B CELL STIMULATORY FACTOR 1 (INTERLEUKIN 4) IS SUFFICIENT FOR THE PROLIFERATION AND DIFFERENTIATION OF LECTIN-STIMULATED CYTOLYTIC T LYMPHOCYTE PRECURSORS

BY JOHN D. PFEIFER, DOUGLAS T. MCKENZIE, SUSAN L. SWAIN, AND RICHARD W. DUTTON

From the Department of Biology, University of California, San Diego, La Jolla, California 92093

The in vitro generation of splenic CTL requires both proliferation and differentiation of inactive precursors. Beyond the requirement for antigen or mitogenic lectin, the details of CTL induction remain controversial. Accessory cells are involved (1) and Th cells provide soluble mediators. IL-2 promotes the proliferation of CTL precursors (2), but various CTL differentiation factors (3–5) may also have a role. Recent reports (6, 7) have demonstrated that IL-2 alone is sufficient for proliferation and differentiation of lectin or alloantigen-stimulated CTL precursors into fully active CTL.

B cell stimulatory factor 1 (BSF-1/IL-4),[1] a Th cell–derived lymphokine with a well-characterized spectrum of activities on B cells, has recently been shown to exhibit a number of activities on T cells as well. Thus, IL-4 has been shown to support the proliferation of long-term T cell lines (8, 9), to be a potent costimulant for normal resting T lymphocytes (10), and to mediate the autocrine growth of Th cell lines after antigenic stimulation (11). In this report we demonstrate that purified IL-4 is also sufficient to stimulate both the proliferation and differentiation of splenic CTL precursors stimulated with the mitogenic lectin Con A.

Materials and Methods

Animals. (C57BL/6 × DBA/2)F_1 hybrids were raised in our colony from breeding pairs originally obtained from The Jackson Laboratory, Bar Harbor, ME. Mice were used as spleen donors at 2–3 mo of age.

Monoclonal Antibodies. The cell line making anti-L3T4, LICR.LAU.RL172.4, was a kind gift of Dr. Susan Webb (Scripp Clinic and Research Foundation, La Jolla, CA), and the cell line producing anti-IL-2-R, 7D4, was a kind gift of Dr. Tom Malek (Univ. of Miami, School of Medicine, Miami, FL). The cell line producing the anti-$Ia^{b,d}$ was a generous gift of Dr. Sue Tonkonogy (School of Veterinary Medicine, North Carolina State University, Raleigh, NC). Antibodies from the above lines were obtained from

This work was supported by grants from the National Institute of General Medical Sciences, National Research Service Award 07198 from the Medical Scientist Training Program, School of Medicine, University of California, San Diego; the American Cancer Society (IM-1T); the National Institutes of Health (AI-08795 and AI-23287); and the University Wide Task Force on AIDS (86SD004).

[1] *Abbreviation used in this paper:* BSF-1, B cell stimulatory factor 1/IL-4.

 J. EXP. MED. © The Rockefeller University Press · 0022-1007/87/11/1464/07 $2.00
Volume 166 November 1987 1464–1470

ascites collected from pristane-injected mice inoculated with tumor cells. The cell producing the mAb 11B11 to IL-4 was obtained from Maureen Howard (DNAX Research Institute, Palo Alto, CA); culture supernatant from the 11B11 hybridoma, pressure concentrated and partially purified on an HPLC ion-exchange column, served as the source of antibody.

Factors. rIL-2 (human) was obtained from Cetus Corp., Emeryville, CA. IL-4/B cell stimulatory factor 1 (BSF-1) was purified to homogeneity from the serum-free culture supernatants of the cell line D10.G4.1, a conalbumin-specific Ia^k-restricted T cell line of the TH2 type (12, 13). The D10 cells were stimulated with Con A for 48 h, and crude culture supernatants absorbed onto and eluted from controlled-pore glass according to the method of Henderson et al. (14). BSF-1 containing material eluted from controlled-pore glass was dialyzed against 10 mM Tris, pH 7.0, and applied to a 250 × 10 cm high-pressure ion-exchange column (SynChrom, Inc., Linden, IN). The column was eluted with a 0–250-mM sodium phosphate, pH 7.0, gradient. The active fractions were pooled, concentrated by ultrafiltration, and applied to a Vydac C4 reverse-phase HPLC column (The Separations Group, Hesperia, CA) equilibrated in 0.1% TFA, 30% acetonitrile. The column was developed with a linear gradient of 30–80% acetonitrile in 0.1% TFA over 80 min. The IL-4 activity was recovered at ~39% acetonitrile. The purified IL-4 contained a single major protein on SDS-PAGE and contained no detectable IL-1, IL-2, IL-3, or BCGF-II/IL-5, (McKenzie, D. T., H. I. Filutowicz, S. L. Swain, and R. W. Dutton, manuscript submitted for publication). Recombinant IL-4 used in some experiments was obtained from DNAX Research Institute.

1 U of IL-4 is defined as the amount of IL-4 needed for half-maximal stimulation of $[^{125}I]$UdR incorporation by resting B cells stimulated with rat anti-mouse IgM mAb obtained from the supernatant of the hybridoma Bet2 (15). The half-maximal stimulation of proliferation of the cloned IL-2-dependent T cell line NK (16) requires 0.03 U/ml of IL-4.

Lyt-2⁺ Subset Isolation. Splenic T lymphocytes were isolated as described by Julius et al. (17). The nylon column-purified cells recovered from this procedure were >90% Thy-1.2^+. Cells were incubated for 30 min on ice with the anti-L3T4 mAb RL172.4 and the anti-$Ia^{b/d}$ mAb D3.137, followed by incubation twice with guinea pig and rabbit complement for 30 min at 37°C. In control experiments, thymocytes prepared in this way were <1% $L3T4^+$ as determined by flow cytofluorometry.

Cell Culture. 5×10^4 Ia^-,$L3T4^-$ splenic T cells isolated as described above were stimulated with IL-2 or IL-4 in the presence of 1 μg/ml Con A. Cells were cultured in U-bottomed microtiter plates (No. 3799; Costar, Cambridge, MA) in RPMI 1640 supplemented with 5% FCS, 200 IU/ml penicillin, 200 mg/ml streptomycin, 4 mM glutamine, 50 mM 2-ME and 25 mM Hepes. Microcultures contained a final volume of 200 μl medium per well, and were incubated at 37°C in a humidified 5% CO_2 in air atmosphere.

Assay for Cytolytic Activity. Cells were assayed for cytolytic activity on day 5 in the presence of 10 μg/ml Con A. Effector cells were incubated for 4 h with 2,000 ^{51}Cr-labeled P815 tumor targets in 96-well V-bottomed microtiter plates (No. 3896, Costar). To obtain sufficient numbers of cells for effector titrations, cells from replicate microculture wells were pooled. The apparent specific lysis was calculated as 100 × [(experimental release − spontaneous release)/(total release − spontaneous release)] in which spontaneous release is the cpm release in the absence of effector cells and total release is determined by adding Triton X-100 to a final concentration of 0.4%.

Assay for Cell Proliferation. At 24-h intervals after initiation of culture, 50 μl of cell suspension from each of triplicate microcultures was transferred to 96-well flat-bottomed microtiter plates (No. 3596, Costar), to which $[^{125}I]$UdR was immediately added. $[^{125}I]$-UdR incorporation was determined after incubation for an additional 24 h.

Results

Preparation of IL-4/BSF-1. The purified IL-4 used for these studies contained a single protein on SDS-PAGE and contained no detectable IL-1, IL-2, IL-3, or

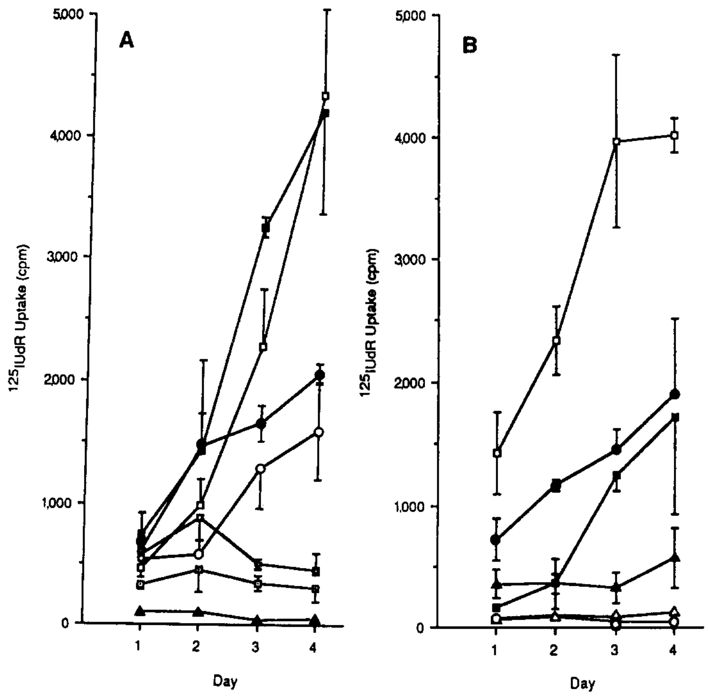

FIGURE 1. Proliferative response of lectin-stimulated Lyt-2$^+$ splenic T cells to IL-4 (*A*) or IL-2 (*B*). At 24-h intervals, [^{125}I]UdR was added to 50 μl of cell suspension removed from each of triplicate microcultures initiated with the indicated concentration of lymphokine. [^{125}I]UdR incorporation was determined after incubation for an additional 24 h. All cultures contained Con A at 1 μg/ml. 7D4 ascites was used at a final concentration of 1:3,200. Error bars represent ±1 SD. See Materials and Methods for experimental details. (*A*) (■) 15 U/ml IL-4; (●) 5 U/ml IL-4; (■) 1.5 U/ml IL-4; (□) 15 U/ml IL-4 + 7D4 ascites; (○) 5 U/ml IL-4 + 7D4 ascites; (⊡) 1.5 U/ml IL-4 + 7D4 ascites; (▲) Con A alone. (*B*) (□) 90 U/ml IL-2; (●) 30 U/ml IL-2; (▲) 10 U/ml IL-2; (■) 90 U/ml IL-2 + 7D4 ascites; (○) 30 U/ml IL-2 + 7D4 ascites; (△) 10 U/ml IL-2 + 7D4 ascites.

BCGF-II/IL-5 activity (McKenzie, D. T., H. I. Filutowicz, S. L. Swain, and R. W. Dutton, manuscript submitted for publication). The sample had an approximate specific activity of 16 U/ng and at a dilution of 1:2 × 10^4 exhibited a half-maximal stimulation of proliferation of resting B cells stimulated with rat monoclonal anti-mouse IgM antibody (data not shown).

Lectin and IL-4 Will Induce the Proliferation of Splenic Lyt-2$^+$ CTL Precursors. As is shown in Fig. 1*A*, addition of Con A and IL-4 together results in a vigorous proliferative response by splenic Lyt-2$^+$ T cells, a proliferative response that has not yet reached a plateau by day 5. At the concentrations used here, Con A alone or IL-4 alone (data not shown) does not cause significant proliferation. As a positive control, Fig. 1*B* demonstrates that IL-2 and Con A will also cause a strong proliferative response of Lyt-2$^+$ splenic T cells, as previously reported (6).

Fig. 1*B* demonstrates that addition of the anti-IL-2-R antibody at high concentration results in ~75% inhibition of the proliferative response to Con A and IL-2 at the highest concentration of IL-2 used, and the complete inhibition of the proliferative response at lower concentrations of IL-2. Fig. 1*A* shows that addition of anti-IL-2-R blocking antibody results in only a slight, statistically insignificant inhibition of the proliferative response to IL-4 plus Con A.

Lectin and IL-4 Will Induce the Generation of Cytolytic Activity in Splenic Lyt-2⁺ CTL Precursors. Fig. 2*A* demonstrates that after culture for 5 d with IL-4 and Con A, splenic Lyt-2^+ cells have developed marked cytolytic activity. Culture with Con A alone does not induce the generation of cytolytic cells (data not shown), although culture with IL-4 does induce a small but reproducible generation of cytolytic activity at high concentrations of the lymphokine. In additional experiments (Fig. 2*D*), similar responses were obtained using rIL-4 and Con A.

Fig. 2*B* shows the cytolytic activity of cultures of Lyt-2^+ splenic T cells stimulated with IL-2 plus Con A. Despite the equivalent proliferative response of cells stimulated with IL-2 plus Con A and cells stimulated with IL-4 plus Con A, it is noteworthy that 15 U/ml IL-4 induces a markedly higher level of cytolytic activity per cell than 90 U/ml IL-2. Higher cytolytic cell responses were obtained with higher doses of rIL-2 (data not shown).

Addition of the mAb, 7D4, at high concentration inhibits the generation of cytolytically active cells by IL-2 plus Con A, as shown in Fig. 2*B*, but has no significant effect on the generation of cytolytically active cells in cultures induced with IL-4 plus Con A, as shown in Fig. 2*A*. Fig. 2*C* demonstrates that the IL-4-dependent responses, but not the IL-2-dependent responses, are blocked by 11B11, the mAb to IL-4. The cytolytic activity of the effector cells on day 5 was completely abolished by treatment with anti-Lyt-2 and complement before assay, demonstrating that the effector CTL are $CD8^+$ (data not shown).

Discussion

BSF-1/IL-4 has been shown to exhibit a number of activities on various T cell populations in addition to its activities on B cells. IL-4 will support the proliferation of long-term T cell lines (8, 9), is able to mediate the autocrine growth of some helper T cell lines after antigenic stimulation (11), and is a potent costimulant for normal resting T lymphocytes (10). A recent report (18) also demonstrates that IL-4 enhances the CTL response to alloantigen within mixed lymphocyte cultures.

We demonstrate here that Lyt-2^+ splenic T cells will proliferate to IL-4 together with the mitogenic lectin Con A, and that the proliferating Lyt-2^+ cells will differentiate into CTL. The recent report (10) that both the L3T4^+ and Lyt-2^+ mesenteric lymph node T cell populations proliferate vigorously to IL-4 together with PMA is in agreement with our results.

Previous reports (6, 7) have demonstrated that IL-2 alone is sufficient for the proliferation and differentiation of lectin- or antigen-stimulated CTL precursors. These results, when coupled with the costimulatory effects of IL-4 on Th cells (10, 11) and the presence of an IL-2-producing T cell subset within the Lyt-2^+ population (19, 20), made it necessary to rule out any contribution by endogenously produced IL-2 to the proliferation and differentiation of cytolytically active cells observed in the cultures. The lack of any significant effect by the blocking anti-IL-2 (21, 22) receptor antibody argues that the stimulatory effects of the purified IL-4 are a property of the IL-4 itself.

It was noteworthy that levels of IL-4 that support a significantly smaller

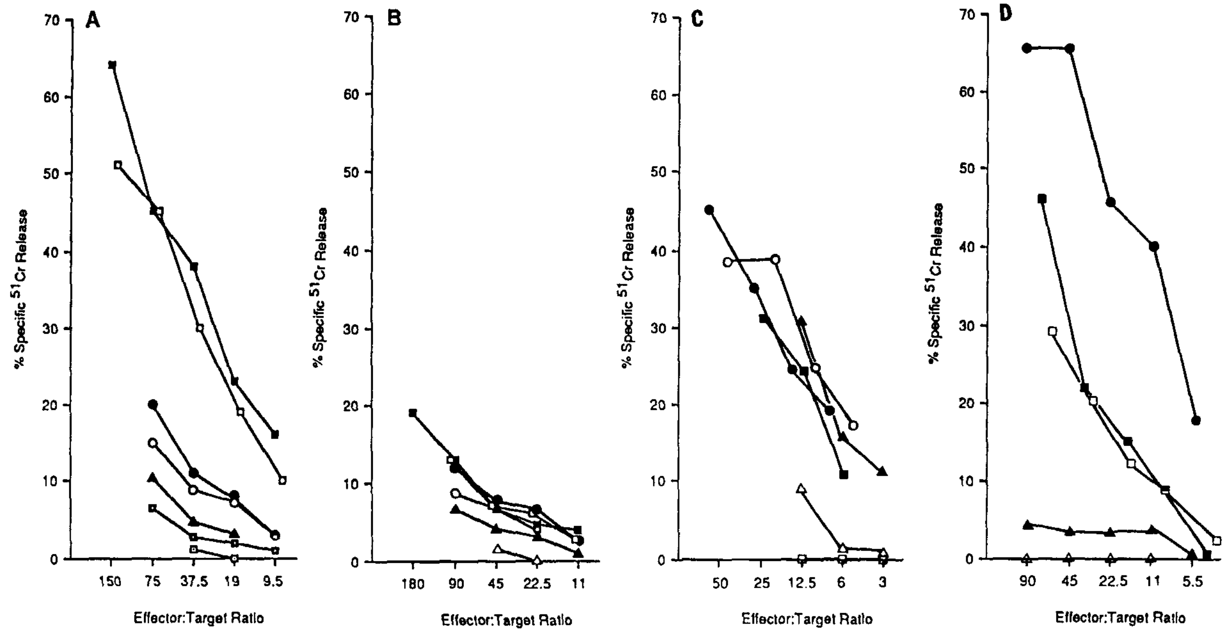

FIGURE 2. Cytolytic activity of Lyt-2$^+$ splenic T cells costimulated with lectin and purified IL-4 (*A*, *C*); rIL-2 (*B*, *C*); rIL-4 (*D*). Microcultures initiated with Con A at 1 μg/ml and the indicated concentration of lymphokine were assayed for cytolytic activity on day 5 in the presence of 10 μg/ml Con A. Cells from replicate microcultures were pooled to obtain sufficient numbers of cells for effector titrations. 7D4 ascites was used at a final concentration of 1:3,200 (*A*, *B*); purified 11B11 culture SN was used at a final concentration of 1:300 (*C*). See Materials and Methods for experimental details. (*A*) (■) 15 U/ml IL-4; (●) 5 U/ml IL-4; (◘) 1.5 U/ml IL-4; (□) 15 U/ml IL-4 + 7D4 ascites; (○) 5 U/ml IL-4 + 7D4 ascites; (⊡) 1.5 U/ml IL-4 + 7D4 ascites; (▲) 15 U/ml IL-4 without Con A. (*B*) (■) 90 U/ml IL-2; (●) 30 U/ml IL-2; (▲) 10 U/ml IL-2; (□) 90 U/ml IL-2 + 7D4 ascites; (○) 30 U/ml IL-2 + 7D4 ascites; (△) 10 U/ml IL-2 + 7D4 ascites. (*C*) (●) 200 U/ml IL-2; (○) 200 U/ml IL-2 + 11B11; (▲) 400 U/ml IL-4; (△) 400 U/ml IL-4 + 11B11; (■) 130 U/ml IL-4; (□) 130 U/ml IL-4 + 11B11. (*D*) (●) 600 U/ml rIL-4; (■) 200 U/ml rIL-4; (▲) 70 U/ml rIL-4; (□) 400 U/ml purified IL-4; (△) 1 μg/ml Con A alone.

proliferative response than observed with a given concentration of IL-2 are more efficacious in stimulating the differentiation of Lyt-2^+ cells into mature cytolytically active cells. The ability of IL-4 to drive differentiation of Lyt-2^+ cells into mature CTL is so marked that we propose a model in which IL-2 is primarily responsible for CTL precursor proliferation but in which IL-4 is the lymphokine primarily responsible for CTL precursor differentiation. This suggests that although Th1 or Th2 cells (as defined by Mossman et al. in reference 13) can independently produce a lymphokine that is sufficient for mature CTL generation, the combined action of the lymphokines produced by Th1 and Th2 cells may be most efficient for CTL generation.

Finally it will be of interest to establish the relationship of IL-4 to other previously described CTL differentiation factors (3–5).

Summary

In this report, we demonstrate that IL-4 is sufficient to stimulate both the proliferation and differentiation of Lyt-2^+, Ia^- splenic CTL precursors stimulated with the mitogenic lectin Con A. The response to IL-4 and Con A was not dependent on a putative endogenous production of IL-2 within the cultures, as demonstrated by an absence of an inhibitory effect by an anti-IL-2-R blocking mAb. Our results indicate that IL-2 and IL-4 can support an equivalent proliferative response by lectin-stimulated Lyt-2^+ T lymphocytes, while IL-4 is more efficacious in stimulating their differentiation into mature cytolytically active cells.

We thank Terry Aaron for her excellent secretarial assistance.

Received for publication 30 March 1987 and in revised form 26 May 1987.

References

1. Hünig, T., M. Loos, and A. Schimpl. 1983. The role of accessory cells in polyclonal T cell activation. I. Both induction of interleukin-2 production and of interleukin-2 responsiveness by concanavalin A are accessory cell dependent. *Eur. J. Immunol.* 13:1.
2. Farrar, J. J., W. R. Benjamin, M. L. Hilfiker, M. Howard, W. L. Farrar, and J. Fuller-Farrar. 1982. The biochemistry, biology, and role of interleukin 2 in the induction of cytotoxic T cell and antibody-forming B cell responses. *Immunol. Rev.* 63:129.
3. Raulet, D. H., and M. J. Bevan. 1982. A differentiation factor required for the expression of cytotoxic T-cell function. *Nature* (*Lond.*). 296:754.
4. Wagner, H., C. Hardt, B. T. Rouse, M. Rollinghoff, P. Scheurich, and K. Pfizenmaier. 1982. Dissection of the proliferative and differentiative signals controlling murine cytotoxic T lymphocyte responses. *J. Exp. Med.* 155:1876.
5. Garman, R. D., and D. P. Fan. 1983. Characterization of helper factors distinct from interleukin 2 necessary for the generation of allospecific cytolytic T lymphocytes. *J. Immunol.* 130:756.
6. Erard, F., P. Corthesy, M. Nabholtz, J. W. Lowenthal, P. Zaech, G. Plaetinck, and H. R. MacDonald. 1985. Interleukin 2 is both necessary and sufficient for the growth and differentiation of lectin-stimulated cytolytic T lymphocyte precursors. *J. Immunol.* 134:1644.
7. Erard, F., M. Nabholtz, and H. R. MacDonald. 1985. Antigen stimulation of cytolytic

T lymphocyte precursors: minimal requirements for growth and acquisition of cytolytic activity. *Eur. J. Immunol.* 15:798.

8. Grabstein, K., J. Eisenman, D. Mochizuki, K. Shanebeck, P. Conlon, T. Hopp, C. March, and S. Gillis. 1986. Purification to homogeneity of B cell stimulating factor. A molecule that stimulates proliferation of multiple lymphokine-dependent cell lines. *J. Exp. Med.* 163:1405.
9. Mossman, T. R., M. W. Bond, R. L. Coffman, J. Ohara, and W. E. Paul. 1986. T-cell and mast cell lines respond to B-cell stimulatory factor 1. *Proc. Natl. Acad. Sci. USA.* 83:5654.
10. Hu-Li, J., E. M. Shevach, J. Mizuguchi, J. Ohara, T. Mossman, and W. E. Paul. 1987. B cell stimulatory factor 1 (interleukin 4) is a potent costimulant for normal resting T lymphocytes. *J. Exp. Med.* 165:157.
11. Fernandez-Botran, R., V. M. Sanders, K. G. Oliver, Y.-W. Chen, P. H. Krammer, J. W. Uhr, and E. S. Vitetta. 1986. Interleukin 4 mediates autocrine growth of helper T cells after antigenic stimulation. *Proc. Natl. Acad. Sci. USA.* 83:9689.
12. Kaye, J., S. Porcelli, J. Tite, B. Jones, and C. A. Janeway, Jr. 1983. Both a monoclonal antibody and antisera specific for determinants unique to individual cloned helper T cell lines can substitute for antigen and antigen-presenting cells in the activation of T cells. *J. Exp. Med.* 158:836.
13. Mossman, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clones. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348.
14. Henderson, L. E., J. F. Hewetson, R. F. Hopkins, III, R. C. Sowder, R. H. Newbauer, and H. Rabin. 1983. A rapid, large scale purification procedure for gibbon interleukin 2. *J. Immunol.* 131:810.
15. 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.
16. Swain, S. L., G. Dennert, J. F. Warner, and R. W. Dutton. 1981. Culture supernatants of a stimulated T cell line have helper activity that synergizes with IL-2 in the response of B cells to antigen. *Proc. Natl. Acad. Sci. USA.* 78:2157.
17. Julius, M. H., E. Simpson, and L. S. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* 3:645.
18. Widmer, M. B., and K. H. Grabstein. 1987. Regulation of cytolytic T-lymphocyte generation by B-cell stimulatory factor. *Nature (Lond.).* 326:795.
19. Sprent, J., and M. Schaefer. 1985. Properties of purified T cell subsets. I. In vitro responses to class I vs. class II H-2 alloantigens. *J. Exp. Med.* 162:2068.
20. Swain, S. L. 1981. Significance of Lyt phenotypes: Lyt antibodies block activities of T cells that recognize class I major histocompatability complex antigens regardless of their function. *Proc. Natl. Acad. Sci. USA.* 78:7101.
21. Malek, T. R., G. Ortega R., J. P. Jakway, C. Chan, and E. M. Shevach. 1984. The murine IL 2 receptor. II. Monoclonal anti-IL 2 receptor antibodies as specific inhibitors of T cell function *in vitro*. *J. Immunol.* 133:1976.
22. Gillis, S., A. E. Gillis, and C. S. Henney. 1981. Monoclonal antibody directed against interleukin 2. I. Inhibition of T lymphocyte mitogenesis and the *in vitro* differentiation of alloreactive cytotoxic T cells. *J. Exp. Med.* 154:983.