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Interleukin 4 mediates autocrine growth of helper T cells after antigenic stimulation

(interleukin 2/lymphokine/T-cell clones)

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ABSTRACT **The role of interleukin 4 (IL-4) (previously called B-cell stimulatory factor 1) in the response of T helper (T_H) cells to antigen presented by antigen-specific B cells or splenic adherent cells was investigated. Antigenic stimulation of either a keyhole-limpet-hemocyanin-specific T_H-cell line or two keyhole-limpet-hemocyanin-specific T-cell clones resulted in the secretion of IL-4 but not interleukin 2 (IL-2). The secretion of IL-4 was first detected in the culture supernatant 6–8 hr after antigenic stimulation. Induction of IL-4 secretion was antigen specific and major histocompatibility complex restricted. Antigenic stimulation also resulted in increased responsiveness of the T_H cells to exogenously added or endogenously produced IL-4. The antigen-induced proliferation of the T_H cells could be inhibited by an anti-IL-4 antibody but not by an anti-IL-2-receptor antibody. These results suggest that IL-4 mediates the proliferation of some T_H cells by an antigen-induced autocrine mechanism. Taken together with past results, these data indicate that, during T-cell–B-cell interactions involving some soluble protein antigens, IL-4 and not IL-2 is the critical lymphokine for activating resting B cells and inducing proliferation of the T_H cells.**

Interleukin 4 (IL-4), previously called B-cell stimulatory factor 1 (BSF-1), acts on B cells at several phases during their activation and differentiation (1–8). Recently, it has become evident that IL-4 also induces the proliferation of mast cells and several interleukin 2 (IL-2)- and interleukin 3 (IL-3)-dependent cell lines (9, 10, 38).

In a recent paper (11), we reported that IL-4 promoted the proliferation of clones or lines of antigen-specific T helper (T_H) cells but not alloreactive or cytotoxic T cells, suggesting a regulatory role for IL-4 in the generation of T-cell-dependent humoral immune responses.

In the present study, we demonstrate that some antigen-specific T_H-cell lines and clones respond to antigen in the presence of adherent cells or antigen-specific B cells with the release of IL-4 but not IL-2. After antigen stimulation, these T_H cells show increased responsiveness to endogenously produced or exogenously added IL-4. The antigen-induced IL-4-mediated proliferation of these T cells can be inhibited by a monoclonal anti-IL-4 antibody but not by a monoclonal anti-IL-2 receptor (anti-IL-2R) antibody.

MATERIALS AND METHODS

Animals. Female BALB/c, (C57BL/6 × DBA/2)F_1 (BDF_1), and (C57BL/6 × A/J) (B6A) mice, all 7–10 weeks of age, were purchased from The Jackson Laboratory.

Cell Lines. The IL-2-dependent T-cell lines, HT-2 (12) and CTLL-2 (13), were maintained by using supernatant of concanavalin A (Con A)-stimulated murine spleen cells as source of IL-2 (11). The keyhole limpet hemocyanin (KLH)-reactive T-cell clones, 8-5 (14) and A-12, were kindly provided by R. Hodes (National Institutes of Health, Bethesda, MD). The KLH-specific T-cell line, T-286, was prepared in our laboratory from the lymph nodes of KLH-immunized BALB/c mice, following the method described by Kimoto and Fathman (15). All antigen-specific T_H-cell clones and lines were maintained by a 4- to 5-day stimulation period in the presence of irradiated syngeneic spleen cells, KLH, and supernatant of Con A-stimulated rat spleen cells, followed by a 7- to 14-day rest period. T cells were harvested for assays during the rest period by centrifugation over Ficoll to remove feeder cells and dead cells. Under conditions of cognate interaction, the two T-cell clones and one T-cell line provided major histocompatibility complex (MHC)-restricted, antigen-specific help to B cells, as measured by the generation of plaque-forming cells (PFC) and by IgM secretion (Table 1 and data not shown).

Reagents and Monoclonal Antibodies. Monoclonal anti-IL-4 antibody (11B11) (18) and its isotype-matched control (50C1) were kindly provided by J. Ohara and W. Paul (National Institutes of Health, Bethesda, MD). The monoclonal anti-IL-2R antibody was prepared by ammonium sulfate precipitation of the culture supernatant of the 7D4 hybridoma (19) (American Type Culture Collection). Recombinant human IL-2 (rIL-2) was purchased from AMGen Biologicals (Thousand Oaks, CA). IL-4 was purified from the supernatant of the Con-A-stimulated PK 7.1 T-cell line (20) according to the method described by Ohara *et al.* (21). KLH was purchased from Calbiochem. Ovalbumin (OVA) was from Sigma.

Enrichment of 2,4,6-Trinitrophenyl (TNP)-Antigen-Binding Cells (TNP-ABC). Procedures for the enrichment of TNP-ABC from spleens of nonimmunized mice were adapted from Snow *et al.* (22) as modified by Myers *et al.* (23). Enriched B-cell populations were 65–85% TNP specific. Phenotypic and functional characterizations of TNP-ABC have been described previously (22–24).

Proliferation Assay for Antigen-Specific T_H Cells. Proliferation of KLH-specific T_H-cell lines was induced by antigen-pulsed splenic adherent cells (SAC). [^{3}H]Thymidine was added (1 μCi per well; 1 Ci = 37 GBq) after 48 hr of culture and the cells were harvested 16 hr later.

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Abbreviations: APC, antigen-presenting cell(s); B6A, (C57BL/6 × A/J)F_1; BDF_1, (C57BL/6 × DBA/2)F_1; Con A, concanavalin A; IFN-γ, interferon γ; IL-2, interleukin 2; IL-4, interleukin 4; IL-2R, interleukin 2 receptor; KLH, keyhole limpet hemocyanin; MHC, major histocompatibility complex; OVA, ovalbumin; PFC, plaque-forming cell(s); SAC, spleen adherent cell(s); TNP, 2,4,6-trinitrophenyl; TNP-ABC, TNP-antigen-binding cell(s); T_H cell(s), T helper cell(s).

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Table 1. Functional characteristics of the KLH-specific T-cell line, T-286

Antigen	B-cell proliferation, cpm per culture*	B-cell differentiation†	
		PFC/10^6 cells	IgM, ng/ml
None added	603	0	17
TNP-KLH	35,517	9733	14,000
TNP-OVA + KLH	5,385	667	370

γ-irradiated [1500 rads (1 rad = 0.01 gray)] T-286 cells (5×10^4) were incubated with 5×10^4 2,4,6-trinitrophenyl (TNP)-antigen-binding cells (TNP-ABC) and antigens at final concentrations of 1 μg/ml. OVA, ovalbumin.

*Cultures were pulsed with [^{3}H]thymidine during the last 18 hr of the 72-hr culture period.

†On day 5 of culture, IgM levels in the supernatant were determined by using an IgM-specific RIA (16), and cells were enumerated for anti-TNP antibody-secreting cells by using a direct PFC assay (17).

Proliferation Assay for HT-2 or CTLL-2 Cells. Although both IL-2 and IL-4 induce proliferation of HT-2 cells, IL-2 is much more effective at inducing proliferation of CTLL-2 cells (11). Thus, IL-2 and IL-4 activities in a supernatant could be determined by using these two cell lines in a [^{3}H]thymidine uptake assay. In each case, monoclonal anti-IL-4 antibodies were used to block the IL-4-mediated response to confirm its presence.

Induction of Hyper-Ia Expression. IL-4 activity in the antigen-specific T_H-cell SN was also measured by its ability to induce hyper-Ia expression on resting B cells as described by Noelle *et al.* (1). IL-2 does not induce hyper-Ia expression (K.G.O. and E.S.V., unpublished data).

Analysis of IL-2- and IL-4-Specific RNAs. Dot-blot analyses of IL-2- and IL-4-specific RNAs were performed according to White and Bancroft (25). Cell lysates containing 5×10^5 and 1×10^5 T-cell equivalents were blotted onto nitrocellulose filter paper with a filtration manifold (Schleicher & Schuell). To detect IL-2-specific RNA, the filter was probed with a ^{32}P-labeled 1.0-kilobase pair *Bam*HI–*Bam*HI fragment of pGEM-3-IL-2 containing the coding sequence of the murine IL-2 gene. pGEM-3-IL-2 was a generous gift from David Lafrenz (Veterans Administration Medical Center, Iowa City, IA) and DNAX Research Institute of Molecular and Cellular Biology (Palo Alto, CA). To detect IL-4-specific RNA, the filter was probed with a ^{32}P-end-labeled synthetic 40-mer spanning positions −6 to −45 (relative to the methionine codon) of the IL-4 cDNA clone sequence (9, 26). The synthetic oligonucleotide was prepared by the Macromolecular Synthesis Core Facility in The University of Texas Health Science Center. The hybridization and washing conditions for the filter probed for IL-2-specific RNA have been described previously (27). The final wash was carried out at 68°C for 1 hr. For the synthetic IL-4-specific 40-mer, the filter was hybridized overnight at 42°C in 0.90 M NaCl/0.090 M trisodium citrate (6× SSC) in Denhardt's solution (0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin) containing sonicated herring sperm DNA (100 μg/ml) and was washed three times for 10 min each with 6× SSC containing 0.1% $NaDodSO_4$ at room temperature. The filter was then washed for 1 hr at 56°C in 2× SSC containing 0.1% $NaDodSO_4$. The filter was exposed to x-ray film (Fuji Photo Film, Tokyo) with an intensifying screen (Lightning Plus, DuPont) at −70°C for 1 or 4 days.

RESULTS

T_H Cells Secrete IL-4 in Response to Antigenic Stimulation. The role of IL-4 in regulating B-cell activation and differentiation (1–8) as well as its ability to induce proliferation of T_H-cell lines (11) prompted us to investigate whether IL-4 was *produced* by T_H cells in response to antigenic stimulation. KLH-specific T_H cells were stimulated with antigen (TNP-KLH) presented by TNP-ABC and the culture supernatants were assayed for the presence of IL-4 and IL-2. Although both lymphokines induce proliferation of HT-2 cells, in comparison with IL-2, IL-4 is only marginally effective at inducing the proliferation of CTLL-2 cells (11). In contrast, IL-4, but not IL-2, induces the increased expression of Ia antigens on small resting B cells (1, 2). By comparing the patterns of proliferation of HT-2 and CTLL-2 cells, the induction of Ia antigens on B cells, and the inhibition of both effects by a monoclonal anti-IL-4 antibody (18), it was possible to distinguish between IL-4 and IL-2 activities. As seen in Fig. 1 *A–C* and as determined by the assays described above, IL-4 was first detected in the culture supernatant of antigen-stimulated T-286 cells 6–8 hr after antigenic stimulation, reaching maximum levels between 24 and 48 hr. The KLH-specific T_H-cell clones responded similarly. For example, supernatants harvested from 8-5 cells exposed to TNP-KLH (1 μg/ml) and TNP-ABC for 12 hr strongly stimulated the proliferation of HT-2 cells (stimulation index, 59.4 ± 10.4), whereas these supernatants induced only a modest proliferation in CTLL-2 cells (stimulation index: 2.03 ± 0.3). Pulsing the TNP-ABC with antigen prior to the addition of T-286 cells decreased the time at which IL-4 was first detected to 4 hr (results not shown). The release of IL-4 by the antigen-specific T_H cells was dependent upon antigen presentation by TNP-ABC, since T cells cultured with antigen in the absence of TNP-ABC did not secrete IL-4. The secretion of IL-4 was also dependent upon the concentration of antigen and the nature of the APC, as shown in Fig. 1 *D–F*. When SAC or peritoneal macrophages were substituted for TNP-ABC as APC, essentially the same patterns of lymphokine activity and kinetics of secretion were obtained, except that adherent cells required approximately 1000-fold more antigen to stimulate IL-4 production by the T_H cells. Moreover, stimulation of the T_H cells with Con A (3 μg/ml) also induced the release of IL-4. Taken together, these results demonstrate that IL-4 is the predominant, if not the sole, T-cell growth-promoting lymphokine secreted after stimulation of the T_H cells with APC and antigen. Consistent with this interpretation, all IL-4-mediated activities (including the modest proliferation of CTLL-2 cells) could be blocked by anti-IL-4 antibodies.

The secretion of IL-4 by T_H cells was antigen specific and MHC restricted, since stimulation with an irrelevant antigen such as OVA in the context of syngeneic APC, or the appropriate antigen, KLH, presented in the context of allogeneic APC from B6A mice ($Ia^{b/k}$), did not result in the secretion of IL-4 by the T_H cells (Table 2).

Analyses of Cytoplasmic IL-2- and IL-4-Specific RNAs. Although no IL-2 could be detected in the supernatant of the antigen-stimulated KLH-specific T_H cells, it was possible that low levels of IL-2 might have escaped detection. Additionally, the T_H cells might have been synthesizing and utilizing IL-2 at the same rate. To test this possibility, we assayed cytoplasmic IL-2-specific RNA in antigen-stimulated T-286 cells. As shown in Fig. 2*A*, antigen did not induce the T-286 cells to express detectable levels of IL-2-specific RNA. Similarly, dot-blot analysis of KLH-specific T_H cell clones, 8-5 and A-12, also failed to show detectable levels of IL-2-specific RNA in the T_H cells after antigenic or mitogenic stimulation (data not shown). These data add further support to the notion that IL-2 is not produced by these cells. In contrast, IL-4-specific RNA was readily detected in antigen-stimulated T-286 cells by using a synthetic IL-4-specific 40-mer probe (Fig. 2*B*). The KLH-specific T_H cell clones, 8-5 and A-12, were also positive for IL-4-specific RNA (data not shown).

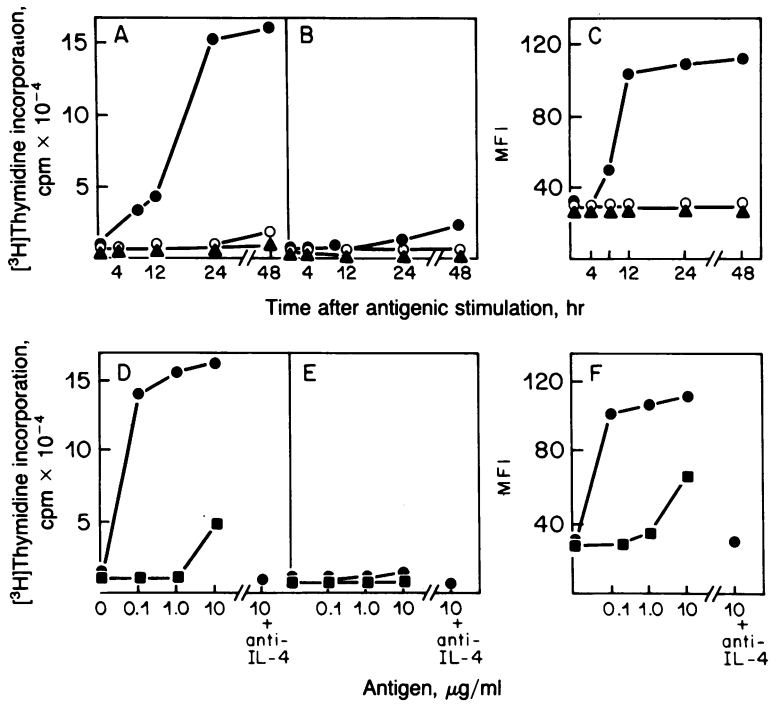

FIG. 1. Time course and antigen concentration-dependency of IL-4 secretion by T-286 cells after stimulation by antigen and TNP-ABC. T-286 cells (1×10^5) were cultured for 1–48 hr with TNP-ABC (1×10^5) and TNP-KLH at 0.1–10 μg/ml. Supernatants were analyzed for their ability to induce proliferation of HT-2 cells (*A* and *D*) or CTLL-2 cells (*B* and *E*) and to induce Ia antigen expression on normal resting B cells (*C* and *F*). ●, T_H + TNP-ABC + TNP-KLH; ○, T_H + TNP-KLH; ▲, TNP-ABC + TNP-KLH; ■, T_H + SAC + TNP-KLH. MFI, mean fluorescence intensity of Ia as determined on the fluorescence-activated cell sorter. (*A–C*) Kinetics of IL-4 production by cells cultured with TNP-KLH (10 μg/ml). (*D–F*) IL-4 production as a function of the concentration of TNP-KLH and the nature of the antigen-presenting cell (APC). Cells were cultured for 24 hr. Monoclonal anti-IL-4 antibody (11B11) (18) was added where indicated at 20 μg/ml. Control antibodies at the same concentration had no effect.

Increase in Responsiveness to IL-4 After Antigenic Stimulation. Since T_H cells are able to secrete and respond to IL-4 (11), we next determined whether antigenic stimulation would render the cells more responsive to exogenously added IL-4 and whether they would proliferate in response to their endogenously produced IL-4. T-286 cells were cultured with SAC in the presence or absence of antigen for 24 hr and then tested for their ability to proliferate in response to IL-4 either immediately or after 24–48 additional hours of culture in the *absence* of antigen. As shown in Fig. 3*A*, when T_H cells were utilized at the end of the 14-day rest period, they responded very poorly to exogenously added purified IL-4. However, after being stimulated with antigen, their responsiveness to IL-4 increased and was maintained 48 hr after stimulation. Interestingly, responsiveness to exogenously added IL-2 also increased in parallel, although these cells did not secrete IL-2 (results not shown). As shown in Fig. 3*A*, T_H cells continued to proliferate 48 hr after antigen stimulation even when no source of exogenous IL-4 was added to the cultures. This suggested that T_H cells were using their endogenously produced IL-4 to support their proliferation in an "autocrine"

Table 2. Production of IL-4 by T-286 cells is antigen specific and MHC restricted

T cells	SAC*	Antigen	Proliferation of HT-2 cells, cpm per culture
−	—	KLH	623 ± 55
+	—	KLH	966 ± 260
+	BALB/c	—	1,876 ± 631
+	BALB/c	KLH	26,870 ± 6010
+	BALB/c	OVA	2,913 ± 720
+	B6A	—	1,486 ± 206
+	B6A	KLH	2,057 ± 101
+	B6A	OVA	1,864 ± 199

T-286 cells were cultured at 5×10^4 cells per well with SAC from BALB/c (Ia^d) or B6A ($Ia^{b/k}$) mice in the presence or absence of KLH (50 μg/ml) or OVA (50 μg/ml). After 24 hr, supernatants were tested for IL-4 activity by using the HT-2 proliferation assay.

*SAC from the indicated mouse strain were prepared by incubating an irradiated (3300 rads) spleen cell suspension at 7.5×10^5 cells per well for 2 hr at 37°C and then removing nonadherent cells by aspiration followed by two washes with Hanks' balanced salt solution containing 2% fetal calf serum.

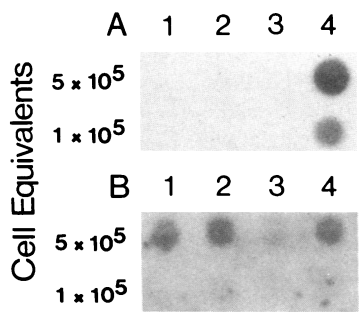

FIG. 2. Dot-blot analysis of IL-2-specific (*A*) and IL-4-specific (*B*) cytoplasmic RNA in antigen-stimulated T-286 cells. Cells (1×10^6) were cultured in the presence of TNP-ABC (1×10^6) and TNP-KLH (1 μg/ml) for 24 or 48 hr. The cells were collected and B-cell-containing T-cell cultures were treated with rabbit antibody to mouse immunoglobulin and complement. Lysates from the T cells were prepared and processed as described in the text. Lane 1, T-286 cells + TNP-ABC + TNP-KLH, 24 hr; lane 2, T-286 cells + TNP-ABC + TNP-KLH, 48 hr; lane 3, EL-4 cells; lane 4, EL-4 cells stimulated by phorbol 12-myristate 13-acetate (positive control for IL-2). The filters were probed with an IL-2-specific cDNA fragment (*A*) or a synthetic IL-4 40-mer (*B*).

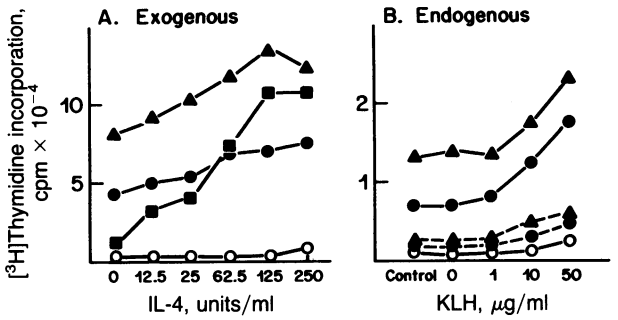

FIG. 3. Responsiveness of antigen-stimulated T-286 cells to exogenously added or endogenously produced IL-4. (*A*) T-286 cells (2×10^4 per well) were cultured with irradiated (3300 rads) SAC in the presence (●, ▲, ■) or absence (○) of KLH (50 μg/ml) for 24 hr. The cells were then harvested, extensively washed, and tested for their ability to proliferate in response to exogenously added IL-4, as measured by a 48- hr [^{3}H]thymidine incorporation assay, on the same day (●, ○) or after culture in the absence of SAC and antigen for additional 24 (▲) or 48 (■) hr. (*B*) T-286 cells (2×10^4 per well) were cultured with irradiated (3300 rads) SAC in the presence (●, ▲) or absence (○) of KLH at 1, 10, and 50 μg/ml for 24 hr. The supernatants were collected and filtered and the cells from the cultures that had been stimulated with 10 (●) or 50 (▲) μg of KLH per ml (phase 1), were harvested, extensively washed, and tested for their ability to proliferate in response to the supernatants produced at different antigen concentrations in the presence (broken lines) or absence (solid lines) of the monoclonal anti-IL-4 antibody, 11B11, at 10 μg/ml (phase 2).

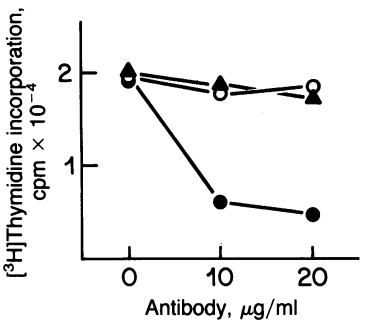

FIG. 4. Effect of monoclonal anti-IL-4 and anti-IL-2R antibodies on the proliferation of T-286 cells stimulated with antigen. T-286 cells (5×10^4 per well) were cultured with irradiated SAC and KLH (10 μg/ml) for 72 hr, in the presence of the indicated concentrations of the monoclonal anti-IL-2 receptor antibody (7D4; ○), the monoclonal anti-IL-4 antibody (11B11; ●), or normal rat Ig (▲). [^{3}H]Thymidine was added during the last 18 hr of culture.

fashion. The results shown in Fig. 3*B* support this possibility. When T_H cells were stimulated with antigen and SAC for 24 hr (phase 1), washed, and then stimulated with supernatants generated from the same cells (phase 2), they proliferated in a concentration-dependent manner. Cells cultured in the absence of antigen (phase 1) proliferated very poorly in response to the same supernatants (phase 2). Moreover, during phase 2 of culture, the proliferation of the stimulated T_H cells could be inhibited by a monoclonal anti-IL-4 antibody. Similar experiments with the KLH-specific T_H-cell clones, 8-5 and A-12, showed increased responsiveness to exogenously added or endogenously produced IL-4 after antigenic stimulation. For example, 8-5 and A-12 cells incubated with antigen and then exposed to IL-4 (62.5 units/ml) displayed stimulation indices (based on [^{3}H]thymidine uptake) of 114.2 ± 12.7 and 46.1 ± 6.3, respectively.

Effect of Anti-IL-4 and Anti-IL-2R Antibodies on the Antigen-Specific Proliferation of T_H Cells. The previous results suggest a model in which T_H cells proliferate in response to IL-4 in an autocrine fashion after antigenic stimulation. To further confirm these results, we determined whether monoclonal anti-IL-4 (16) or anti-IL-2R antibodies (17), which block IL-4- and IL-2-mediated proliferation of HT-2 cells, respectively (11), inhibited antigen-induced proliferation of T-286 cells. As shown in Fig. 4, monoclonal anti-IL-4 antibody (11B11), but not monoclonal anti-IL-2R antibody (7D4), or the control, normal rat Ig, inhibited the proliferation of T-286 cells stimulated with antigen and SAC. This finding provides additional support for the notion of IL-4-mediated, antigen-induced proliferation of T_H cells.

DISCUSSION

We have studied the role of IL-4 in the response of T_H cells to KLH, utilizing two KLH-specific T_H-cell clones, 8-5 and A-12, and one KLH T_H-cell line, T-286. The responses of the three cell types were identical. The T_H-cell line and clones respond to antigenic stimulation with the secretion of IL-4 but not IL-2. In addition, they respond to IL-4, indicating that this interaction can function in an autocrine fashion. The responsiveness to IL-4 is increased after antigenic stimulation and could be the result of up-regulation in the number or affinity of receptors for IL-4 in a manner similar to the up-regulation of IL-2 receptors on T cells after mitogenic or antigenic stimulation (28).

The secretion of IL-4 is dependent upon MHC-restricted presentation of specific antigen. Presentation of KLH by either adherent cells or TNP-ABC results in the secretion of IL-4 (but not IL-2) with essentially similar kinetics. However, TNP-ABC can stimulate T_H cells at antigen concentrations that are 1/1000th of those required to stimulate T_H cells with antigen-pulsed SAC. The ability of B cells to present antigen to T cells is well established (29–34). IL-2 secretion by antigen-stimulated T-286 cells was not detected, irrespective of whether TNP-ABC or adherent cells were used as APC. Moreover, stimulation of the same T_H cells with Con A induced the secretion of IL-4 but not IL-2. Dot-blot analysis of cytoplasmic IL-2-specific RNA was negative on antigen-stimulated T-286, 8-5, and A-12 cells, whereas the same cells were positive for IL-4-specific RNA, thus supporting the hypothesis that IL-4 and not IL-2 is the lymphokine secreted after mitogenic or antigenic stimulation of KLH-specific T_H cells.

Recently, Mossmann *et al.* (35) have described two types of murine T_H-cell clones. Both types can provide help to B cells, but they differ in the pattern of lymphokines secreted. One type (T_H1) secretes IL-2, IL-3, and interferon-γ (IFN-γ), whereas the second type (T_H2) does not secrete IL-2 but secretes IL-4. By this definition, T-286 cells are representative of the T_H2 type. It is unclear whether only T_H2 cells respond to KLH or our culture system preferentially selects for one particular T_H-cell type. In this regard, it appears that most T_H1 clones are generated in response to cell-bound but not soluble antigens (35).

The increased responsiveness to IL-4 in the antigen-stimulated T-286 cells is accompanied by a parallel increase in responsiveness to IL-2, even though T-286 cells do not produce IL-2. In fact, when IL-2 was added to T-286 cells cultured with either antigen or exogenously added IL-4, the proliferative response was potentiated (results not shown). *In vivo*, the source of IL-2 could be another antigen-specific T_H cell (such as a T_H1 cell) that acts in concert with the IL-4-producing T_H2 cell.

The results reported here support a model for an antigen-induced autocrine IL-4 pathway for T_H cells acting in concert with an IL-4-mediated B-cell activation pathway. We propose that these IL-4-mediated events occur during the physical interaction between antigen-specific T and B cells. Regulatory mechanisms that enhance or down-regulate the growth of the stimulated cells could be provided by other T_H

cells, such as those that produce IL-2, a stimulator of T-cell growth, or IFN-γ, a powerful inhibitor of many IL-4-mediated activities (7, 36, 37).

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