INTERACTIONS BETWEEN RECEPTORS FOR INTERLEUKIN 2 AND INTERLEUKIN 4 ON LINES OF HELPER T CELLS (HT-2) AND B LYMPHOMA CELLS (BCL_1)

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IL-4 is a T cell-derived lymphokine that has multiple effects on a variety of cells (reviewed in reference 1). Previous reports (2, 3) have demonstrated that T helper (Th) cells of both Th1 and Th2 subtypes respond to IL-4 *and* IL-2 after antigenic stimulation. Of particular interest, when IL-2 and IL-4 are added together to cultures of Th1 or Th2 cells, both subsets respond in a synergistic manner (3). Both lymphokines must be present simultaneously in order for synergy to occur. These results suggest that the pathways of IL-2- and IL-4-mediated proliferation in T cells are interrelated, either at the level of the receptors for the respective lymphokines and/or at the level of intracellular signaling.

In the present study, we have examined the effect of IL-4 on the number and affinity of IL-2 receptors (IL-2Rs) on a T cell and B cell line. Our results indicate that although IL-4 does not directly compete with IL-2 for binding to IL-2Rs, preincubation of HT-2 or BCL_1-3B3 cells with IL-4 for 60 min at 4°C or 37°C results in a decrease in the number of high affinity IL-2Rs on the cell surface as demonstrated by Scatchard analysis and by crosslinking studies with ^{125}I-IL-2. In addition, since the IL-4-pretreated cells bind less IL-2, they internalize less, consistent with a reduction (down-modulation) in the number of high affinity IL-2Rs. Taken together, these results suggest that IL-4Rs interact with IL-2Rs and that the synergy between IL-2 and IL-4, as well as other effects of IL-4 on T cells, could result, in part, from this interaction.

Materials and Methods

Cell Lines. The IL-2/IL-4-responsive T cell line, HT-2 (4), was maintained in RPMI-1640 medium containing 25 mM Hepes (Gibco Laboratories, Grand Island, NY), 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (10 μg/ml), L-glutamine (2 mM), 2-ME (50 μM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM) and human rIL-2 (10 U/ml) (AMGen, Thousand Oaks, CA). The in vitro-adapted BCL_1 leukemia subline, 3B3 (5), was maintained in RPMI-1640 medium containing 5% heat-inactivated FCS and supplements, as described for the culture of HT-2 cells, with the exception of IL-2. BCL_1-3B3 cells express high affinity IL-2Rs and show an increased proliferation and secretion of IgM when cultured with IL-2

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(6). BCL_1-3B3 cells express IL-4-Rs, as evidenced by specific binding of radiolabeled rIL-4 (Fernandez-Botran, R., J. W. Uhr, and E. S. Vitetta, unpublished observations).

Antibodies, Lymphokines, and Reagents. The anti-IL-4 mAb 11B11 (7) was prepared by ammonium sulfate precipitation of hybridoma culture supernatants (SNs).[1] IL-4 was purified from SNs of Con A-stimulated T-286 cells (which do not synthesize IL-2) (8, 9). IL-4 preparations lacked IL-2 and IFN-γ and their stimulatory effect on HT-2 cells could be completely inhibited by the anti-IL-4 mAb 11B11 (10). 1 U of IL-4 was defined as the reciprocal of the dilution of SN causing 50% of the maximal stimulatory activity of a standard IL-4 preparation in the HT-2 proliferation assay. Murine rIL-4 was generously provided by Dr. T. Mosmann (DNAX, Palo Alto, CA) and Drs. J. Ohara and W. Paul (National Institutes of Health, Bethesda, MD); human rIL-1β was purchased from Cistron Technology (Pine Brook, NJ). Murine granulocyte/monocyte colony-stimulating factor (GM-CSF) and IL-3 were purchased from Genzyme Corp. (Boston, MA). Purified mouse anti-human IL-2 antibody, N7-21, was kindly provided by Dr. A. Jurado (CIBA GEIGY, Basel, Switzerland). Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem-Behring Corp. (La Jolla, CA). 3,3'-dithiobis (propionic acid *N*-hydroxysuccinimide ester) (DTSP) was obtained from Sigma Chemical Co. (St. Louis, MO).

Radiolabeled IL-2. 3-^{125}I-iodotyrosyl-rIL-2 (human) (60 μCi/μg) was purchased from Amersham Corp. (Arlington Heights, IL). It contained <5% free ^{125}I-iodide and retained >90% of its biological activity. Alternatively, human rIL-2 (AMGen) was iodinated following the procedure described by Lowenthal et al. (11) and repurified using affinity chromatography on anti-human IL-2-Sepharose (N7-21) and elution by 50 mM citrate/150 mM NaCl buffer, pH 2.0, followed by immediate neutralization with Tris buffer, pH 9.0. BSA was then added to a final concentration of 1.66 mg/ml.

The concentration of the iodinated material was determined based on its biological activity in the HT-2 proliferation assay (10) as compared with a rIL-2 standard. Specific activity of the iodinated material was 4×10^5 cpm/pmol.

Binding Assays. Binding of ^{125}I-IL-2 to HT-2 and BCL_1-3B3 cells was determined using 1×10^{-12} to 5×10^{-10} M ^{125}I-rIL-2. Briefly, HT-2 cells were harvested at the end of their normal culture period (3 d). At this time, little free IL-2 remains in the medium and proliferation has stopped (data not shown). After harvesting the cells, they were washed three times with balanced salt solution (BSS)/1% FCS, resuspended in RPMI-1640/10% FCS and used for the binding assay (BCL_1-3B3) or were further preincubated 1 h at 37°C to allow internalization of receptors still occupied by IL-2 (HT-2). Cells were washed two more times and incubated at $3\text{-}5 \times 10^5$/tube with increasing concentrations of ^{125}I-IL-2 (1×10^{-12} to 5×10^{-10} M) in the presence or absence of a 100-fold molar excess of unlabeled IL-2 in a final volume of 100 μl in polypropylene Eppendorf tubes. After 60 min of incubation at 4°C with periodic shaking, the cell mixtures were overlayed onto a 0.2 ml mixture of 45% dibutylphthalate (Sigma Chemical Co.) and 55% dioctyl-phthalate (Aldrich Chemical Co., Milwaukee, WI) and centrifuged for 30 s in an Eppendorf microfuge. The tips of the tubes containing the cell pellets were then cut off and counted. The concentration of the free ligand was determined by subtracting cell bound counts from total counts. Specific binding at each concentration of ^{125}I-IL-2 was calculated by subtracting the mean cpm bound in the presence of a 100-fold excess of unlabeled IL-2 (nonspecific binding) from the mean cpm bound in its absence (total binding). Affinity and receptor number were calculated by Scatchard analysis (12) of the equilibrium binding data.

Internalization of ^{125}I-IL-2. Internalization of ^{125}I-IL-2 on control and IL-4-pretreated cells was measured following the procedure described by Weissman et al. (13) based on the development of acid resistance as an indicator for internalization. Briefly, cells were preincubated for 60 min at 4°C with ^{125}I-IL-2 ($2\text{-}5 \times 10^{-11}$ M) in the presence and absence of a 100-fold molar excess of unlabeled IL-2. Aliquots of cells were then either maintained at 4°C or transferred to a 37°C water bath for 30 min. After recooling to 4°C, acid-resistant radioactivity

[1] *Abbreviations used in this paper:* BSS, balanced salt solution; EGF, epidermal growth factor; KLH, keyhole limpet hemocyanin; GM-CSF, granulocyte/macrophage colony-stimulating factor; PDGF, platelet-derived growth factor; SN, supernatant.

was determined by resuspending aliquots of cells in two volumes of 0.5 M acetic acid/0.5 M NaCl buffer, pH 2.0, and centrifuging and counting as described for the binding assay. Total cell-associated radioactivity was determined similarly, except that BSS/1% FCS was substituted for the low pH buffer. Specific binding was calculated by subtracting nonspecific counts from total counts.

Crosslinking of ^{125}I-IL-2 to High Affinity IL-2Rs. HT-2 cells prepared as described for the binding assay were resuspended at 10^7 cells/ml in RPMI-1640/10% FCS and preincubated in the absence or presence of IL-1β (10 U/ml), IL-2 (1×10^{-10} M, 10 U/ml) or IL-4 (50 U/ml) for 60 min at 37°C. The lymphokines were removed by washing the cells three times in BSS/1% FCS. Cells were resuspended at 1×10^7/ml in BSS/1% FCS, and ^{125}I-IL-2 was added to a final concentration of 5×10^{-11} M. Most high affinity, but very few low affinity, IL-2Rs are occupied at this concentration (14). After incubation at 4°C for 60 min, the cells were washed once with PBS and the bifunctional crosslinking reagent, DTSP, was added at a final concentration of 0.5 mM in PBS (Final DMSO concentration of 0.5%). The cells were incubated for 30 min at 4°C and the crosslinker was inactivated and removed by washing once with PBS containing 20 mM L-lysine (pH 7.25) and twice with PBS-azide. The cells were then lysed by incubation in 150 μl of PBS/1% NP-40 containing PMSF (1 mM) and EDTA (2 mM) for 30 min at 4°C. The lysates were centrifuged in an Eppendorf microfuge for 5 min in order to separate nuclei and insoluble debris. The SNs were mixed with an equal volume of 2× Laemmli's sample buffer (15), incubated for 3 min at 100°C and subjected to SDS-PAGE using 7.5% gels and a constant current of 25 mA. After electrophoresis, the gels were fixed in 50% methanol, dried, and exposed to Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY) with a Lightning Plus intensifying screen (DuPont Co., Wilmington, DE) at −70°C for 3–7 d.

Results

Synergy between IL-2 and IL-4 on HT-2 Cells. In a previous study (3) we reported that the addition of both IL-2 and IL-4 to clones of activated Th1 or Th2 cells resulted in a synergistic proliferative response. Delayed addition and "wash-out" experiments indicated that the lymphokines had to be present simultaneously in order for synergy to occur. These findings suggested that the mechanisms involved in the signaling by IL-2 and IL-4 are related. To investigate a possible biochemical basis for this relationship, we chose to study the IL-2/IL-4-responsive, Th cell line, HT-2 (4), because HT-2 cells express relatively high numbers of IL-4Rs (11, 16) and because their responsiveness to IL-2 and IL-4 is not dependent upon antigenic stimulation. We later extended our observations to a neoplastic B cell line, BCL_1-3B3, which also expresses IL-2 and IL-4 receptors (5, 6, and unpublished observations).

We first investigated whether the simultaneous addition of IL-2 and IL-4 to HT-2 cells induced a synergistic proliferative response. As reported previously for clones of Th1 and Th2 cells (3), the addition of a mixture of IL-2 and IL-4 to HT-2 cells induced a proliferative response that was greater than that observed with either lymphokine alone or from that calculated by adding the responses to both lymphokines (when they were added separately) (Table I).

Effects of IL-4 on the Expression of IL-2Rs. The binding of ^{125}I- or ^{35}S-labeled IL-4 to a variety of cells of hemopoietic origin has been reported (11, 16–18) and this binding was *not* affected by the presence of IL-2 or other lymphokines (11, 16–18). This finding, together with the fact that the effect of IL-4 on T cell proliferation cannot be blocked by anti-IL-2R antibodies (9, 10), suggested that IL-4Rs are different from IL-2Rs. However, because of previous reports of synergy and receptor/receptor interactions among several hormones and hemopoietic growth factors (19–21) and the synergy between IL-2 and IL-4 observed in our studies using Th cells (3), we

TABLE I
Synergy between IL-2 and IL-4

Lymphokine added	Units/milliliter	Proliferation of HT-2 cells (cpm/culture)
None		1,296 ± 222
IL-2	1	36,372 ± 4,277
IL-4	5	4,148 ± 313
IL-4	25	17,460 ± 1,519
IL-2 + IL-4	1 + 5	70,625 ± 5,110
IL-2 + IL-4	1 + 25	73,069 ± 11,748

HT-2 cells were cultured in 96-w plates at a density of 5×10^4 cells/ml with the indicated concentrations of lymphokines (IL-2, IL-4) for 24 h at 37°C. The cells were then pulsed with [^{3}H]thymidine (1 μCi/ml) and harvested after 16 h. Results represent the mean of triplicate experiments + SEM.

hypothesized that IL-4R/IL-2R interactions might occur in some subsets (or lines) of T cells. To study this possibility, we preincubated HT-2 cells with IL-4 and determined whether the affinity and/or number of high affinity IL-2Rs were altered. In such experiments, HT-2 cells were preincubated at 4°C for 60 min with IL-2 (1×10^{-10} M), with medium alone, or with either recombinant or purified IL-4 (50 U/ml). After washing away the unbound lymphokines, the affinity and number of high affinity IL-2Rs were determined by analyses of the binding of ^{125}I-IL-2 to HT-2 cells under conditions that would detect mainly high affinity receptors (10^{-12} M to 5×10^{-10} M). The binding data were analyzed using a Scatchard plot (12).

Preincubation of HT-2 cells with IL-4 resulted in a decrease in the levels of specific binding of ^{125}I-IL-2 to such cells when compared with cells preincubated with medium alone (control) (Fig. 1). This decrease was most pronounced at lower concentrations (2.5×10^{-12} to 1×10^{10} M) of ^{125}I-IL-2, where most of the specific binding should involve high affinity receptors. The IL-4-mediated reduction in the binding of ^{125}I-IL-2 was always partial, even after preincubation with higher doses of IL-4. In nine experiments, the decrease in the binding of ^{125}I-IL-2 to HT-2 cells after preincubation with IL-4 ranged from 20.2 to 53.0% (mean ± SD: 39.2 ± 6.7) and from 12.7 to 43.0% (mean ± SD: 28.2 ± 7.1%) when binding was measured at ^{125}I-IL-2 concentrations of 1×10^{-11} M and 5×10^{-11} M, respectively. In contrast, preincubation with unlabeled IL-2 (1×10^{-10} M) almost completely inhibited subsequent binding of ^{125}I-IL-2.

The IL-4-induced decrease in the specific binding of ^{125}I-IL-2 to HT-2 cells was observed irrespective of the source of IL-4 (purified or recombinant) and was completely reversed if anti-IL-4 antibody (11B11) (7) was added to cells incubated with IL-4 (results not shown). In addition, the effect of IL-4 on binding of ^{125}I-IL-2 was not temperature dependent, since it occurred after preincubation at either 4°C or 37°C (Fig. 1, *A, B, D, E*). Preincubation of IL-4R$^+$/IL-2R$^+$ BCL$_1$-3B3 cells with IL-4 at 4°C also resulted in a reduction of the specific binding of ^{125}I-IL-2 (Fig. 1, *C, F*).

When the binding data (Fig. 1, *D–F*) for HT-2 cells (preincubated at 4°C and 37°C) and BCL$_1$-3B3 cells were analyzed by means of a Scatchard plot, curvilinear plots were obtained, suggesting the presence of two populations of IL-2-binding sites

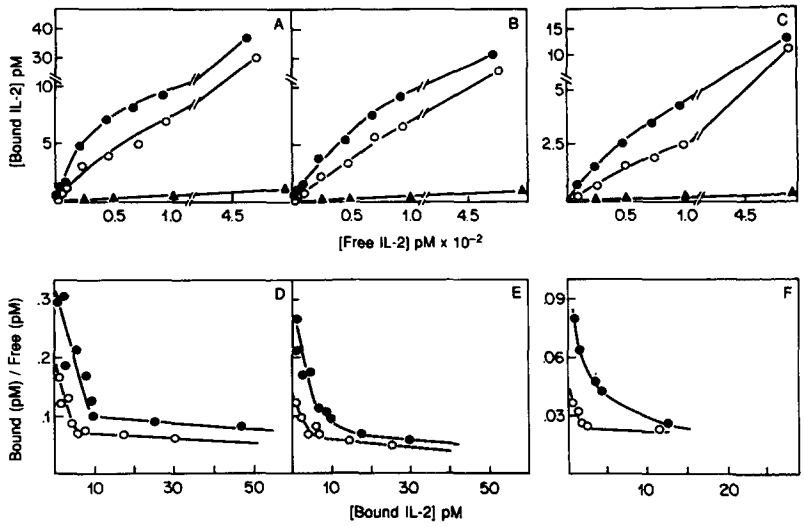

FIGURE 1. Binding of ^{125}I-IL-2 to HT-2 (*A, B, D, E*) and BCL_1-3B3 cells (*C, F*) after preincubation with IL-2 or IL-4 at 4°C (*A, C, D, F*) or 37°C (*B, E*). HT-2 or BCL_1-3B3 cells (3×10^5 cells/group) were preincubated with medium (●), IL-4 (50 U/ml) (○) or IL-2 (0.1 nM) (▲) for 60 min at 4°C or 37°C. The cells were washed (2×) with BSS-1% FCS to remove the unlabeled lymphokines and subsequently incubated at 4°C for 60 min with varying amounts of ^{125}I-IL-2 in the presence and absence of a 100-fold excess unlabeled IL-2 as described in Materials and Methods. Cell-bound ligand was separated from free ligand by centrifugation of the cells through an oil mixture (dibutyl-phtalate/dioctyl-phtalate). The tips of the tubes containing the cell pellets were then cut off and counted. (*A-C*) The specific binding of ^{125}I-IL-2 to cells as a function of the free ligand concentration. (*D-F*) Scatchard plots from binding data obtained from *A-C*, respectively. Graphs show the results of a representative experiment of nine performed. Percent error was <10% for all points.

of high and low affinities as previously reported (14). Pretreatment with IL-4 at 4°C and 37°C affected the high affinity IL-2Rs on both HT-2 and BCL_1-3B3 cells. Linear regression analysis of points representing the high affinity sites showed that although the slopes of the curves were not altered significantly by IL-4, their intercept at the abscissa was reduced, suggesting that the effect of IL-4 was mediated primarily by a decrease in the number of high affinity sites. Table II lists the dissoci-

TABLE II

Effect of Preincubating Cells with IL-4 on the Dissociation Constant and Number of High Affinity IL-2Rs on HT-2 and BCL_1-3B3 Cells

Cells	Dissociation constant*		Receptor number/cell	
	Control	IL-4	Control	IL-4
	$K_d \times 10^{-11}$ M			
HT-2 (4°C)	4.30	4.38	2,810	1,680 (-40.2%)
HT-2 (37°C)	5.65	7.24	2,670	1,646 (-38.4%)
BCL_1-3B3 (4°C)	10.60	10.30	1,676	955 (-43.0%)

* Values for the K_d and receptor number were obtained by linear regression analysis of the Scatchard plots shown on Fig. 1, *D-F*.

ation constant (K_d) and receptor numbers obtained after Scatchard analysis of the ^{125}I-IL-2-binding data. HT-2 cells incubated with medium alone (control) had 2,810 high affinity IL-2Rs per cell with a K_d of 4.5×10^{-11} M. Preincubation with IL-4 for 60 min at 4°C gave a partial decrease (40%) of receptors to 1,680/cell with no apparent change in their affinity (K_d: 4.38×10^{-11} M). Similarly, the number of receptors and their K_d values for HT-2 cells preincubated in the absence or presence of IL-4 for 60 min at 37°C were 2,670 receptors per cell; K_d: 5.65×10^{-11} M and 1,646 receptors per cell; K_d: 7.24×10^{-11} M, respectively. Thus at both temperatures, pretreatment of cells with IL-4 induced a partial decrease in the number of high affinity receptors with no appreciable change in their affinity. This phenomenon was not confined to HT-2 cells, since preincubation of B lymphoma cells (BCL_1-3B3) with IL-4 also resulted in a partial reduction of the number, but not affinity, of the high affinity IL-2Rs. The binding studies did not measure ^{125}I-IL-2 binding at doses higher than 500 pM; thus, an accurate calculation of receptor number and K_d values for low affinity IL-2Rs could not be performed. Nonetheless, after extrapolation of available data (Fig. 1) for control and IL-4-pretreated HT-2 cells, K_d values of $1.5–2 \times 10^{-9}$ M and $3.1–3.8 \times 10^4$ receptors/cell were obtained for both groups, suggesting that the number and/or affinity of low affinity IL-2Rs were not appreciably affected by preincubation with IL-4.

The IL-4-mediated decrease in the specific binding of ^{125}I-IL-2 to high affinity IL-2Rs was relatively selective, because preincubation of HT-2 cells with other lymphokines, including IL-1β, GM-CSF, and IL-3, did not affect the binding of ^{125}I-IL-2 (results not shown). IL-1β induces a synergistic proliferative response on HT-2 cells in the presence of IL-2 or IL-4, indicating that IL-1 binds to these cells (results not shown). We do not know, however, whether GM-CSF and IL-3 synergize with IL-2 and indeed whether our HT-2 cells express receptors for these lymphokines.

Effect of IL-4 on the Internalization of ^{125}I-IL-2. A number of laboratories have reported that internalization of IL-2 is mediated exclusively through high affinity IL-2R (13, 22–25). Therefore, we hypothesized that if pretreatment of HT-2 cells with IL-4 results in a decrease in the number of high affinity sites, the ability of such cells to internalize IL-2 should be diminished when compared with control cells preincubated in medium alone. To test this prediction, HT-2 cells were preincubated with medium alone, IL-4 (50 U/ml) or unlabeled IL-2 (5×10^{-10} M) for 60 min at 4°C; the cells were washed and incubated with ^{125}I-IL-2 (5×10^{-11} M) for an additional hour at 4°C after which aliquots of cells were maintained at 4°C or warmed to 37°C for 30 min. At the end of this incubation period, the cells were cooled to 4°C and the total and acid-resistant (internalized) cell-associated radioactivity was determined by washing the cells with BSS/1% FCS or a high salt/low pH buffer, respectively, followed by centrifugation through an oil cushion.

As shown in Fig. 2, preincubation of HT-2 cells with IL-4 partially decreased the binding of ^{125}I-IL-2 and diminished the internalization of IL-2 when they were warmed to 37°C. Preincubation of HT-2 cells with unlabeled IL-2, on the other hand, completely abolished specific binding and internalization of ^{125}I-IL-2. No ^{125}I-IL-2 was internalized by cells maintained at 4°C. Although the absolute amount of ^{125}I-IL-2 that was internalized after incubation at 37°C was reduced in IL-4-pretreated cells when compared with controls, the amount of IL-2 internalized by both groups of cells relative to the total cell-associated IL-2 was similar (33% and 31% for control and IL-4-pretreated cells, respectively); suggesting that the de-

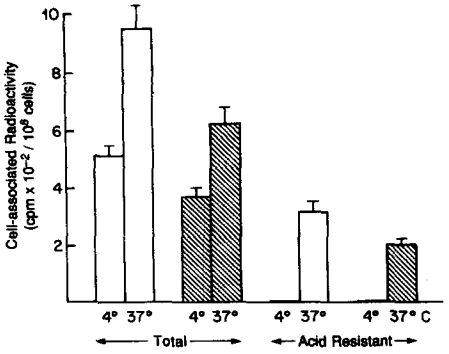

FIGURE 2. Internalization of ^{125}I-IL-2 by HT-2 cells after preincubation with IL-4. HT-2 cells were preincubated with medium alone (control, □) or IL-4 (50 U/ml, ▨) for 60 min at 4°C. After two washes with BSS/1% FCS, both groups of cells were incubated (60 min at 4°C) with ^{125}I-IL-2 at the final concentration of 5×10^{-11} M in the presence or absence of a 100-fold molar excess of unlabeled IL-2. Aliquots of cells were then warmed up to 37°C or maintained at 4°C for 30 min and total and acid-resistant, cell-associated radioactivity were determined by centrifugation through oil, as described in Materials and Methods. Graphs show a representative experiment of three performed. Bars represent mean of triplicate determinations ± SD.

creased internalization of IL-2 in IL-4-pretreated cells was a reflection of reduced binding and not interference with the process of internalization. These results strengthen the notion that preincubation of HT-2 cells with IL-4 results in a decrease in the number of high affinity IL-2Rs, thereby decreasing specific binding and internalization of IL-2.

Crosslinking of IL-2R by ^{125}I-IL-2. The high affinity IL-2R is composed of at least two different subunits, with M_r of 70–75,000 (p70) and 55,000 (p55) (26–30). Both p70 and p55 can independently bind IL-2 with intermediate ($0.5–1 \times 10^{-9}$ M) and low ($\sim 1 \times 10^{-8}$ M) affinities, respectively. Together, p70 and p55 bind IL-2 with high affinity ($\sim 1 \times 10^{-11}$ M). These IL-2 subunits can be visualized by autoradiography of cell lysates on SDS gels after the covalent crosslinking of ^{125}I-IL-2 to cells under conditions of high affinity binding (26–30).

Scatchard analyses of the binding of ^{125}I-IL-2 to cells after preincubation with IL-4 at 4°C and 37°C demonstrated a decrease in the number of high affinity IL-2Rs. Accordingly, we determined whether preincubation of HT-2 cells with IL-4 would result in a partial decrease in the density of bands corresponding to the p70 and p55 subunits of the IL-2R after SDS-PAGE of lysates of cells crosslinked with ^{125}I-IL-2.

As shown in Fig. 3, preincubation of HT-2 cells with unlabeled IL-4 at 37°C fol-

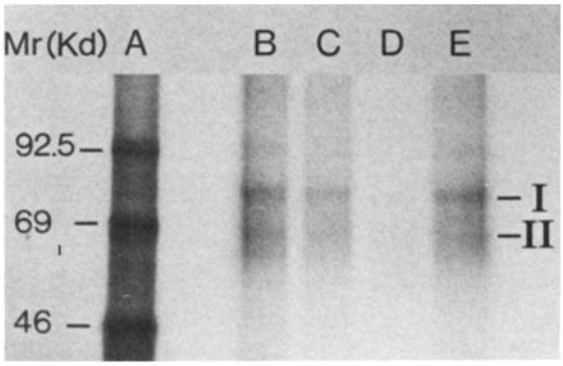

FIGURE 3. SDS-PAGE analysis of lysates of HT-2 cells preincubated with different lymphokines and crosslinked to ^{125}I-IL-2. HT-2 cells (2×10^7 cells/group) were preincubated with (*B*) medium (control), (*C*) IL-4 (50 U/ml), (*D*) IL-2 (0.1 nM), or (*E*) IL-1β (10 U/ml) for 60 min at 37°C in a total volume of 1 ml. After three washes with PBS/azide (10 mM), the cells were incubated with ^{125}I-IL-2 (5×10^{-11} M) for 60 min at 4°C, crosslinked with DTSP and lysed as described in Materials and Methods. After electrophoresis, the gel was fixed, dried, and exposed to X-ray film for 3–7 d. The migration of the standards (*A*) is indicated. The figure depicts one representative experiment of four that were performed.

TABLE III

Scanning Densitometry Analysis of SDS-PAGE Gels of Lysates of HT-2 Cells Crosslinked to ^{125}I-IL-2

Lymphokine added (preincubation)	Area OD		Percent of control	
	Peak I (p70)	Peak II (p55)	Peak I (p70)	Peak II (p55)
	mm			
Medium (control)	2.33 ± 0.13	4.16 ± 0.30	100	100
IL-4 (50 U/ml)	1.03 ± 0.21	2.00 ± 0.45	44.2	48.1
IL-2 (0.1 nM)	0.02 ± 0.01	0.02 ± 0.01	0.9	0.5
IL-1β (10 U/ml)	2.30 ± 0.35	3.92 ± 0.51	98.7	94.2

HT-2 cells (2×10^7/group) were preincubated with the indicated concentration of lymphokines for 60 min at 37°C. After three washes, the cells were incubated with ^{125}I-IL-2, crosslinked and lysed as described in Materials and Methods. After autoradiography of the gels, autoradiograms were scanned on a video densitometer (Bio-Rad Laboratories, Richmond, CA). Peaks I and II correspond to the p70 and p55 subunits of the high affinity IL-2R, respectively, crosslinked to one molecule of ^{125}I-IL-2 (Mr = 15,000), i.e., Peak I (75–90 K_d); Peak II (50–75 K_d). Results represent the mean ± SEM of four experiments.

lowed by binding and crosslinking of ^{125}I-IL-2 resulted in the decreased labeling of the bands corresponding to p70 and p55. As a positive control, preincubation with cold IL-2 followed by crosslinking with ^{125}I-IL-2 virtually abolished the labeling of these two bands, whereas preincubation with IL-1β followed by crosslinking with ^{125}I-IL-2 had no effect compared with the medium control. No binding (or bands) was observed using cells lacking high affinity IL-2Rs (data not shown). When the bands corresponding to p70 (plus ^{125}I-IL-2) (band I) and p55 (plus ^{125}I-IL-2) (band II) were scanned by densitometry, preincubation with IL-4 resulted in a 52–56% decrease in the densities of both bands (Table III). Additional but less distinct bands of higher M_r were also detected and their density was also decreased by preincubation with either IL-4 or IL-2. The nature of these additional bands is not known, but they may represent either multimers of p55 and/or p70 associated with each other or with other surface molecules, or additional IL-2R-associated proteins, such as those described recently by Saragovi and Malek (31, 32).

Discussion

The impetus for the present studies was our observation that IL-4 and IL-2 induce a synergistic proliferative response in both a T cell (HT-2) (3) and a B cell (BCL_1-3B3) line. In this report, evidence is presented to indicate that binding of IL-4 to its receptor induces an interaction between IL-2Rs and IL-4Rs on both HT-2 and BCL_1-3B3 cells. Preincubation of cells with IL-4 at either 4°C or 37°C resulted in a 40–50% reduction in the number of high affinity IL-2 binding sites and, hence, in the amount of ^{125}I-IL-2 internalized. There was no significant change in either the affinity of the remaining IL-2Rs or in the number or affinity of low affinity IL-2Rs. The effect on IL-2Rs appears to be relatively selective since preincubation of HT-2 cells with other lymphokines, such as IL-1β, GM-CSF, and IL-3, did not show any effect on the affinity and/or number of IL-2Rs.

Previous studies have shown that either IL-4 or IL-2 can support proliferation of activated Th cell lines or clones in the absence of other lymphokines (3, 10). In

addition, the receptors for these two lymphokines appear to be distinct as judged by blocking studies with anti-receptor antibodies and by their physicochemical properties (9–11, 16–18, 31–35). Thus, antibodies to IL-2Rs do not block the effects of IL-4 on susceptible cell populations (9, 10, 33–35). A single class of IL-4Rs with a dissociation constant of $\sim 0.5\text{–}1 \times 10^{-10}$ M has been described (11, 16–18). In contrast, high affinity IL-2Rs are composed of at least two different subunits (26–30), both of which bind IL-2 independently and with different association rates (36, 37). The smaller subunit (p55) binds IL-2 with low affinity ($K_d = 1 \times 10^{-8}$ M), whereas the larger subunit (p70) binds IL-2 with intermediate affinity ($K_d = 0.5 \times 10^{-9}$ M). Together, the two subunits form the high affinity IL-2R ($K_d = 1\text{–}5 \times 10^{-11}$ M) which is generally responsible for internalization of IL-2 and for the induction of a proliferative response (22–25, 38). However, p70 is able to mediate signaling and internalization of IL-2 in cells lacking high affinity IL-2Rs (25, 39–41). In summary, IL-2 and IL-4, through their distinctive cell surface receptors, can independently signal cells to proliferate. Hence, the synergy between these two lymphokines could be explained by events taking place intracellularly or at the level of surface receptors. The present studies suggest that interactions between the two cell surface receptors take place and that these interactions may explain the observed synergy.

Receptor/receptor interactions have been documented in a number of hormone receptor systems (reviewed in references 19, 20 and 21, 42–46). Thus, the addition of one hormone can induce a decrease in the affinity (trans-modulation) or the number (down-modulation) of receptors for other hormones (reviewed in reference 19). For example, addition of platelet-derived growth factor (PDGF) or fibroblast growth factor to fibroblasts, can induce trans-modulation of epidermal growth factor (EGF) receptors (reviewed in references 19 and 44). Similarly, although distinct receptors for the different CSFs (IL-3, GM-CSF, granulocyte [G]-CSF and monocyte [M]-CSF) have been identified on immature hematopoietic cells (reviewed in references 20 and 21), there is evidence to suggest that there are interactions among these receptors. For example, Walker et al. (21) have reported a hierarchical pattern of receptor down-modulations in which the CSFs with the broadest range of activities (IL-3, GM-CSF) downregulate the expression of receptors for the more specific cytokines (G-CSF, M-CSF).

There are several possible mechanisms to explain the IL-4-mediated decrease in the number of high affinity IL-2Rs: (*a*) IL-4 could induce an enzymatic change, such as phosphorylation of IL-2Rs, that results in their downregulation; (*b*) IL-4R and IL-2R could share a common subunit; (*c*) The binding of IL-4 to its receptor could induce a direct interaction with IL-2Rs; (*d*) The binding of IL-4 to its receptor could induce an IL-4R-associated molecule or cytoskeletal structure to interact with IL-2Rs.

Larsen et al. (47) have recently reported that activators of protein kinase C, e.g., PMA and 1-oleoyl-2-acetyl-rac-glycerol (OAG), can downregulate high affinity IL-2Rs on mitogen-activated human T cells; this occurs at 37°C but not at 4°C. Since the IL-4-mediated decrease in the number of high affinity IL-2Rs occurs at both 4°C and 37°C, this observation argues against, but does not disprove, such a mechanism.

With regard to sharing a common subunit, crosslinking experiments with labeled IL-4 have assigned the IL-4R an M_r of 60–75 K_d (16, 17), which is close to the M_r of the p70 subunit of the IL-2R (26–32). Scanning densitometry analysis of SDS-

PAGE gels from lysates of cells previously treated with IL-4 and then crosslinked with ^{125}I-IL-2 revealed an equivalent decrease in the density of the bands corresponding to both p55 and p70 when compared with controls. These results suggest a loss of both subunits of the high affinity IL-2R after treatment of the cells with IL-4. Hence, if IL-4Rs interact with IL-2Rs, it is likely that this interaction involves the p70 subunit, or the combination of the p70 and p55 subunits, but not the p55 subunit alone. The presence of p70 subunits has been recently demonstrated on resting T cells (39, 48), which also display similar numbers of IL-4Rs (500–1,000 receptors per cell) (11, 16–18). The availability of an mAb to IL-4R or p70 could definitively settle this issue.

We have considered the possibility that binding of IL-4 to its receptor might induce a conformational change in the receptor and expose a new interaction site for IL-2R or for another molecule that interacts with it. Indeed, crosslinking experiments using ^{125}I-IL-2 and HT-2 cells reveal the presence of additional bands of higher M_r other than those corresponding to the p55 and p70 subunits of IL-2Rs. Bands of similar M_r have recently been reported by Saragovi and Malek (31, 32) in another murine cell line (p55-transfected EL-4 cells). These results suggest that high affinity IL-2Rs might have an even more complex subunit structure or that the p70 and p55 subunits might be closely associated with other membrane proteins. Thus, it is possible that interactions between IL-2Rs and IL-4Rs could take place through one or more of these IL-2R-"associated" proteins.

The observation that the binding of IL-4 to its receptor is able to trigger an interaction with IL-2Rs, raises the possibility that IL-4 might indirectly activate cells via IL-2Rs. Hence, some of the effects of IL-4 on T cells, such as the induction of T cell proliferation (9, 10, 49, 50), the generation of LAK cells (51) and the generation of CTLs (52–54), could be due, at least in part, to the activation of IL-2Rs via interactions with IL-4Rs. Preliminary studies with two sublines of HT-2 cells that differ in their ability to proliferate in response to IL-4, have suggested that the subline that proliferates more effectively to IL-4 undergoes a greater reduction in the number of high affinity IL-2Rs (after treatment with IL-4) when compared with the other subline. This suggests that the ability of IL-4 to downregulate high affinity IL-2Rs on some cells might be related to the responsiveness of such cells to IL-4. In conclusion, it is possible that receptor/receptor interactions are a common occurrence among the different lymphokine receptors and might explain why different lymphokines can synergize or mediate seemingly identical effects on some of their target cells.

Summary

IL-2 and IL-4 induce a synergistic proliferative response in HT-2 cells, suggesting that IL-2Rs and IL-4Rs may interact. The purpose of this study was to examine the effect of IL-4 on the expression and function of IL-2Rs. Preincubation of HT-2 and BCL_1-3B3 cells with IL-4 for 60 min at 4°C or 37°C resulted in a partial decrease in the number, but not the affinity of high affinity IL-2Rs as evidenced by Scatchard analysis of binding data. The decrease in the number of high affinity receptors correlated with decreased internalization of IL-2. After preincubation with IL-4, crosslinking of ^{125}I-IL-2 to high affinity IL-2Rs also demonstrated a ~50% reduction in the number of high affinity IL-2Rs. Another lymphokine, IL-1, which acts

on HT-2 cells, had no measurable effect on the affinity or number of IL-2Rs. Taken together, these results indicate that IL-4 downregulates the expression of high affinity IL-2Rs on some cells. It is not known whether this occurs by a direct ligand-mediated receptor interaction, by the sharing of a common receptor subunit, or by interaction of the two receptors with another membrane molecule or cytoskeletal component.

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References

1. Paul, W. E., and J. Ohara. 1987. B cell-stimulatory factor-1/interleukin-4. *Annu. Rev. Immunol.* 5:429.
2. Vitetta, E. S., A. Bossie, R. Fernandez-Botran, C. D. Myers, K. G. Oliver, V. M. Sanders, and T. L. Stevens. 1987. Interaction and activation of antigen-specific T and B cells. *Immunol. Rev.* 99:193.
3. Fernandez-Botran, R., V. M. Sanders, T. R. Mosmann, and E. S. Vitetta. 1988. Lymphokine-mediated regulation of the proliferative response of clones of Th1 and Th2 cells. *J. Exp. Med.* 168:543.
4. Watson, J. 1979. Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. *J. Exp. Med.* 150:1510.
5. Brooks, K., D. Yuan, J. W. Uhr, P. H. Krammer, and E. S. Vitetta. 1983. Lymphokine-induced IgM secretion by clones of neoplastic B cells (BCL_1). *Nature (Lond.).* 302:825.
6. Brooks, K. H., and E. S. Vitetta. 1986. Recombinant IL-2 but not recombinant interferon-γ stimulates both proliferation and IgM secretion in a $Ly1^+$ clone of neoplastic murine B cells (BCL_1). *J. Immunol.* 137:3205.
7. Ohara, J., and W. E. Paul. 1985. Production of a monoclonal antibody to and molecular characterization of B cell stimulatory factor-1. *Nature (Lond.).* 315:333.
8. Ohara, J., S. Lahet, J. Inman, and W. E. Paul. 1985. Partial purification of murine B cell stimulatory factor (BSF-1). *J. Immunol.* 135:2518.
9. 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 (IL-4) mediates autocrine growth of helper T cells after antigenic stimulation. *Proc. Natl. Acad. Sci. USA.* 83:9689.
10. Fernandez-Botran, R., P. H. Krammer, T. Diamantstein, J. W. Uhr, and E. S. Vitetta. 1986. B cell stimulatory factor-1 promotes growth of helper T cell lines. *J. Exp. Med.* 164:580.
11. Lowenthal, J. W., B. E. Castle, J. Christiansen, J. Schreurs, D. Rennick, N. Arai, P. Hoy, Y. Takebe, and M. Howard. 1988. Expression of high affinity receptors for murine interleukin-4 (BSF-1) on hemopoietic and nonhemopoietic cells. *J. Immunol.* 140:456.
12. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci.* 51:660.
13. Weissman, A. M., J. B. Harford, P. B. Svetlik, W. J. Leonard, J. M. Depper, T. A. Waldmann, W. C. Greene, and R. D. Klausner. 1986. Only high affinity receptors for interleukin-2 mediate internalization of ligand. *Proc. Natl. Acad. Sci. USA.* 83:1463.
14. Robb, R. J., W. C. Greene, and C. M. Rusk. 1984. Low and high affinity cellular receptors

for interleukin-2. Implications for the level of Tac antigen. *J. Exp. Med.* 160:1126.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
16. Ohara, J., and W. E. Paul. 1987. Receptors for B cell stimulatory factor-1 expressed on cells of hematopoietic lineage. *Nature (Lond.).* 325:537.
17. Park, L. S., D. Friend, K. Grabstein, and D. L. Urdal. 1987. Characterization of the high affinity cell surface receptor for murine B cell-stimulating factor-1. *Proc. Natl. Acad. Sci. USA.* 84:1669.
18. Nakajima, K., T. Hirano, K. Koyama, and T. Kishimoto. 1987. Detection of receptors for murine B cell-stimulatory factor-1 (BSF-1): presence of functional receptors on CBA/N splenic cells. *J. Immunol.* 139:774.
19. Zachary, I., and E. Rozengurt. 1985. Modulation of the epidermal growth factor receptor by mitogenic ligands: effects of bombesin and role of protein kinase C. *Cancer Surv.* 4:729.
20. Nicola, N. A. 1987. Why do hemopoietic growth factor receptors interact with each other? *Immunol. Today.* 8:134.
21. Walker, F., N. A. Nicola, D. Metcalf, and A. W. Burgess. 1985. Hierarchical down-modulation of hemopoietic growth factor receptors. *Cell.* 43:269.
22. Fujii, M., K. Sugamura, K. Sano, M. Nakai, K. Sugita, and Y. Hinuma. 1986. High affinity receptor-mediated internalization and degradation of interleukin-2 in human T cells. *J. Exp. Med.* 163:550.
23. Lowenthal, J. W., H. R. MacDonald, and B. J. Iacopetta. 1986. Intracellular pathway of interleukin-2 following receptor-mediated endocytosis. *Eur. J. Immunol.* 16:1461.
24. Nabholz, M., M. C. Combe, P. Corthesy, J. Lowenthal, and R. Gabathuler. 1987. Interleukin-2 receptor traffic in a murine cytolytic T cell line. *Eur. J. Immunol.* 17:783.
25. Robb, R. J., and W. C. Greene. 1987. Internalization of interleukin-2 is mediated by the β chain of the high affinity interleukin-2 receptor. *J. Exp. Med.* 165:1201.
26. Sharon, M., R. D. Klausner, B. R. Cullen, R. Chizzonite, and W. J. Leonard. 1986. Novel interleukin-2 receptor subunit detected by cross-linking under high affinity conditions. *Science (Wash. DC).* 234:859.
27. Tsudo, M., R. Kozak, C. K. Goldman, and T. A. Waldman. 1986. Demonstration of a non-Tac peptide that binds interleukin-2: a potential participant in a multichain interleukin-2 receptor complex. *Proc. Natl. Acad. Sci. USA.* 83:9694.
28. Teshigawara, K., H.-M. Wang, K. Kato, and K. A. Smith. 1987. Interleukin-2 high affinity receptor expression depends on two distinct binding proteins. *J. Exp. Med.* 165:223.
29. Robb, R. J., C. M. Rusk, J. Yodoi, and W. C. Greene. 1987. Interleukin-2 binding molecule distinct from the Tac proteins: analysis of its role in formation of high affinity receptors. *Proc. Natl. Acad. Sci. USA.* 84:2002.
30. Dukovich, M., Y. Wano, L.-T. Bich Thuy, P. Katz, B. R. Cullen, J. H. Kehrl, and W. C. Greene. 1987. A second human interleukin-2 binding protein that may be a component of high affinity interleukin-2 receptors. *Nature (Lond.).* 327:518.
31. Saragovi, H., and T. R. Malek. 1987. The murine interleukin-2 receptor: irreversible cross-linking of radiolabeled interleukin-2 to high affinity interleukin-2 receptors reveals a noncovalently-associated subunit. *J. Immunol.* 139:1918.
32. Saragovi, H., and T. R. Malek. 1988. Direct identification of the murine IL-2 receptor p55-p75 heterodimer in the absence of IL-2. *J. Immunol.* 141:476.
33. Lichtman, A. H., E. A. Kurt-Jones, and A. K. Abbas. 1987. B cell stimulatory factor-1 and not interleukin-2 is the autocrine growth factor for some helper T lymphocytes. *Proc. Natl. Acad. Sci. USA.* 84:824.
34. Severinson, E., T. Naito, H. Tokumoto, D. Fukushima, A. Hirano, K. Hanna, and T. Honjo. 1987. Interleukin-4 (IgG_1 induction factor): a multifunctional lymphokine acting also on T cells. *Eur. J. Immunol.* 17:67.
35. Brown, M., J. Hu-Li, and W. E. Paul. 1988. IL-4/B cell stimulatory factor-1 stimulates

T cell growth by an IL-2-independent mechanism. *J. Immunol.* 131:504.
36. Wang, H.-M., and K. A. Smith. 1987. The interleukin-2 receptor. Functional consequences of its bimolecular structure. *J. Exp. Med.* 166:1055.
37. Lowenthal, J. W., and W. C. Greene. 1987. Contrasting interleukin-2 binding properties of the α (p55) and β (p70) protein subunits of the human high affinity interleukin-2 receptor. *J. Exp. Med.* 166:1156.
38. Smith, K. A. 1984. Interleukin-2. *Annu. Rev. Immunol.* 2:319.
39. Bich-Thuy, L. T., M. Dukovich, N. J. Peffer, A. J. Fauci, J. H. Kehrl, and W. C. Greene. 1987. Direct activation of human resting T cells by IL-2: the role of an IL-2 receptor distinct from the Tac protein. *J. Immunol.* 139:1550.
40. Siegel, J. P., M. Sharon, P. L. Smith, and W. J. Leonard. 1987. The IL-2 receptor β chain (p70): role in mediating signals for LAK, NK, and proliferative activities. *Science (Wash. DC).* 238:75.
41. Kehrl, J. H., M. Dukovich, G. Whalen, P. Katz, A. S. Fauci, and W. C. Greene. 1988. Novel interleukin-2 (IL-2) receptor appears to mediate IL-2-induced activation of natural killer cells. *J. Clin. Invest.* 81:200.
42. Cruise, J. L., S. Cotecchia, and G. Michalopoulos. 1986. Norepinephrine decreases EGF binding in primary rat hepatocyte cultures. *J. Cell. Physiol.* 127:39.
43. Assoian, R. K. 1985. Biphasic effects of type β growth factor on epidermal growth factor receptors in NRK fibroblasts. Functional consequences for epidermal growth factor-stimulated mitosis. *J. Biol. Chem.* 260:9613.
44. Bowen-Pope, D. R., P. E. DiCorleto, and R. Ross. 1983. Interactions between the receptors for platelet-derived growth factor and epidermal growth factor. *J. Cell Biol.* 96:679.
45. Osborne, R., and A. H. Tashjian, Jr. 1982. Modulation of peptide binding to specific receptors on rat pituitary cells by tumor-promoting phorbol esters: decreased binding of thyrotropin-releasing hormone and somatostatin as well as epidermal growth factor. *Cancer Res.* 42:4375.
46. Zeggari, M., C. Susini, N. Viguerie, J. P. Esteve, N. Vaysse, and A. Ribet. 1985. Tumor promoter inhibition of cellular binding of somatostatin. *Biochem. Biophys. Res. Commun.* 128:850.
47. Larsen, C. S., N. O. Christiansen, and V. Esmann. 1988. Modulation of high-affinity interleukin-2 receptors on activated human T lymphocytes by activators of protein kinase C. *Scand. J. Immunol.* 28:167.
48. Sharon, M., J. P. Siegel, G. Tosato, J. Yodoi, T. L. Gerrard, and W. J. Leonard. 1988. The human interleukin-2 receptor β chain (p70). Direct identification, partial purification, and patterns of expression on peripheral blood mononuclear cells. *J. Exp. Med.* 167:1265.
49. Mosmann, 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.
50. Hu-Li, J., E. M. Shevach, J. Mizuguchi, J. Ohara, T. Mosmann, 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.
51. Mule, J. J., C. A. Smith, and S. A. Rosenberg. 1987. Interleukin-4 (B cell-stimulatory factor-1) can mediate the induction of lymphokine-activated killer cell activity directed against fresh tumor cells. *J. Exp. Med.* 166:792.
52. Widmer, M. B., R. B. Acres, H. M. Sassenfeld, and K. H. Grabstein. 1987. Regulation of cytolytic cell populations from human peripheral blood by B cell-stimulatory factor-1 (interleukin-4). *J. Exp. Med.* 166:1447.
53. Widmer, M. B., and K. H. Grabstein. 1987. B cell-stimulatory factor regulates the generation of cytolytic T lymphocytes. *Nature (Lond.).* 326:795.
54. Pfeifer, J. D., D. T. McKenzie, S. L. Swain, and R. W. Dutton. 1987. B cell-stimulatory factor-1 (interleukin-4) is sufficient for the proliferation and differentiation of lectin-stimulated cytolytic T lymphocyte precursors. *J. Exp. Med.* 166:1464.