LYMPHOKINE-MEDIATED REGULATION OF THE PROLIFERATIVE RESPONSE OF CLONES OF T HELPER 1 AND T HELPER 2 CELLS

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T helper (Th) cells are functionally heterogeneous (1-6), and in the murine system, Mosmann et al. (4) have classified clones of Th cells into two major subsets, termed Th1 and Th2. This classification is based, in part, on the pattern of lymphokines that the two types of Th clones secrete after mitogenic or antigenic stimulation. Th1 cells secrete IL-2 and IFN-γ, whereas Th2 cells secrete IL-4 and IL-5 (4, 7). In addition, such clones differ in their ability to support a delayed-type hypersensitivity reaction (5) and in the type of help provided to B cells (4), including the differential induction of the secretion of certain Ig isotypes (8). Similar subsets of rat Th cells have been defined. Using a monoclonal anti-CD45 (lymphocyte-common antigen, T-200), MRC-OX22, Arthur and Mason (9) have shown that MRC-OX22+ Th cells produce IL-2, and MRC-OX22− Th cells help B cells. More recently, antibodies recognizing epitopes on the human (10) and murine (Birkcland, M. L., J. Metlay, V. M. Sanders, R. Fernandez-Botran, E. S. Vitetta, R. M. Steinman, and E. Pure, submitted for publication) CD45 family of molecules have been reported to distinguish these two types of functionally different Th cells.

The mechanisms that control the preferential activation of normal analogs of one subset of Th cells vs. the other and the regulatory interactions between the two cell types in vivo are not yet known. Work from our laboratory (11) and others (12, 13) has demonstrated that clones of Th1 and Th2 cells use IL-2 or IL-4, respectively, as autocrine growth factors in vitro. In addition, Th2 cells can also use IL-2 as a paracrine growth factor, and their responsiveness to IL-2 and IL-4 increases concomitantly after antigenic stimulation (14). These findings suggest that the proliferation of normal Th2 cells could be regulated, at least in part, by Th1 cells. In the present study, we have examined the effects that lymphokines produced by a clone of Th1 cells (IL-2 and IFN-γ), by a clone of Th2 cells (IL-4) or by APC (IL-1) have on the antigen-induced proliferation of both clones.

Our results indicate that antigenic stimulation results in the acquisition of responsiveness by both types of clones to the proliferative effects of both IL-2 and IL-4.

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Responsiveness to both lymphokines decreases with time after initial activation, although the Th1 clone loses its responsiveness to IL-4 more rapidly and to IL-2 more slowly than does the Th2 clone. Together, IL-2 and IL-4 induce a synergistic proliferative response in both types of Th clones. Both lymphokines must be present at the same time in order for synergy to occur. Finally, in Th2 cells, IFN-γ partially inhibits the proliferative responses induced by IL-4 and IL-2, whereas IL-1 synergizes with IL-2 and IL-4. However, neither IFN-γ nor IL-1 affect the IL-2- or IL-4-mediated proliferation of Th1 cells. Taken together, these data suggest that the antigen-induced proliferation of a particular subset of Th cells may be partially modulated by lymphokines produced by the other subset and that the pathways of IL-2- and IL-4-mediated proliferation are related.

**Materials and Methods**

**Animals.** Female BALB/c mice, 7-10 wk of age, were purchased from Cumberland Farms (Clinton, TN) or were bred in the animal facility of the Department of Microbiology, University of Texas Southwestern Medical Center at Dallas.

**Cell Lines.** The IL-2/IL-4-responsive T cell line, HT-2 (15), was maintained in RPMI-1640 medium containing 25 mM Hepes (Gibco Laboratories, Grand Island, NY), 10% heat-activated 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 keyhole-limpet hemocyanin (KLH)-specific, I-A<sup>d</sup>-restricted Th line, T-286 (11), was generated from the lymph nodes of KLH-immunized BALB/c mice according to the procedure described by Kimoto and Fathman (16). T-286 cells were maintained by a 4-5 day stimulation period in the presence of irradiated (3,300 rad) syngeneic spleen cells, KLH (50 μg/ml), and supernatant (SN) from Con A-stimulated rat spleen cells, followed by a 7-14-d rest period. The T-286 cell is a Th2 cell as defined by its production after antigenic or mitogenic stimulation of IL-4, but not IL-2 or IFN-γ (11). A second KLH-specific Th clone, HDK-1 (7), has been classified as a Th1 cell based on its production of IL-2 and IFN-γ, but not IL-4, after antigenic or mitogenic stimulation (7). The IFN-γ-sensitive B lymphoma cell line, WEHI-279 (17) was kindly provided by Mr. Anwar Michael (Department of Pathology, University of Texas Southwestern Medical Center at Dallas) and was maintained in RPMI 1640 medium with 10% FCS and other supplements as described for the culture of HT-2 cells, except for rIL-2.

**Antibodies, Lymphokines, and Reagents.** The monoclonal anti-IL-4 antibody, 11B11 (18), was prepared by ammonium sulfate precipitation of hybridoma culture SNs. Affinity-purified anti-IL-2 mAb, S4B6 (4) and anti-murine IFN-γ, XMG1.2 (7) have been described. The anti-IL-2-R antibody (7D4) was prepared by ammonium sulfate precipitation of culture SNs from the 7D4 hybridoma (American Type Culture Collection, Rockville, MD). Rabbit anti-human IL-1β, which is crossreactive with mouse IL-1β, was generously provided by Dr. R. Newton (DuPont Glenolden Laboratory, Glenolden, PA). A polyclonal antibody (rabbit) to IL-1α was purchased from Cistron Technology (Pine Brook, NJ). A monoclonal directed against natural human IL-1, which is crossreactive with murine IL-1, was purchased from Genzyme (Boston, MA). IL-4 was purified from SNs of Con A-stimulated T286 cells according to the method described by Ohara, et al. (20). IL-4 preparations lacked IL-2 and IFN-γ activities and their stimulatory effect on the proliferation of HT-2, T286, and HDK-1 cells could be completely inhibited by the monoclonal anti-IL-4 antibody, 11B11. 1 U of IL-4 was defined as the reciprocal of the dilution of SN containing 50% of the maximal stimulatory activity of a standard IL-4 preparation in the HT-2 proliferation assay. Murine rIL-4

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1 *Abbreviations used in this paper: KLH, keyhole-limpet hemocyanin; p, maximal proliferation; SAC, splenic adherent cells; SN, supernatant.*
and rIFN-γ were prepared at DNAX (Palo Alto, CA); human rIL-2 was purchased from AMGen (Thousand Oaks, CA), and human rIL-1α and β were obtained from Cistron Technology.

**Lymphokine Assays.** IL-2 and IL-4 activities were assayed by their ability to induce the proliferation of HT-2 cells, as described (21) and by specific inhibition by anti-IL-2 or anti-IL-4 antibodies, respectively. Briefly, HT-2 cells were plated in 96-well plates (5 x 10³ cells/well) together with different dilutions of Th cell SNs in the presence and absence of anti-IL-2 (S4B6) or anti-IL-4 (11B11) antibodies. After 24 h of incubation at 37°C/7.5% CO₂, [³H]thymidine (1 μCi/well) was added, the plates were cultured for an additional 16 h, harvested, and counted. IFN-γ was assayed by its ability to inhibit the proliferation of WEHI-279 cells (17). In this assay, WEHI-279 cells were plated in 96-well plates at a density of 10⁶ cells/ml with different dilutions of Th cell SNs or a standard rIFN-γ preparation in the presence or absence of the anti-IFN-γ antibody, XMG1.2 (7). [³H]Thymidine (1 μCi/well) was added after 24 h of incubation, and the cells were harvested and counted 16 h later.

**Proliferation of Th Cells.** Stimulation of T-286 (Th2) or HDK-1 (Th1) cells was accomplished by culturing the Th cells (24-well plates) at a density of 5 x 10⁵/ml in the presence of KLH (50–100 μg/ml) and syngeneic splenic adherent cells (SAC) for 24 h at 37°C. The SAC were prepared by removing the nonadherent cell fraction from culture wells previously seeded with 7.5 x 10⁶ irradiated spleen cells and incubated for 2 h at 37°C. After 24 h of stimulation, the T cells were washed three times with balanced salt solution, 1% FCS, separated from SAC and dead cells by centrifugation through Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden), and subsequently cultured at a density of 10⁶/ml in RPMI-1640-10% FCS medium with no added lymphokines. Cells were harvested at different times during the secondary culture, and their proliferative response to IL-2 or IL-4 in the presence or absence of other lymphokines (IFN-γ, IL-1) was measured using a 36-h proliferation assay. Briefly, T-286 (Th2) or HDK-1 (Th1) cells were harvested, washed twice in BSS-1% FCS, and incubated in 96-well plates at a density of 2 x 10⁵ cells/ml in the presence of the indicated concentrations of lymphokines. [³H]Thymidine (1 μCi/well) was added after 24 h of culture, and the cells were incubated for an additional 16 h at 37°C. The cells were then harvested and counted. [³H]Thymidine incorporation was taken as a measure of proliferation.

**Staining.** Cells were stained for IL-2-R after the indicated times of culture. Briefly, the cells were incubated for 30 min at 4°C with biotinylated (B) anti-IL-2R (7D4) antibodies in PBS-azide (10 mM)-1% FCS, followed by three washes. Cells were incubated with FITC-avidin (Vector Laboratories, Burlingame, CA), counterstained with propidium iodide (5 μg/ml) (in order to exclude dead cells), and were analyzed on the FACS.

**Results**

**Th1 and Th2 Clones.** Two clones of similar antigenic specificity (KLH) and MHC restriction (I-Aβ), but belonging to different Th subtypes, were chosen for comparison. HDK-1 and T-286 clones have been classified as Th1 and Th2 subtypes, respectively, based on their pattern of lymphokine secretion after antigenic or mitogenic stimulation (4, 7). Th1 cells secrete IL-2 and IFN-γ, whereas Th2 cells secrete IL-4 (7, 11). Table I shows the lymphokine levels secreted after antigenic stimulation, as detected by biological assays and inhibition with monoclonal anti-lymphokine antibodies. Results have also been confirmed by hybridization of cytoplasmic RNA with lymphokine-specific cDNA probes (7, 11). Both clones are able to provide antigen-specific, MHC-restricted help to B cells under conditions of cognate interaction (8).

**Responsiveness of Th1 and Th2 Cells to IL-2 and IL-4 after Antigenic Stimulation.** In previous experiments, we have observed that after antigenic stimulation, Th2 cells secrete IL-4 and proliferate in response to both IL-4 and IL-2 (11, 14). Although Th1 cells would be expected to acquire responsiveness to IL-2 in a similar manner, it was not clear whether Th1 cells would respond to IL-4 after antigenic stimulation.
TABLE I
Lymphokines Secreted by Two Types of Th Cell Clones after Antigenic Stimulation

| Cell lines     | IL-2 | IL-4 | IFN-γ |
|---------------|------|------|-------|
| HDK-1 (Th1)   | 25   | <0.1 | 23.5  |
| T-286 (Th2)   | <0.1 | 37.5 | <0.01 |

Culture SN from antigen-stimulated HDK-1 and T-286 cells were collected after a 24-h incubation of the T cells (T) at a density of $5 \times 10^5$ cells/ml with KLH (50 μg/ml) and irradiated (3,300 rad) SAC from BALB/c mice. After filtration (0.22 μm), the T cell SNs were tested for the presence of IL-2, IL-4, and IFN-γ as described in Materials and Methods. Results are from a representative experiment.

We thus compared the responsiveness of Th1 and Th2 cells with both IL-2 and IL-4 at various times after antigenic stimulation. In addition, we examined the maintenance of responsiveness as a function of the time in culture after removal of both the antigen and the secreted lymphokines.

As shown in Fig. 1, antigenic stimulation resulted in the acquisition of responsiveness by the two types of Th clones to both IL-2 (Fig. 1 A and C) and IL-4 (B and D), although the maximal level of proliferation attained at optimal doses of IL-4 was always lower than that observed with IL-2. The absolute degree of proliferation ([3H]Thymidine incorporation; cpm/culture) to IL-4 at day 1 after stimulation was similar for both types of clones; however, when the maximal proliferation with IL-4 (pIL-4) was expressed as a fraction of the maximal proliferation with IL-2 (pIL-2), Th1 cells were relatively less responsive to IL-4 (pIL-4/pIL-2 = 0.23) than Th2 cells (pIL-4/pIL-2 = 0.43). In the absence of additional stimuli, responsiveness to both IL-2 and IL-4 could be completely inhibited by the monoclonal anti-IL-4 antibody, 11B1 (18), but not by the monoclonal anti-IL-2 antibody, S4B6 (4).
FERNANDEZ-BOTRAN ET AL.

TABLE II
The Proliferation of Th1 Cells to IL-4 Is not Dependent on IL-2

| Lymphokine added | Antibody added | \[^{3}H\]Thymidine incorporation | cpm/culture |
|------------------|----------------|---------------------------------|------------|
| None             | -              | -                               | 1,411 ± 465 |
| None             | +              | -                               | 1,370 ± 184 |
| None             | -              | +                               | 1,814 ± 199 |
| IL-2 (1 U/ml)    | -              | -                               | 91,086 ± 907 |
| IL-2             | +              | -                               | 1,170 ± 71  |
| IL-2             | -              | +                               | 83,739 ± 2,000 |
| IL-4 (25 U/ml)   | -              | -                               | 11,255 ± 720 |
| IL-4             | +              | -                               | 10,519 ± 47  |
| IL-4             | -              | +                               | 1,007 ± 112  |

HDK-1 cells (Th1) were stimulated with KLH (50 μg/ml) and SAC. 2 d later, the Th cells were harvested and tested for their ability to proliferate to IL-2 or IL-4 in the presence and absence of anti-IL-2 (S4136, 2 μg/ml) or anti-IL-4 (11B11, 2 μg/ml). Results represent the mean of triplicate experiments ± SEM.

lymphokines decreased with time after initial antigenic stimulation, although Th1 cells lost their responsiveness to IL-4 more rapidly and to IL-2 more slowly than Th2 cells. In fact, after 4 d of culture, Th1 cells were no longer responsive to IL-4, but retained their responsiveness to IL-2. In contrast, in concomitant assays, Th2 cells showed a parallel decrease in their responsiveness to both lymphokines. After the same stimulation and culture protocol, analysis of the expression of IL-2-Rs by indirect immunofluorescence with the anti-IL-2R antibody, 7D4 (19), demonstrated that the levels of expression of IL-2R, as measured by the mean fluorescence intensity, followed a similar course to that described for IL-2 responsiveness. IL-2-R levels increased as a result of antigenic stimulation and then gradually returned to baseline levels (results not shown).

The IL-4-mediated Proliferation of Th1 Cells Is not Mediated by Endogenously-produced IL-2. Although it is clear that HT-2 and Th2 cells respond to IL-4 and IL-2 independently (11, 21), the possibility existed that the proliferation of Th1 cells observed in the presence of IL-4 was the result of endogenous production and utilization of IL-2 as a consequence of IL-4 stimulation. To determine whether this was the case, Th1 cells were harvested 1–2 d after initial activation and assayed for their ability to proliferate in response to IL-4 in the presence of anti-IL-2 or anti-IL-4 antibodies. As shown in Table II, the presence of anti-IL-2 antibodies at concentrations that completely inhibited IL-2-mediated proliferation had no effect on the IL-4-mediated proliferation of the Th1 cells, while the anti-IL-4 antibodies completely inhibited such responses. These results suggest that the IL-4-mediated proliferation of Th1 cells cannot be attributed to the endogenous production of IL-2, unless IL-2 can deliver a signal to the receptor inside the cell before exposure to the antibody.

Effects of IFN-γ on Th1 and Th2 Proliferation. Although IFN-γ is known to inhibit the effects of IL-4 on B cells (17, 22–26), no effects on the IL-4-mediated proliferation of Th cells have been reported. In this regard, the ability of Th1 and Th2 cells to proliferate in response to IL-2 or IL-4 was measured in the presence of increasing concentrations of rIFN-γ (1–50 U/ml). As shown in Fig. 2 (A and B), rIFN-γ had
LYMPHOKINE REGULATION OF CLONES OF T HELPER CELLS

Effect of murine rIFN-γ on the proliferation of Th1 (HDK-1) (A and B) or TH2 (T-286) (C and D) cells to IL-2 (A and C) and IL-4 (B and D). Th cells were stimulated with antigen and irradiated SAC as described. After 2–5 d of culture in the absence of antigen or lymphokines, proliferation of the Th cells to IL-2 or IL-4 was measured in the presence of rIFN-γ at the final concentrations of 0 (○), 1 (●), 10 (▲), and 50 U/ml (■). Results represent the mean of triplicate experiments.

Figure 3. Effect of rIL-1 on the proliferation of Th1 (A and B) and Th2 (C and D) cells to IL-2 (A and C) and IL-4 (B and D) as a function of the time elapsed after antigenic stimulation. Th cells were stimulated and cultured as described. Proliferation to 5 (●, ○) and 0.5 U/ml (▲, △) of IL-2 or 25 (●, ○) and 10 U/ml (▲, △) IL-4 was tested in the presence (O, △) or absence (●, ▲) of human rIL-1β at 10 U/ml at different times after antigenic stimulation. Results represent the mean of triplicate experiments.

little effect, if any, on the IL-2- or IL-4-mediated proliferation of Th1 cells. In contrast, rIFN-γ partially inhibited (30–60%) both the IL-2- and IL-4-mediated proliferation of Th2 cells (Fig. 2, C and D). The inhibition of proliferation observed with rIFN-γ was never complete even at higher concentrations. Furthermore, the fact that different concentrations of rIFN-γ did not cause a significant shift in the dose-response curves to IL-2 or IL-4, but did cause a change in the level of proliferation, suggests that rIFN-γ acts as a noncompetitive antagonist in the IL-2- or IL-4-mediated proliferation of Th2 cells. Th2 cells harvested at different times after stimulation showed
TABLE III
The Effect of Anti-IL-1 Antibodies on the Proliferation of Th2 Cells to IL-2 or IL-4

| Lymphokine      | Anti-IL-1 | \[^{3}H\]Thymidine incorporation |
|-----------------|-----------|---------------------------------|
|                 |           | cpm/culture                      |
| None            | –         | 1,104 ± 105                      |
| IL-2(1 U/ml)    | –         | 59,326 ± 3,989                   |
| IL-4 (10 U/ml)  | –         | 22,836 ± 1,534                   |
| IL-2            | +         | 56,303 ± 2,375                   |
| IL-4            | +         | 20,910 ± 677                     |
| IL-1 (5 U/ml)   | –         | 1,202 ± 122                      |
| IL-2 + IL-1     | –         | 101,125 ± 4,353                  |
| IL-4 + IL-1     | –         | 40,757 ± 2,965                   |
| IL-2 + IL-1     | +         | 55,401 ± 2,375                   |
| IL-4 + IL-1     | +         | 21,337 ± 1,625                   |

T-286 (Th2) cells were stimulated and cultured as described. 2-3 d after culture in the absence of antigen, the cells were harvested and tested for their ability to proliferate to IL-2 or IL-4 in the presence and absence of rIL-1α and/or anti-IL-1α/β antibody. Results represent the mean of triplicate experiments ± SEM.

similar degrees of inhibition by IFN-γ, suggesting that responsiveness to this lymphokine did not follow the same time course as responsiveness to IL-2 or IL-4.

Effects of IL-1 on the Proliferation of Th1 and Th2 Clones. The requirement for IL-1 in the responses of Th cells to antigens (27-29) and, more recently, to IL-4 has been reported by several groups (13, 30, 31). This prompted us to investigate the effects of IL-1 on the proliferation of Th1 and Th2 cells after antigenic stimulation and to determine whether IL-1 was required for IL-4-mediated proliferation. rIL-1α or rIL-1β alone did not show any proliferative activity at doses up to 10 U/ml. However, both forms of IL-1 synergized with IL-2 or IL-4 in the proliferation of Th2 cells. In contrast, rIL-1 (α or β) had very little effect on the proliferation of Th1 cells. Fig. 3 (A-D) shows the effects of IL-1 on the proliferative response of Th1 and Th2 cells to IL-2 and IL-4. Interestingly, the enhancement of proliferation was most evident when the cells were harvested 2 d after antigenic stimulation.

The addition of anti-IL-1 antibodies at doses that completely inhibited IL-1 activity (α and β) (10 U/ml) in a thymocyte proliferation assay (32), had no effect on the proliferation of HDK-1 or T-286 cells to IL-4. In fact, even when such antibodies inhibited the synergy between IL-1 and IL-4 on T-286 cells, they were unable to inhibit proliferation beyond that attained with IL-4 alone (Table III). These results suggest that although IL-1 is able to synergize with IL-4, IL-1 is not an absolute requirement for the proliferation of some Th2 clones.

Synergy between IL-2 and IL-4. Since both Th1 and Th2 cells are able to proliferate in response to IL-2 and IL-4 shortly after antigenic stimulation, we next investigated the effects of a mixture of IL-2 and IL-4 on the proliferation of both Th1 and Th2 cells. As shown in Fig. 4 (A and B), both HDK-1 and T-286 cells responded in a synergistic fashion to the simultaneous addition of IL-2 and IL-4. Furthermore, in accord with the greater sensitivity of Th2 cells to IL-4, T-286 cells were able to synergize in a more efficient manner (4.2-fold) than HDK-1 cells (2.7-fold). When
increasing doses of IL-2 were titrated against increasing doses of IL-4 (Fig. 5, A and B), it was evident that neither lymphokine changed the concentration of the other which was required to elicit a half-maximal level of proliferation, but rather increased the maximal level of proliferation. These results suggest that IL-2 and IL-4 do not synergize by increasing the affinity of their respective receptors. The synergistic effects of IL-2 and IL-4 were demonstrated with both recombinant and purified IL-4 (results not shown). The synergy between the two lymphokines was more evident 4–5 d after antigenic stimulation when responsiveness to either lymphokine alone had decreased considerably.

**Kinetics of the Synergy between IL-2 and IL-4: Delayed Addition.** In B cells, IL-4 acts before a secondary stimulus, such as anti-Ig or LPS, to induce B cell proliferation (33–35) or IgG1 secretion (35, 36), respectively. We thus determined whether IL-4 could act before IL-2 (or vice versa) at inducing a synergistic proliferative response in Th cells. In these experiments, we studied the synergistic response of Th2 (T-286) cells because of their higher response to IL-4. Th2 cells were harvested 4–5 d after activation (when cells are no longer proliferating) and were cultured in microtiter wells with medium alone or with either IL-2 or IL-4 at concentrations giving similar proliferative responses (time 0). At different times, IL-2 and IL-4 were added to wells containing medium alone, IL-4 was added to wells containing IL-2, and IL-2 was added to wells containing IL-4. The cells were then cultured for 36 h and proliferation was measured. As shown in Figure 6, synergistic responses (when compared with cells cultured with IL-2 or IL-4 only) were observed irrespectively of which lymphokine was added first. Cells cultured with either IL-2 or IL-4 during the first phase of the culture gave a better proliferative response than those receiving medium alone, suggesting that the presence of either lymphokine was required to maintain
initially with IL-2 (group B) received IL-4 (10 U/ml) (C) and incubated for a total of 36 h using a two-phase culture. At the initiation of phase I (time 0), group A was cultured with medium alone ( ), group B with IL-2 (1 U/ml) ( ), and group C with IL-4 (10 U/ml) ( ). At different times after initiation of phase I (x-axis), cells that were cultured with medium alone (group A) received a mixture of IL-2 and IL-4 ( ); IL-2 alone ( ); or IL-4 alone ( ). Control cells cultured initially with IL-2 (group B) received IL-4 ( ) and cells cultured initially with IL-4 (group C) received IL-2 ( ) (phase II). After 36 h in culture, the cells were pulsed with 1 μCi/well of [3H]thymidine and harvested 12 h later.

synergy. Although the degree of proliferation decreased by delaying the addition of the second lymphokine, when compared with cells cultured with only one lymphokine, a synergistic effect was observed throughout the experiment.

**Kinetics of the Synergy between IL-2 and IL-4: Wash-out Experiment.** To investigate whether the signal provided by the first lymphokine could be separated from the signal provided by the second, T-286 cells were cultured as described for the delayed addition experiment, except that the first lymphokine was washed out before the second lymphokine was added. In control cultures, cells were incubated in the absence of lymphokines during the first phase of culture and with both or either lymphokine during the second phase of culture. As shown in Fig. 7, and in contrast to the previous experiment, synergy was observed only when both lymphokines were provided simultaneously. If the first lymphokine was removed before the addition of the second, synergy was not observed and proliferation was comparable with that attained in the presence of the second lymphokine alone. These results suggest that in order for IL-2 and IL-4 to synergize, both must be present at the same time.

**Discussion**

In the present report, we have compared the IL-2- and IL-4-mediated responses of two KLH-specific, I-A<sup>ê</sup>-restricted Th clones, one of the Th1 type, and the other of the Th2 type. We have also studied the effects of IFN-γ and IL-1 on the IL-2-
and IL-4-mediated responses. Our major findings are as follows. (a) Both types of Th cells proliferate in response to either IL-2 or IL-4 after antigenic stimulation. However, Th2 cells were more responsive to the effects of IL-4. (b) Responsiveness to IL-2 and IL-4 decreases rapidly in both Th subsets as a function of time after activation. (c) IL-2 and IL-4 synergize in inducing proliferation of both activated Th clones but synergy requires the simultaneous presence of both lymphokines. (d) In contrast to Th2 cells, Th1 cells were not susceptible to either the inhibitory effects of IFN-γ or the synergistic effects between IL-1 and either IL-2 or IL-4.

Secretion of lymphokines by both Th subsets is not constitutive, but is dependent on mitogenic or antigenic stimulation (4, 7, 11). Moreover, when harvested at the end of their normal rest period (7–14 d), cells are unresponsive to the growth-promoting effects of IL-2 or IL-4, indicating that activation through the TCR induces lymphokine secretion and responsiveness. This responsiveness is probably mediated by the up-regulation of membrane receptors for IL-2 and IL-4. In vivo, such proliferation should be self limited, allowing for the controlled expansion of the activated Th cells. Indeed, after activation in vitro, lymphokine release is complete by 24–48 h (37), and our results have shown a progressive decrease in responsiveness to both IL-2 and IL-4 associated with a down-regulation in the density of IL-2-R.

The relationship between the two types of Th cell subsets in vivo is not yet understood. It is unclear whether the two subsets represent different lineages or different stages of the same lineage. In either case, in vivo immunization would probably lead to the activation of antigen-specific Th cells from both subsets, even though the precursor frequencies might differ depending on the antigen used. The fact that Th2 cells up-regulate IL-2-R and proliferate in response to IL-2 to a greater degree than to IL-4, suggests that in order for an optimal response to occur, Th1 cells would also have to be activated. Based on our results, we would predict that shortly after antigenic stimulation, IL-2 and IL-4 produced by Th1 and Th2 cells, respectively, would not only be used by both autocrine and paracrine mechanisms, but that their combined effects would be synergistic, especially when the responsiveness of both subsets decreases and less than optimal levels of lymphokines are available. On the other hand, IFN-γ production by Th1 cells would regulate Th2 cell function by down-regulating proliferation and would also inhibit the effects of IL-4 on B cells (17, 22–26).

Our results are in agreement with those of Kurt-Jones et al. (31) who demonstrated that Th2 cells respond more effectively to IL-4 than do Th1 cells. In contrast to our results, however, their Th1 clones did not respond to IL-4 at all. This discrepancy can be explained by the fact that they assayed the responsiveness of their clones to IL-4 after 8 d of culture. Thus, our Th1 cells, although responsive to IL-4 soon after antigenic stimulation, lose their responsiveness to IL-4 rapidly, becoming unresponsive after 4 d, while still retaining responsiveness to IL-2.

The decrease in the responsiveness of Th1 and Th2 cells to IL-2 correlates with a concomitant decrease in the levels of IL-2-Rs. However, the antibody used to detect IL-2-R reacts with both high- and low-affinity IL-2-R (p55 subunit) (38) and, as such, a precise estimation of the number of biologically relevant high-affinity IL-2-R (39) that are responsible for the proliferative response, cannot be made. The responsiveness of Th1 and Th2 cells to IL-4 is probably related to their expression of IL-4-Rs. Along these lines, Ohara and Paul (40) have reported an increase in
the number of IL-4-R on splenic T cells after mitogenic stimulation, and Kurt-Jones et al. (31) have reported a relatively higher number of IL-4-R on Th2 cells than on Th1 cells. In light of the differences in IL-2- and IL-4-responsiveness between Th1 and Th2 cells, one could speculate that differences also exist in the manner in which these two Th subsets regulate their receptors for IL-2 and IL-4.

The antagonistic effects of IFN-γ on the IL-4-mediated activities on B cells are well-documented (17, 22-27, 41). With regard to Th2 cells, the partial inhibitory effect of IFN-γ was not restricted to IL-4-, but also to IL-2-mediated proliferation, suggesting that, at least in T cells, IFN-γ does not act by inhibiting only IL-4-mediated signals. Indeed, Snapper and Paul (41) have reported that in LPS-stimulated B cells, the effects of IFN-γ are independent of those of IL-4. The inhibition of IFN-γ on the IL-2- and IL-4-mediated proliferation of Th2 cells was only partial (30-60%) and could not be increased by increasing the levels of IFN-γ. Similarly, Oliver et al. (26) have also found partial inhibitory effects of IFN-γ on the IL-4-mediated increase in Ia antigens on B cells. The reasons for the partial inhibition are unclear at present. A possibility for the decreased proliferation of Th2 cells in the presence of IFN-γ could be the result of a toxic effect of IFN-γ on these cells. However, the fact that the inhibitory effects of IFN-γ were more evident at high concentrations of IL-2 or IL-4 rather than at low concentrations, suggests that IFN-γ might interfere with cell proliferation rather than cell viability. In contrast to Th2 cells, IFN-γ was unable to inhibit IL-4- or IL-2-mediated proliferation of Th1 cells, suggesting that the latter cells may lack or have lower numbers of IFN-γ receptors, or that their growth machinery is not susceptible to inhibition by IFN-γ.

IL-1 has been reported to be a cofactor required for the responses of certain Th cells to antigenic stimulation (27-29). More recently, several Th2 cells such as D.10G4 (42) and CDC25 (43) have also been reported to require the presence of IL-1 in order to respond to IL-4 (13, 31). In our initial experiments, however, we observed significant proliferation of our Th1 or Th2 cells in the absence of added IL-1. The possibility exists, however, that contaminating macrophages from the APC cells used during stimulation, provide a source of IL-1. Arguing against this, the addition of anti-IL-1 antibodies at concentrations that inhibited IL-1 activity (α and β) in a thymocyte proliferation assay were unable to affect the response of Th1 or Th2 cells to IL-4, suggesting that not all Th2 cells require IL-1 in order to respond to IL-4. Nevertheless, although the failure of anti-IL-1 antibodies to inhibit the IL-4-mediated proliferation of Th2 cells would argue against the need for soluble IL-1, we cannot rule out a potential role for membrane-bound IL-1 in IL-4-mediated proliferation of Th cells. An alternative explanation is that shortly after activation, Th2 cells are able to respond to IL-4 alone, but as they lose their responsiveness to this lymphokine, IL-1 can act as cofactor enabling them to respond to IL-4. In fact, Kupper et al. (13) reported that antigen-stimulated D.10G4 cells are able to respond to IL-4 in the absence of IL-1 shortly after stimulation. Manger et al. (44) have also reported that activated human T cells do not require IL-1 for induction of proliferation (after anti-CD3 stimulation) while resting T cells do require IL-1. Moreover, the addition of IL-1 to the T-286 cells, even when not absolutely required, had synergistic effects on the IL-2- and IL-4-mediated proliferation, indicating that IL-1 can potentiate the effects of IL-4 (and IL-2). Such synergistic effects have also been observed in
D10G4 cells (13). In contrast to Th2 cells, IL-1 had little or no effect on the IL-2- or IL-4-mediated proliferation of Th1 cells. Such results are in agreement with those of Kurt-Jones et al. (31), who also reported that Th2, but not Th1, cells are responsive to IL-1, and suggested that Th2 cells express higher numbers of high-affinity IL-1-R.

From a functional point of view, the synergy between IL-2 and IL-4 could provide a mechanism for a cooperative response between the two Th cell subtypes. From a biochemical perspective, it suggests that the pathways of IL-2- and IL-4-mediated proliferation are interrelated. Results from wash-out experiments indicate that IL-2 and IL-4 must be present simultaneously in order for synergy to occur. It is possible that synergy requires simultaneous signalling through both IL-2-R and IL-4-R, either independently of each other or through some type of receptor/receptor interaction. Indeed, hormone-induced receptor interactions, as manifested by changes in the affinity or number of receptors for a second hormone, are often accompanied by synergy between the two hormones or by imitation of the effects of one hormone by the other (reviewed in reference 45). Along these lines, Walker et al. (46) have demonstrated a hierarchical pattern of receptor interactions, as evidenced by receptor down-regulation, among receptors for several hemopoietic growth factors, including IL-3, granulocyte-monocyte colony stimulating factor (GM-CSF), granulocyte-CSF (G-CSF) and monocyte-CSF (M-CSF). We have obtained evidence for an IL-4-mediated down-regulation of IL-2-Rs, suggesting a potential IL-4-R/IL-2-R interaction (Fernandez-Botran, R., V. M. Sanders, and E. S. Vitetta, manuscript in preparation). There are, therefore, several mechanisms that could operate in vivo to amplify initial stimulation of both Th subsets and, subsequently, turn-off their responses. A hypothetical scenario would be that antigenic stimulation in the context of cognate interaction induces lymphokine secretion (IL-2, IL-4, and IL-1) and upregulation in the number of IL-2-R and IL-4-R, resulting in the acquisition of responsiveness to the secreted lymphokines and leading to increased proliferation of both Th subsets (to IL-2 and IL-4 as a result of synergy amongst the three lymphokines). IFN-γ would act by regulating both Th2 proliferation and the effects of IL-4 on B cells. Antigen depletion would then result in cessation of lymphokine secretion and decreased responsiveness due to a reduction in the number of IL-2 and IL-4 receptors. These effects would result in a return of the T cells to a non-dividing state.

In addition to the reported effects of IL-2, IL-4, IFN-γ, and IL-1 on the proliferation of Th1 and Th2 cells, such lymphokines might also affect another aspect of Th cell function, such as lymphokine secretion. The possibility is currently being investigated. Moreover, regulation at the level of the Th cells will eventually lead to regulation at the level of B cell activation and differentiation. Indeed, both Th clones used in these studies are able to provide help to B cells (4) and induce the secretion of different IgG isotypes (8).

Interpretation of the data reported here could be more complicated if there is additional heterogeneity among Th cell subtypes or differences between cloned Th cells and normal Th cells (6). Recently, different laboratories have reported the existence in human (9) and mouse (10) of cell surface markers which may distinguish Th cell subsets based on reactivity with antibodies recognizing certain epitopes on
the CD45 surface molecules (lymphocyte common antigen, T-200). The use of such reagents should facilitate studies of how Th cell subsets are regulated in vivo.

Summary

Murine Th1 and Th2 subsets differ not only in the lymphokines they produce, but also functionally. It is not clear what factors influence the preferential activation of one subset versus the other and what regulatory interactions exist between them. The purpose of this study was to examine the effect of lymphokines produced by clones of Th1 cells (IL-2 and IFN-γ), Th2 cells (IL-4), and APC (IL-1) on the proliferative response of Th1 and Th2 cells after antigenic stimulation. Activation of both types of clones in the presence of antigen and APC resulted in the acquisition of responsiveness to the proliferative effects of both IL-2 and IL-4, although Th2 cells were more responsive to IL-4 than Th1 cells. Responsiveness of Th1 and Th2 cells to both lymphokines decreased with time after initial antigenic activation; Th1 cells lost their responsiveness to IL-4 more rapidly and to IL-2 more slowly than Th2 cells. IFN-γ partially inhibited the IL-2- and IL-4-mediated proliferation of Th2, but not Th1 cells. Although the presence of IL-1 was not required for the response of Th1 or Th2 cells to IL-4, its presence resulted in a synergistic effect with IL-2 or IL-4 in Th2 but not in Th1 cells. Both subsets responded to a mixture of IL-2 and IL-4 in synergistic fashion. Delayed addition and wash-out experiments indicated that both IL-2 and IL-4 had to be present simultaneously in order for synergy to occur. These results suggest that Th cell subsets might regulate each other via the lymphokines that they secrete and that the pathways of IL-2- and IL-4-mediated proliferation are interrelated.

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Note added in proof: Recent studies by Gajewski and Fitch (47) have shown that the IL-1-dependent response of Th2, but not Th1, clones is suppressed by IFN-γ. These results agree with our finding that IFN-γ acts on Th2, but not Th1, cells.

References

1. Swierkosz, J. E., P. Marrack, and J. Kappier. 1979. Functional analysis of T cells expressing antigens. J. Exp. Med. 150:1293.
2. Imperiale, M. J., D. A. Faherty, J. F. Sproviero, and M. Zauderer. 1982. Functionally distinct helper T cells enriched under different culture conditions cooperate with different B cells. J. Immunol. 129:1843.
3. Kim, J., A. Woods, E. Becker-Dunn, and K. Bottomly. 1985. Distinct functional phenotypes of cloned Ia-restricted helper T cells. J. Exp. Med. 162:188.
4. Mosmann, 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.
5. Cher, D. J., and T. R. Mosmann. 1987. Two types of murine helper T cell clones. II. Delayed type hypersensitivity is mediated by Th1 clones. J. Immunol. 138:3688.
6. Powers, G. D., and R. A. Miller. 1987. Heterogeneity among T helper cells. Interleukin-2 secretion and help for immunoglobulin secretion. J. Immunol. 139:2567.
7. Cherwinski, H. M., J. H. Schumacher, K. D. Brown, and T. R. Mosmann. 1987. Two types of mouse helper T cell clones. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J. Exp. Med. 166:1229.
8. Stevens, T. L., A. Bossie, V. Sanders, R. Fernandez-Botran, R. L. Coffman, T. R. Mosmann, and E. S. Vitetta. 1988. Subsets of antigen-specific helper T cells regulate isotype secretion by antigen-specific B cells. Nature (Lond). In press.
9. Arthur, R. P., and D. Mason. 1986. T cells that help B cell responses to soluble antigen are distinguishable from those producing interleukin 2 on mitogenic or allogenic stimulation. J. Exp. Med. 163:74.
10. Rudd, C. E., C. Morimoto, L. L. Wong, and S. F. Schlossman. 1987. The subdivision of the T4 (CD4) subset on the basis of the differential expression of L-C/T200 antigens. J. Exp. Med. 166:1758.
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 (IL-4) mediates autocrine growth of helper T cells after antigenic stimulation. Proc. Natl. Acad. Sci. USA. 83:9689.
12. 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.
13. Kupper, T., M. Horowitz, F. Leo, R. Robb, and P. M. Flood. 1987. Autocrine growth of T cells independent of interleukin-2: identification of interleukin-4 (IL-4, BSF-1) as an autocrine growth factor for a cloned antigen-specific helper T cell. J. Immunol. 138:4280.
14. 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.
15. Watson, J. 1979. Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. J. Exp. Med. 150:1510.
16. 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.
17. Reynolds, D. S., W. H. Boom, and A. K. Abbas. 1987. Inhibition of B lymphocyte activation by interferon-γ. J. Immunol. 139:767.
18. 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.
19. Malek, T. R., R. J. Robb, and E. M. 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.
20. 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.
21. 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.
22. Mond, J. J., F. D. Finkelman, C. Sarma, J. Ohara, and S. Serrate. 1986. Recombinant interferon-γ inhibits the B cell proliferative response stimulated by soluble but not by Sepharose-bound anti-immunoglobulin antibody. J. Immunol. 135:2513.
23. Rabin, E. M., J. J. Mond, J. Ohara, and W. E. Paul. 1986. Interferon-γ inhibits the action of B cell stimulatory factor (BSF-1) on resting B cells. J. Immunol. 137:1573.
24. Coffman, R. L., and J. Carty. 1986. A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-\(\gamma\). J. Immunol. 136:949.
25. Mond, J. J., J. Carman, C. Sarma, J. Ohara, and F. D. Finkelman. 1986. Interferon-\(\gamma\) suppresses B cell stimulation factor (BSF-1) induction of class II MHC determinants on B cells. J. Immunol. 137:3534.
26. Oliver, K., P. H. Krammer, P. W. Tucker, and E. S. Vitetta. 1987. The effects of cytokines and adherent cells on the interleukin-4 (IL-4)-mediated induction of Ia antigens on resting B cells. Cell. Immunol. 106:428.
27. Mizel, S. B. 1982. Interleukin-1 and T cell activation. Immunol. Rev. 63:51.
28. Williams, J. M., D. Deloria, J. A. Hansen, C. A. Dinarello, R. Loertscher, N. M. Shapiro, and T. B. Strom. 1985. The events of primary T cell activation can be staged by use of Sepharose-bound anti-T3 (64.1) monoclonal antibody and purified interleukin-1. J. Immunol. 135:2249.
29. Meuer, S. C., and K.-H. Meyer zum Buscheufelde. 1986. T cell receptor triggering induces responsiveness to IL-1 and interleukin-2 but does not lead to T cell proliferation. J. Immunol. 136:4106.
30. Ho, S. N., R. T. Abraham, A. Nilson, B. S. Handwerger, and D. J. McKeon. 1987. Interleukin-1-mediated activation of interleukin-4 (IL-4)-producing T lymphocytes. Proliferation by IL-4-dependent and IL-4-independent mechanisms. J. Immunol. 139:1532.
31. Kurt Jones, E. A., S. Hamberg, J. Ohara, W. E. Paul, and A. K. Abbas. 1987. Heterogeneity of helper/inducer T lymphocytes. I. Lymphokine production and lymphokine responsiveness. J. Exp. Med. 166:1774.
32. Mizel, S. B. 1980. Studies on the purification and structure-function relationships of murine lymphocyte activating factor (interleukin-1). Mol. Immunol. 17:571.
33. Oliver, K., R. J. Noelle, J. W. Uhr, P. H. Krammer, and E. S. Vitetta. 1985. B cell growth factor (BCGF I or BSFpI) is a differentiation factor for resting B cells and may not induce cell growth. Proc. Natl. Acad. Sci. USA. 82:2465.
34. Rabin, E. M., J. Ohara, and W. E. Paul. 1985. B cell-stimulatory factor (BSF-1) activates resting B cells. Proc. Natl. Acad. Sci. USA. 82:2935.
35. Rabin, E. M., J. J. Mond, J. Ohara, and W. E. Paul. 1986. B cell stimulatory factor (BSF-1) prepares resting B cells to enter S phase in response to anti-IgM and to lipopolysaccharide. J. Exp. Med. 164:517.
36. Snapper, C., and W. E. Paul. 1987. B cell stimulatory factor-1 (interleukin-4) prepares resting murine B cells to secrete IgG\(_1\) upon subsequent stimulation with bacterial lipopolysaccharide. J. Immunol. 139:10.
37. Mosmann, T. R., and R. L. Coffman. 1987. Two types of mouse helper T cell clones: implications for immune regulation. Immunol. Today. 8:233.
38. Moreau, J. L., M. Nabholz, T. Diamantstein, T. Malek, E. Shevach, and J. Theze. 1987. Monoclonal antibodies identify three epitope clusters on the mouse p55 subunit of the interleukin-2 receptor: relationship to the interleukin-binding site. Eur. J. Immunol. 17:929.
39. Smith, K. A. 1984. Interleukin-2. Annu. Rev. Immunol. 2:319.
40. Ohara, J., and W. E. Paul. 1987. Receptors for B cell stimulatory factor-1 expressed on cells of hematopoietic lineage. Nature (Lond.). 325:537.
41. Snapper, C. M., and W. E. Paul. 1987. Interferon-\(\gamma\) and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science (Wash. DC). 236:944.
42. Kaye, J., S. Gillis, S. B. Mizel, E. M. Shevach, T. R. Malek, C. A. Dinarello, L. B. Lachman, and C. A. Janeway. 1984. Growth of a cloned T helper cell line induced by a monoclonal antibody specific for the antigen receptor. J. Immunol. 133:1339.
43. Tony, H.-P., N. E. Phillips, and D. C. Parker. 1985. Role of membrane immunoglobulin (Ig) crosslinking in membrane Ig-mediated histocompatibility-restricted T cell-B cell cooperation. J. Exp. Med. 162:1695.
558  LYMPHOKINE REGULATION OF CLONES OF T HELPER CELLS

44. Manger, B., A. Weiss, C. Weyand, J. Goronzy, and J. D. Stobo. 1985. T cell activation: differences in the signals required for IL-2 production by nonactivated and activated T cells. *J. Immunol.* 135:3669.

45. 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.

46. Walker, F., N. A. Nicola, D. Metcalf, and A. W. Burgess. 1985. Hierarchical down-modulation of hemopoietic growth factor receptors. *Cell.* 43:269.

47. Gajewski, T. F., and F. W. Fitch. 1988. Anti-proliferative effect of IFN-γ and immunoregulation. I. IFN-γ inhibits the proliferation of the TH2 but not TH1 murine helper T lymphocyte clones. *J. Immunol.* 140:4245.