context of syngeneic spleen cells (Table IV); all three of the lines produced BCGF-I in response to phenyl Sepharose–purified IL-2 (Table IV). In contrast to the above situation with the OVA-reactive T cell line, IL-2 proved equal to or better than presented antigen in inducing BCGF-I

TABLE IV
BCGF-I Production by Other Antigen-reactive T Cell Lines

T cell line‡	BCGF-I		
	+ Irradiated spleen	+ Irradiated spleen + antigen	+ IL-2‖
			U/ml
A2.9	4	48	32
9R.1	<2	24	64
A3	<2	<2	48

* Conditions for BCGF-I production as in Table I.
‡ For details on antigen specificity and genetic haplotype of T cell lines, see Materials and Methods.
‖ IL-2 = phenyl Sepharose–purified mouse IL-2, added at ~10 U/ml. This mixture contained <2 U/ml BCGF-I.

production by these T cell lines (Table IV). As cloning of T cell lines involves an initial IL-2-dependent propagation step, this may simply reflect selection during cloning of T cells that are highly IL-2–responsive.

From these data we conclude that BCGF-I production is a common feature of long-term antigen-reactive T cell lines when stimulated either with presented antigen or IL-2.

Discussion

This study demonstrates the production of murine BCGF-I by antigen-reactive long-term T cell lines and T cell clones following stimulation with either antigen presented in the context of syngeneic spleen cells, or alternatively IL-2. While BCGF-I has been found in supernatants of T cell hybridomas, lymphomas, and alloreactive T cell clones (13, 30–32), this is the first demonstration of its production by normal mouse T cells in response to antigenic stimulus. As most other sources of BCGF-I require induction by PMA or Con A, the current source provides the added advantage of being mitogen-free. The fact that BCGF-I production by the OVA-reactive T cell line used throughout most of this study was substantially greater using presented antigen as the inductant rather than IL-2 can be readily explained by a consideration of the physiology of this type of T cell line. The optimum procedure for growing such lines on alternate cycles of antigen-rest and antigen-exposure is consistent with the fact that T cells lose their IL-2 receptors some time after activation (29). Thus, at the end of a 2-wk antigen-rest period the T cell lines will most likely comprise a mixture of IL-2-receptor-bearing cells and cells that have already lost their IL-2 receptors. Whereas IL-2 induction will only encompass the first of these populations, antigen plus APC has the potential to recruit both i.e., to re-activate expression of IL-2-receptors and to generate IL-2 to act on such receptors. In contrast, the antigen-reactive T cell clones analysed for BCGF-I production showed the reverse preference for IL-2 rather than presented antigen as optimum inductant. As mentioned above, this may reflect selection for enhanced IL-2 responsiveness during the cloning procedure.

Our observation that antibodies to the IL-2 receptor block BCGF-I production

in response to IL-2 but not in response to presented antigen, raises the possibilty that a mechanism independent of engagement of the IL-2 receptor can be used to cause BCGF-I production. Since [^{3}H]thymidine uptake by these cells in response to antigen and APC is also not blocked by the anti-receptor antibody, this would further imply that an IL-2–independent mechanism may exist for proliferation of these T cells. Such a conclusion, however appears to us to be premature. The results might equally well be explained by the induction on T cells of large numbers of IL-2 receptors as a result of stimulation by antigen and APC, making such cells much more difficult to block than cells simply treated with IL-2. Alternatively, antigenic stimulation might induce the T cells to produce IL-2 and IL-2 receptors simultaneously. Under these circumstances receptor-ligand complexes could be formed intracytoplasmically, i.e. before their surface expression, thereby removing all opportunity for an extracellular antibody to compete in the interaction. Thus, antigen-induced proliferation and BCGF-I production by T cells may also be mediated by the IL-2 receptor, despite the fact that both processes are refractory to the blocking effects of the anti-IL-2 receptor antibody.

This paper contributes to our growing knowledge of the lymphokine cascade that apparently regulates the effector mechanisms of the immune response. Others have shown that T cell–derived colony-stimulating-factor induces macrophages to release IL-1, which in turn stimulates T cells to release IL-2 (reviewed in reference 22). Here we demonstrate that IL-2 in turn acts on T cells to release the B cell proliferation cofactor BCGF-I. Thus, IL-2 is a bifunctional mediator capable of inducing T cells to proliferate and/or secrete BCGF-I. Such plurifunction has previously been reported in the case of the biochemically similar lymphokine colony stimulating factor, a mediator that acts on macrophages, inducing both proliferation (33) and effector cell function (34, 35). Whether all B cell–specific cofactors are induced by IL-2 is not yet clear. Antigen stimulation of long-term T cell lines similar to those used in this study is known to cause release of other B cell–specific cofactors (36–38), but the mechanisms governing those events have not yet been elucidated. Preliminary evidence from Swain and colleagues suggests that IL-2 induces an allorective T cell line to produce B cell differentiation factors and the second B cell growth factor BCGF-II (reference 39; S. Swain, personal communication). In contrast however, Asano et al. (40) have demonstrated that antigen-reactive T cell clones are capable of providing helper T cell function in an antibody response at antigen concentrations below which proliferation and thus, presumably IL-2 release are observed. Whether or not IL-2 induces all B cell–specific cofactors, one should not regard it as a universal lymphokine inductant. At least in the case of interferon, lymphokine production by antigen-reactive T cell clones has been segregated from proliferation and IL-2 induction (41). The fact that IL-2 causes production of at least some B cell–specific interleukins may well explain the current controversy regarding the role of IL-2 in B cell immunity (reviewed in reference 18). The current studies demonstrate that as few as 10^4 T cells produce detectable amounts of BCGF-I. While proponents of IL-2 acting directly on B cells may feel confident that their starting B cell population is free of this level of T cell contamination, an even lower level may well expand to such a number in the presence of IL-2

during the 3–5-d culture periods generally used.

The demonstration of BCGF-I production by antigen-reactive MHC-restricted helper T cells prompts some modification of current models that divide B cell triggering into either factor-mediated or involving cognate T cell interactions (discussed in reference 18). In light of the present study, cognate T cell interactions will presumably generate at least some B cell–specific interleukins. Thus it seems more likely that both pathways will use similar, if not the same, cofactors. The apparent difference between those B cells that appear to utilize direct interactions with T cells for their activation and those B cells that do not could be explained at the level of cofactor receptor expression, i.e., that the role of cognate T cell interaction may be to induce a membrane receptor for a B cell–specific interleukin; other B cells may already express this receptor or develop it in response to membrane immunoglobulin cross-linkage by antigen, thus bypassing the need for a cognate T cell signal. Testing of this model and direct demonstration that the cognate interaction pathway utilizes factors will hopefully be possible when antibodies against the factors or their membrane-bound receptors are developed.

Summary

Antigen-activated T lymphocytes produce within 24 h of stimulation a factor that is indistinguishable biochemically and functionally from the B cell co-stimulating growth factor, BCGF-I, originally identified in induced EL4 supernatants: Supernatants from antigen-stimulated T cell lines are not directly mitogenic for resting B cells, but synergize in an H-2–unrestricted manner with anti-Ig activated B cells to produce polyclonal proliferation but not antibody-forming-cell development; biochemical studies reveal the B cell co-stimulating factor present in antigen-stimulated T cell line supernatants is identical by phenyl Sepharose chromatography and isoelectric focusing (IEF) to EL4 supernatant BCGF-I. We thus conclude that normal T cells produce BCGF-I in response to antigenic stimulation.

Analysis of the mechanism of BCGF-I production by antigen-stimulated T cells showed that optimum amounts of BCGF-I were obtained as quickly as 24 h post-stimulation, and that the factor producing cells in the T cell line investigated bore the Lyt-1^+2^- phenotype. As few as 10^4 T cells produced sufficient BCGF-I to support the proliferation of 5×10^4 purified anti-Ig activated B cells. Finally, the activation of normal T cell lines to produce BCGF-I required either antigen presented in the context of syngeneic antigen-presenting cells (APC) or interleukin 2 (IL-2).

Note added in proof: After this paper was submitted, a working group on B cell factors met in Kyoto and proposed that BCGF-I be designated BSF (B cell stimulatory factor).

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References

1. Dutton, R. W., R. Falkoff, J. A. Hirst, M. Hoffman, J. M. Kappler, J. R. Kettman, J. F. Lesley, and D. Vann. 1971. Is there evidence for a non-antigen specific diffusable chemical mediator from the thymus-derived cell in the initiation of the immune response? *Progr. Immunol.* 1:355.
2. Schimpl, A., and E. Wecker. 1975. A third signal in B cell activation given by TRF. *Transplant. Rev.* 23:176.
3. Kishimoto, T., and K. Ishizaka. 1975. Immunologic and physiochemical properties of enhancing soluble factors for IgG and IgE antibody responses. *J. Immunol.* 114:1177.
4. Parker, D. C., J. J. Fothergill, and D. C. Wadsworth. 1979. B lymphocyte activation by insoluble anti-immunoglobulin: induction of immunoglobulin secretion by a T cell-dependent soluble factor. *J. Immunol.* 123:931.
5. Watson, J., S. Gillis, J. Marbrook, D. Mochizuki, and K. A. Smith. 1979. Biochemical and biological characterization of lymphocyte regulatory molecules. I. Purification of a class of murine lymphokines. *J. Exp. Med.* 150:849.
6. Takatsu, K., K. Tanaka, A. Tominaga, Y. Kumahara, and T. Hamaoka. 1980. Antigen-induced T cell-replacing factor (TRF). III. Establishment of T cell hybrid clone continuously producing TRF and functional analysis of released TRF. *J. Immunol.* 125:2646.
7. Andersson, J., and F. Melchers. 1981. T cell-dependent activation of resting B cells: requirement for both nonspecific unrestricted and antigen-specific Ia-restricted soluble factors. *Proc. Natl. Acad. Sci. USA.* 78:2497.
8. Howard, M., S. Kessler, T. Chused, and W. Paul. 1981. Long-term culture of normal mouse B lymphocytes. *Proc. Natl. Acad. Sci. USA.* 78:5788.
9. Leibson, H., P. Marrack, and J. Kappler. 1981. B cell helper factors. I. Requirement for both IL-2 and another 40,000 M_r factor. *J. Exp. Med.* 154:1681.
10. Swain, S., G. Dennert, J. Warner, and R. Dutton. 1981. Culture supernatant of a stimulated T cell line have helper activity that synergizes with IL2 in the response of B cells to antigen. *Proc. Natl. Acad. Sci. USA.* 78:2517.
11. Vaux, D. L., B. L. Pike, and G. J. V. Nossal. 1981. Antibody production by single hapten-specific B lymphocytes: an antigen-driven cloning system free of filler or accessory cells. *Proc. Natl. Acad. Sci. USA.* 78:7702.
12. Pure, E., P. C. Isakson, K. Takatsu, T. Hamada, S. L. Swain, R. W. Dutton, G. Dennert, J. H. Uhr, and E. S. Vitetta. 1981. Induction of B cell differentiation by T cell factors. Stimulation of IgM secretion by products of a T cell hybridomas and a T cell line. *J. Immunol.* 127:1953.
13. Howard, M., J. Farrar, M. Hilfiker, B. Johnson, K. Takatsu, T. Hamaoka, and W. E. Paul. 1982. Identification of a T-cell derived B-cell growth factor distinct from interleukin 2. *J. Exp. Med.* 155:914.
14. Parker, D. C. 1982. Separable helper factors support B cell proliferation and maturation to Ig secretion. *J. Immunol.* 129:469.
15. Isakson, P., E. Pure, E. Vitetta, and P. Krammer. 1982. T cell-derived B cell differentiation factor(s). Effect on the isotype switch of murine B cells. *J. Exp. Med.* 155:734.
16. Nakanishi, K., M. Howard, A. Muraguchi, J. Farrar, K. Takastsu, T. Hamaoka, and W. E. Paul. 1983. Soluble factors involved in B cell differentiation: identification of two distinct T cell replacing factors (TRF). *J. Immunol.* 130:2219.
17. Swain, S. L., M. Howard, J. Kappler, P. Marrack, J. Watson, R. Booth, G. D. Wetzel,

and R. W. Dutton. 1983. Evidence for two distinct classes of murine B cell growth factors with activities in different functional assays. *J. Exp. Med.* 158:822.

18. Howard, M., and W. E. Paul. 1983. Regulation of B cell growth and differentiation by soluble factors. *Annu. Rev. Immunol.* 1:307.
19. Kimoto, M., and C. G. Fathman. 1980. Antigen-reactive T cell clones. I. Transcomplementing hybrid I-A region gene products function effectively in antigen presentation. *J. Exp. Med.* 152:759.
20. Sieckmann, D., R. Asofsky, D. Mosier, I. Zitron, and W. E. Paul. 1978. Activation of mouse lymphocytes by anti-immunoglobulin. I. Parameters of the proliferation response. *J. Exp. Med.* 147:814.
21. Farrar, J., J. Fuller-Farrar, P. Simon, M. Hilfiker, B. Stadler, and W. Farrar. 1980. Thymoma production of T cell growth factor (inteleukin 2). *J. Immunol.* 125:2555.
22. Farrar, J., W. Benjamin, M. Hilfiker, M. Howard, W. Farrar, and J. Fuller-Farrar. 1982. The biochemistry, biology, and role of IL2 in the induction of cytotoxic T cells and antibody-forming B cell responses. *Immunol. Rev.* 63:129.
23. Farrar, J., M. Howard, J. Fuller-Farrar, and W. E. Paul. 1983. Biochemical and physiochemical characterization of mouse B cell growth factor: a lymphokine distinct from interleukin 2. *J. Immunol.* 131:1838.
24. Julius, M., G. Simpson, and L. A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived lymphocytes. *Eur. J. Immunol.* 112:420.
25. Matis, L., D. Longo, S. Hedrick, C. Hannum, E. Margoliash, and R. Schwartz. 1983. Clonal analysis of the major histocompatibility complex restriction and the fine specificity of antigen recognition in the T cell proliferative response to cytochrome c. *J. Immunol.* 130:1527.
26. Ledbetter, J. A., and L. A. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* 47:362.
27. Lanier, L., G. Gutman, D. Lewis, S. Griswold, and N. Warner. 1982. Monoclonal antibodies against rat immunoglobulin kappa chains. *Hybridoma.* 1:125.
28. Malek, T., R. Robb, and E. Shevach. 1983. Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin 2 receptor-ligand complex. *Proc. Natl. Acad. Sci. USA.* 80:5694.
29. Robb, R., A. Munck, and K. Smith. 1981. T cell growth factor receptors. Quantitation, specificity and biological relevance. *J. Exp. Med.* 154:1455.
30. Leanderson, T., E. Lundgren, E. Ruuth, H. Borg, H. Persson, and A. Coutinho. 1982. B cell growth factor (BCGF): distinction from T cell growth factor and B cell maturation factor. *Proc. Natl. Acad. Sci. USA.* 79:7455.
31. Lernhardt, W., C. Corbel, R. Wall, and F. Melchers. 1982. T cell hybridomas which produce B lymphocyte replication factors only. *Nature (Lond.).* 300:355.
32. Pure, E., P. Isakson, J. Kappler, P. Marrack, P. Kramer, and E. Vitetta. 1983. T cell–derived B cell growth and differentiation factors: dichotomy between the responsiveness of B cells from adult and neonatal mice. *J. Exp. Med.* 157:583.
33. Bradley, T. and D. Metcalfe. 1966. The growth of mouse bone marrow cells in vitro. *Aust. J. Exp. Biol. Med. Sci.* 44:287.
34. Handman, E. and A. Burgess. 1979. Stimulation by granulocyte-macrophage colony stimulating factor of *Leishmania tropica* killing by macrophages. *J. Immunol.* 122:1134.
35. Hamilton, J., Stanley, E., Burgess, A. and Shadduck, R. 1980. Stimulation of macrophage plasminogen activator activity by colony-stimulating factors. *J. Cell. Physiol.* 103:435.
36. Melchers, F., J. Andersson, W. Lernhardt, and M. Schreier. 1980. H-2 unrestricted polyclonal maturation without replication of small B cells induced by antigen-activated T cell help factors. *Eur. J. Immunol.* 10:679.

37. Andersson, J., and F. Melchers. 1981. T cell-dependent activation of resting B cells: requirement for both nonspecific unrestricted and antigen-specific Ia-restricted soluble factors. *Proc. Natl. Acad. Sci. USA.* 78:2497.
38. Paige, C., M. Schreier, and C. Sidman. 1982. Mediators from cloned T helper cell lines affect immunoglobulin expression by B cells. *Proc. Natl. Acad. Sci. USA.* 79:4756.
39. Swain, S., G. Wetzel, P., Soubiran, and R. Dutton. 1982. T cell replacing factors in the B cell response to antigen. *Immunol. Rev.* 63:111.
40. Asano, Y., M. Shigeta,C. Fathman, A. Singer, and R. Hodes. 1982. Role of the major histocompatibility complex in T cell activation of B cell subpopulations. A single monoclonal T helper cell population activates different B cell subpopulations by distinct pathways. *J. Exp. Med.* 156:350.
41. Hecht, T., D. Longo, and L. Matis. 1983. The relationship between immune interferon production and proliferation in antigen-specific, MHC-restricted T cell lines and clones. *J. Immunol.* 131:1049.