Generation of Interleukin 4 (IL-4)-producing Cells In Vivo and In Vitro: IL-2 and IL-4 Are Required For In Vitro Generation of IL-4-producing Cells

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Summary

T cell populations derived from naive mice produce very small amounts of interleukin 4 (IL-4) in response to stimulation on anti-CD3-coated dishes. IL-4 production by such cells is mainly found among large- and intermediate-sized T cells and is dependent upon IL-2. Injection of anti-IgD into mice, a stimulus that leads to striking increases in serum levels of IgG1 and IgE, causes a striking increase in the IL-4-producing capacity of T cells. This increase is first observed 4 d after injection of anti-IgD. IL-4 production by T cells from anti-IgD-injected donors is mainly found among large- and intermediate-sized T cells. Small, dense T cells are poor producers of IL-4. The capacity of T cells from anti-IgD-injected donors to produce IL-4 is enhanced by addition of IL-2 and is largely, but not completely, inhibited by neutralization of in situ produced IL-2. These results indicate that the control of IL-4 production in T cells from naive and anti-IgD-injected donors is similar. However, it is possible that a portion of the IL-4-producing activity of T cells from activated donors is IL-2 independent.

Although small T cells from naive donors have a very limited capacity to produce IL-4 in response to stimulation with anti-CD3, even in the presence of added IL-2, they can give rise to IL-4-producing cells upon in vitro culture on plates coated with anti-CD3 if both IL-2 and IL-4 are added. This leads to the appearance of IL-4-producing cells within 2 d. When analyzed after 5 d of culture by harvesting and re-exposure to anti-CD3-coated culture wells and IL-2, these cells have increased their IL-4-producing capacity by ∼100-fold. The development of IL-4-producing cells in response to anti-CD3, IL-2, and IL-4 is not inhibited by interferon γ (IFN-γ), nor does IFN-γ diminish IL-4 production by these cells upon challenge with anti-CD3 plus IL-2.

The regulatory actions of T lymphocytes are largely mediated by the production of a group of potent polypeptides designated lymphokines (1). Among these molecules, IL-4 has been demonstrated to play an important role in the growth and development of B lymphocytes (2) and in the control of the process through which such cells switch to the secretion of IgG1 (3) and IgE (4, 5). Long-term lines of murine $CD4^+$ T cells have been divided into those that produce IL-4 but not IL-2 or IFN-γ (Th2 cells) (6), and those that produce IL-2 and IFN-γ (Th1 cells). Based on their distinctive production of lymphokines, it would be anticipated that Th2 cells would be efficient helpers for antibody responses, while Th1 cells should preferentially favor cellular immune response. Indeed, in experimentally infected mice in which progressive Leishmaniasis develops, antigen-specific T cell clones producing IL-4 predominate, whereas in mice that limit such infections, IL-2-producing clones are mainly found (7, 8). These results strongly suggest that the decision as to which lymphokines are produced is critical to the quality and protective value of an immune response.

Little is known regarding the factors that determine whether a given form of immunization will lead to cells that principally produce IL-2 or IL-4. Indeed, in naive mice, very modest amounts of IL-4 are produced by T cells in response to anti-CD3 antibodies or to mitogenic lectins (9–11). Furthermore, the T cells in naive animals capable of producing IL-4 in response to such stimulation are largely found in the low density population, suggesting that they represent cells that have already been stimulated as a result of environmental immunization (11). By contrast, IL-2 is produced by both small and large T cells, implying that the control of production of the two prototypic lymphokines may be quite different.

Furthermore, production of IL-4 by T cells from naive donors is dependent upon the presence of IL-2 (11).

Here, we examine the increase of IL-4-producing capacity among T cells in mice injected with antibodies to IgD, an in vivo polyclonal activation stimulus known to lead to the production of large amounts of IgG1 and IgE (12), Ig isotypes whose production depends upon the presence of IL-4 (3–5, 13). We show that in such animals there is a striking increase in T cells capable of producing IL-4 in response to anti-CD3, and that this increase is first detected at about day 4, the last day on which injection of anti-IL-4 antibodies can block IgE responses in such mice (14). These results suggest that induction of IL-4-producing cells is critical to IgE production in vivo and further support the concept that the regulation of lymphokine production by T cells has striking biological effects.

We also examine in vitro conditions required for high density (small) T cells, which are very poor IL-4-producing cells, to develop into cells that can be stimulated to produce this lymphokine. Swain et al. (9) have reported that acquisition of IL-4-producing capacity does occur in cultures of stimulated mouse T cells, and were the first to suggest that primed but not resting T cells can produce IL-4. We demonstrate here that IL-4-producing cells can be derived in vitro, in 2–3 d, from small T cells by stimulation with anti-CD3 in the presence of both IL-2 and IL-4. Neither lymphokine alone will act as a cofactor with anti-CD3 for induction of cells capable of producing IL-4. This induction appears to be highly efficient, leading to an ~100-fold increase in IL-4-producing capacity. The presence of IFN-γ during this induction period does not block generation of IL-4-producing T cells, a somewhat surprising result in view of the effect of IFN-γ in preventing the outgrowth of IL-4-producing T cell clones in vitro in response to stimulation with antigen and IL-2 (15).

Materials and Methods

Animals. Virus-free BALB/c female mice, 8–12 wk of age, were obtained from Division of Cancer Therapy Animal Program, National Cancer Institute, Bethesda, MD.

Culture Medium. RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), L-glutamine (2 mM), sodium pyruvate (1 mM), 2-ME (0.05 mM), Hepes (10 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) was used for culturing cells.

Preparation of T Cells. Cell suspensions were prepared from lymph nodes of untreated mice or mice that had been injected with 800 μg of anti-IgD 5 d earlier. The cells were washed two times in HBSS and suspended at 4°C for 1 h with a mixture of fluorescence (Fl)[1]-conjugated antibodies containing 10 μg/ml of anti-B220 (6B2) and anti-Ia^d (MKD6). At the end of the incubation, the cells were washed twice in staining buffer and mixed for 1 h at 4°C with a suspension of magnetic beads coated with sheep anti-Fl antibodies (Advanced Magnetics Inc., Cambridge, MA) (12 ml/10^8 stained cells). Positively staining cells were depleted by two 20-min cycles of exposure to a magnetic field. The remaining cells were washed twice in culture medium and examined for the removal of the B cells by cytometric analysis on a FACScan (Becton Dickinson & Co., Mountain View, CA). In general, residual B cell content was <1%.

Fractionation of Large and Small T Cells. T cells from lymph nodes were layered onto a discontinuous Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient (70, 66, 60, and 50% Percoll) and centrifuged at 1,000 *g* for 20 min at room temperature. The cells at the interface between 50 and 60% Percoll (large), 60 and 66% (medium), and 66 and 70% (small) were collected, washed twice with HBSS, and resuspended in culture medium.

Lymphokines and Cytokines. Human rIL-2 was a gift of Cetus Corp. (Emeryville, CA). IL-2 activity was defined in terms of "Cetus Units". 1 Cetus U is is equivalent to ~0.3 ng and to 6 WHO IU. Synthetic IL-3 was a gift of Dr. Ian Clark-Lewis (Biomedical Research Center, Vancouver, Canada). 1 U of IL-3 was defined as the amount of IL-3 that stimulated half-maximal [^{3}H]thymidine incorporation by FD.C/1 cells. Mouse rIL-4 was obtained from a baculovirus expression system, utilizing a vector into which the IL-4 gene had been inserted by C. Watson, Laboratory of Immunology, NIAID, Bethesda, MD. IL-4 activity was measured using the CT.4S cell line (16), comparing it with a standard that had initially been calibrated on the basis of 10 U/ml being equal to the amount of IL-4 required for half-maximal stimulation of [^{3}H]thymidine uptake by resting B cells stimulated with 5 μg/ml of goat anti-IgM antibody (2, 17). 1 U is equal to ~0.5 pg of protein. Mouse rIFN-γ was a gift of Genentech, Inc., South San Francisco, CA. Mouse rIL-5 was expressed in a baculovirus system, utilizing a vector into which the IL-5 gene had been inserted by C. Watson, D. Kunimoto, and G. Harriman, NIAID.

Igs and mAbs. The following antibodies were prepared by a combination of ammonium sulfate precipitation, DE52 ion exchange chromatography, and Sephadex G200 gel filtration (18); anti-IL-2 (S4.B6) (19); anti-CD3 (2C11) (20); anti-B220 (6B2) (21); and anti-Ia^d (MKD6) (22). Purified Ig was fluoresceinated with FITC, as previously described (23). Purified monoclonal rat anti-mouse IL-4 (11B11) (24) was prepared by Verax Corporation (Hanover, NH). Affinity-purified goat anti-mouse IgD antibody was prepared as previously described (18).

Measurement of Lymphokine Production from Cells Stimulated by Plate-Bound Antibodies. Flat-bottomed 96-well microtiter Immulon 2 plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated by incubation for 4 h at room temperature with purified anti-CD3 antibody (2C11; 10 μg/ml in 50 μl borate buffered saline, pH 8.5). Wells were washed three times with 200 μl HBSS. Cells were added to the antibody-coated wells (in triplicate) in 0.2 ml culture medium. After 36 h in culture, the plates were exposed to 1000 rad in an irradiator (Gamma Cell 40; Atomic Energy of Canada Ltd., Ontario, Canada). Lymphokine-dependent lines (CT.EV [16], an IL-2-dependent line, or CT.4S, an IL-4-dependent line) were then added (5,000 cells/well) to measure the secretion of the individual lymphokines (11, 25). 48 h later, 1 μCi of [^{3}H]thymidine (ICN, Irvine, CA) was added, and after 10 h the cells were harvested. Incorporation of tritium was measured in a liquid scintillation spectrometer. Relative SEs for triplicates had a mean value of 15% or less. The amount of lymphokine was expressed as either the quantity of [^{3}H]thymidine incorporated into the DNA of the indicator cell line or as the equivalent of the amount of lymphokine required to support the same level of thymidine incorporation in the indicator cell line (equivalent units).

IL content of the supernatant of activated cells was assessed by adding 5,000 cells from each of the appropriate cell lines to supernatant of one well (CT.EV for IL-2; CT.4S for IL-4; FD.C/1 [26, 27], an IL-3-dependent line, for IL-3). The extent of IL secretion was determined by incorporation of [^{3}H]thymidine as described

[1] *Abbreviation used in this paper:* Fl, fluorescence.

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earlier in this section. IFN-γ was assayed by its ability to inhibit plaque formation by vesicular stomatitus virus on L929/2G1 cells. The level of IL-5 in the supernatants was determined by an ELISA with two anti-IL-5 mAbs (28). We thank Dr. Robert Coffman (DNAX Institute of Molecular and Cell Biology, Palo Alto, CA) for making these measurements.

Generation of IL-4-producing T Cells In Vitro. Small T cells from lymph nodes of naive mice were incubated on anti-CD3-coated tissue culture dishes ($2-3 \times 10^5$ cells/5-cm dish) in 3 ml culture medium alone or in culture medium supplemented with lymphokines and/or antilymphokine antibodies as specified in the legends. After 3 d in culture, 2 ml of fresh culture medium and the appropriate lymphokines and/or antilymphokine antibodies were added. 2 d later, the cells were collected, washed three times with HBSS, and resuspended in culture medium to further test their IL-4-producing capability on anti-CD3-coated wells. In such experiments, a series of concentrations of live cells were tested for IL-4-producing capacity. Comparisons between groups therefore represent comparisons of IL-4-producing capacity by specified numbers of living cells.

Results

T Cells from Mice Injected with Anti-IgD Antibody Produce More IL-4 than T Cells from Naive Mice. T cells from naive mice produce small amounts of IL-4 in response to stimulation with anti-CD3 coated on the surface of culture wells. Polyclonal stimulation by injection of anti-IgD causes striking increases in serum IgE in 7–8 d that can be inhibited by treatment of these mice with anti-IL-4 antibody, administered as late as 4 d after injection of anti-IgD (14). These results indicate that IL-4 is critical to the elevation of serum IgE that occurs in these mice and suggest that induction of IL-4-producing capacity occurs in such animals. We have previously shown that freshly isolated T cells from anti-IgD-injected mice secrete small amounts of IL-4 without in vitro stimulation (29). To determine whether this polyclonal stimulation resulted in an increase in the capacity of mice to produce IL-4 in response to anti-CD3 coated on culture dishes, lymph node T cells were prepared from mice that had not been injected with anti-IgD and from mice injected 2, 4, 6, 8, and 11 d

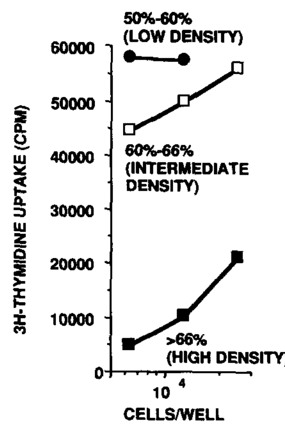

Figure 2. Low density T cells from anti-IgD-injected donors are better IL-4 producers than high density T cells. T cells were prepared from mice that had been injected with anti-IgD 5 d earlier. The cells were separated on Percoll density gradients into cells of low, intermediate, and high density. The separated cells were cultured in wells coated with anti-CD3 in the presence of IL-2 and IL-4 production measured as described in Fig. 1.

earlier with 800 μg of anti-IgD. Since we have shown that the capacity of T cells from naive donors to produce IL-4 in response to anti-CD3 requires the presence of IL-2 (11), human IL-2 (50 U/ml) was added to all cultures. Fig. 1 *A* shows a striking increase in IL-4-producing capacity of lymph node T cells from anti-IgD-injected donors that peaks between 4 and 6 d after injection. In Fig. 1 *B*, the IL-4-producing capacity of T cells from naive donors and from donors injected with anti-IgD 5 d earlier is compared in detail. 5,000 T cells from anti-IgD-injected mice produce more IL-4 in response to anti-CD3 plus IL-2 than 100,000 T cells from naive donors, indicating that the polyclonal activation known to lead to IgE production is marked by a striking increase in the IL-4 producing capacity of the lymph node T cell population.

IL-4-producing T Cells from Primed Donors Have Low and Intermediate Densities. High density T cells from naive donors produce very little IL-4 in response to anti-CD3 and IL-2, even in the presence of exogenous accessory cells. The IL-4-producing cells from these mice are markedly enriched in the low density T cell population (11). Similarly, IL-4-producing T cells from anti-IgD-injected donors are concentrated in cells of low and intermediate density (Fig. 2). Purified T cells were

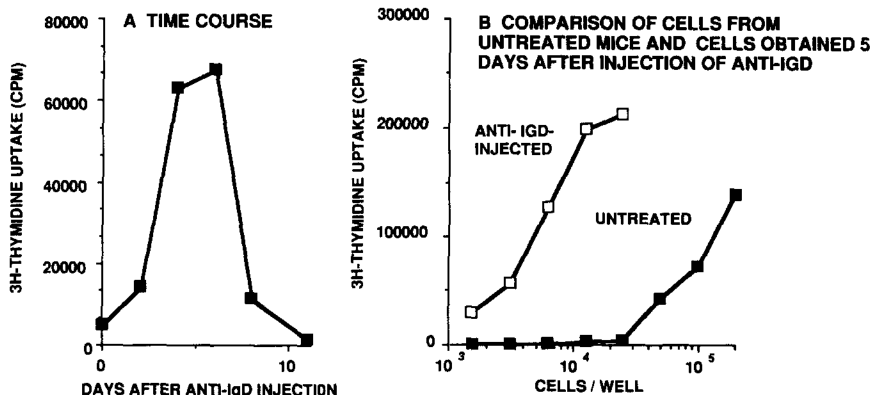

Figure 1. Anti-IgD injection causes an increase in T cell production of IL-4 in response to anti-CD3. (*A*) T cells were prepared from mice that had been injected with 800 μg of anti-IgD 2, 4, 6, 8, and 11 d earlier and from mice that had not been injected with anti-IgD (day 0). Cells were cultured, in the presence of IL-2 (50 U/ml), at 5,000/well on culture plates that had been coated with anti-CD3. Cultures were irradiated (1,000 rad) at 36 h, and 5,000 CT.4S cells were added. After 48 h, [^{3}H]thymidine was added, and incorporation into DNA was measured 10 h later as an index of IL-4 production. (*B*) In a separate experiment, cells obtained from mice injected with anti-IgD 5 d earlier or not injected were cultured in various numbers, in the presence of IL-2, in wells coated with anti-CD3. CT.4S responses, as a measure of IL-4 production, were determined as described above.

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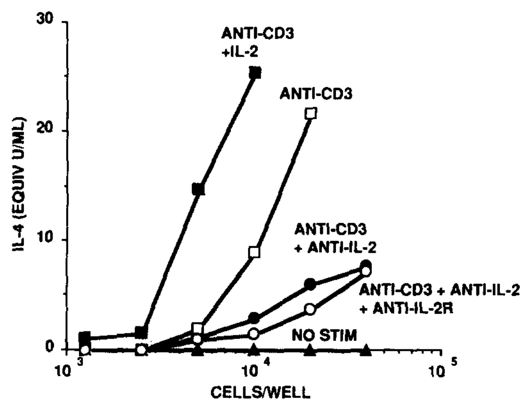

Figure 3. Neutralization of IL-2 diminishes IL-4 production by T cells from mice injected with anti-IgD. T cells were prepared from mice that had been injected with anti-IgD 5 d earlier and cultured on wells that had been coated with anti-CD3. IL-2 (50 U/ml), anti-mouse IL-2 (S4.B6 [10 μg/ml]), anti-mouse IL-2 plus a 1:100 dilution of a mixture of ascitic fluids of the anti-IL-2R mAbs 3C7, 7D4, 2E4, and PC61, or nothing was added to wells. IL-4 production was measured as described in Fig. 1, except that the results are expressed as "equivalent units" rather than as uptake of [^{3}H]thymidine. Equivalent units represents the amount of IL-4 added to CT.4S cells that gives [^{3}H]thymidine uptake equivalent to that observed in the CT.4S coculture assay. Since IL-2 at 50 U/ml, but not at lower concentrations, enhances responses of CT.4S cells to IL-4, the dose-response curve that was used to calculate equivalent units for the group to which IL-2 had been added was derived from responses of CT.4S cells to which IL-4 had been added in the presence of 50 U/ml of IL-2, while the dose-response curve used to calculate equivalent units for the other groups was derived from responses of CT.4S cells to which IL-4 was added in the absence of IL-2.

fractionated by Percoll density gradient centrifugation into low density (50–60% Percoll), intermediate density (60–66%), and high density (>66%) populations. High density cells produced very little IL-4 in response to anti-CD3 in comparison with low and intermediate density cells (Fig. 2). In these experiments, IL-2 was added to all cultures to maximize IL-4 production. These results indicate that among T cells from recently stimulated mice, just as was true of T cells from naive donors, the major IL-4-producing cell population is made up of cells of low and intermediate density. Since resting cells are found mainly in the high density population, it would appear that the small resting T cell population in polyclonally activated as well as in naive donors has very limited capacity to produce IL-4 in response to anti-CD3.

IL-4 Production by T Cells From Anti-IgD-injected Mice Is Inhibited by Neutralization of IL-2. IL-4 production by T cells from naive donors cultured on anti-CD3-coated plates is enhanced by the addition of IL-2 and completely inhibited by the addition of the monoclonal anti-mouse IL-2 antibody S4.B6 (11). The latter result indicates that in situ production of IL-2 is normally required for IL-4 production by cells from naive donors stimulated with anti-CD3. To examine whether T cells that had been "activated" in vivo as a result of injection of anti-IgD also required IL-2 in order to produce IL-4, lymph node T cells from such donors were cultured, in varying numbers, in wells coated with anti-CD3. These cells produced substantial amounts of IL-4 (Fig. 3). In this experiment, the results are reported in terms of "IL-4 equivalent units" rather than [^{3}H]thymidine uptake by CT.4S cells, since IL-2, at 50 U/ml or more, increases the response of CT.4S cells to IL-4 (16). IL-4 equivalent units were calculated from dose-response curves carried out in the presence or absence of exogenous human rIL-2 (50 U/ml), as appropriate.

IL-4 production by T cells from anti-IgD-injected donors, cultured on anti-CD3-coated wells, is substantially enhanced by the addition of human IL-2. On the other hand, the addition of the monoclonal anti-mouse IL-2 antibody S4.B6, at 10 μg/ml, significantly, but incompletely, inhibits IL-4 production by T cells cultured in anti-CD3-coated wells in the absence of exogenous IL-2. This degree of inhibition was only slightly increased by using a mixture of anti-IL-2 with four independent antibodies to the p55 chain of the IL-2R (7D4, 3C7, 2E4, and PC61) (Fig. 3). These results indicate that a major component of IL-4 production by T cells from "recently activated" donors is dependent upon the presence of IL-2. This is particularly striking when one compares IL-4 production in the presence of exogenous IL-2, to maximize IL-4 production, with that in the presence of antibodies that neutralize endogenous IL-2. 5,000 of the former cells produce more IL-4 than 40,000 of the latter. Whether the residual production of IL-4 in the presence of anti-IL-2 and anti-p55 represents an IL-2-independent response or is due to the difficulty of fully blocking the action of endogenously produced IL-2 is not clear.

High Density T Cells from Naive Donors Contain Precursors that Can Be Stimulated to Develop into IL-4-producing Cells. The ability of T cells from primed donors to produce substantially more IL-4 than T cells from naive animals in response to anti-CD3 plus IL-2 suggests that the unprimed cell population contains cells that, under appropriate in vivo conditions, give rise to expanded numbers of IL-4-producing cells. Since high density T cells from naive and anti-IgD-injected mice have very little capacity to produce IL-4, the development of IL-4-producing cells from this population would be particularly striking. Thus, we sought in vitro conditions that would allow the appearance of IL-4-producing cells from the high density T cell population of naive donors. We observed that culturing these cells for 5 d on anti-CD3-coated dishes together with IL-2 and IL-4 led to the appearance of a cell population that produced large amounts of IL-4 in response to anti-CD3 plus IL-2 (Fig. 4). Thus, we observed that 312 cells primed in vitro produced more IL-4 than did 20,000 high density cells from naive donors. This in vitro primed T cell population was the richest source of IL-4 production that we have observed, exceeding that of T cells from anti-IgD-injected donors.

Both IL-2 and IL-4 were essential for the appearance of cells that could produce IL-4 in response to restimulation with anti-CD3 plus IL-2. A priming culture of high density T cells from naive donors with anti-CD3 plus IL-2 and monoclonal anti-IL-4, to neutralize any IL-4 that was produced endogenously, did not result in the emergence of cells that could produce IL-4 in response to anti-CD3 plus IL-2 (Fig. 5 *A*). Similarly, cells cultured with anti-CD3 plus IL-4 and anti-

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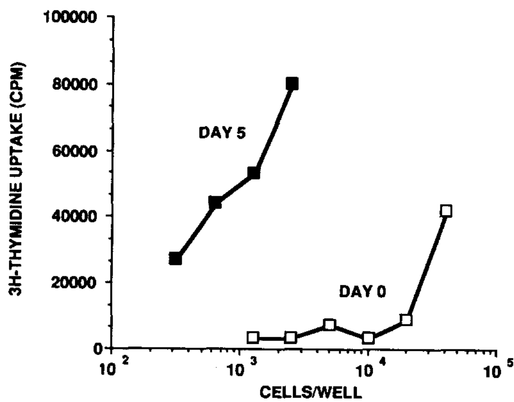

Figure 4. In vitro culture of dense T cells with anti-CD3 plus IL-2 and IL-4 causes a striking increase in IL-4-producing capacity. T cells from naive donors were separated by Percoll density gradient centrifugation. The dense cells (2×10^5) were cultured in a 5-cm dish that had been coated with anti-CD3, in the presence of 3 ml of culture medium containing IL-2 (50 U/ml) and IL-4 (1,000 U/ml). After 3 d of culture, 2 ml of lymphokine-containing medium was added. At 5 d, the cells were harvested and transferred to 96-well culture plates that had been coated with anti-CD3 and cultured in the presence of IL-2. IL-4 production was measured as described in Fig. 1. Dense T cells from naive mice were freshly prepared and cultured in 96-well plates coated with anti-CD3 at the same time as the cells that had been cultured for 5 d were harvested and recultured.

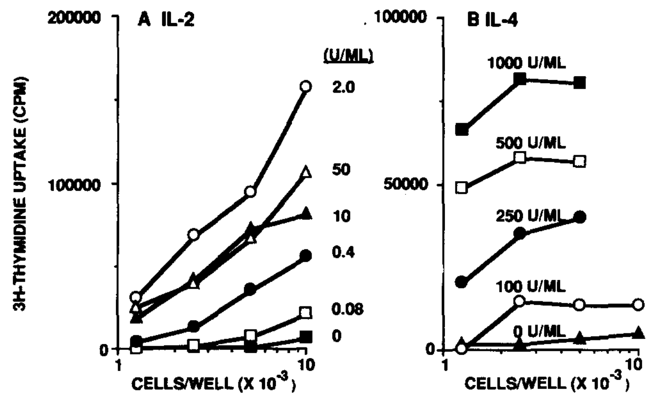

Figure 6. Concentrations of IL-2 and IL-4 required for induction of IL-4-producing T cells. Dense T cells from naive donors were cultured on anti-CD3-coated culture dishes. (*A*) Experiments in which 1,000 U/ml of IL-4 and variable amounts of human IL-2 were added to dishes. Anti-mouse IL-2 (S4.B6) was added to these cultures to neutralize endogenously produced IL-2. (*B*) Experiments to which 50 U/ml of IL-2 and varying amounts of IL-4 were added to culture dishes. After 5 d of culture, cells were harvested and transferred to 96-well plates that had been coated with anti-CD3 and cultured in the presence of IL-2 to determine IL-4-producing capacity. *A* and *B* illustrate results from different experiments.

IL-2 antibody also failed to produce IL-4 in the secondary culture. If the monoclonal anti-IL-4 antibody was not included in the group cultured with anti-CD3 plus IL-2, little or no IL-4 was produced in the second culture, presumably because high density T cells produce very little IL-4 in response to anti-CD3 plus IL-2 in the primary culture. By contrast, if anti-IL-2 was not added to cells cultured with anti-CD3 plus IL-4, this cell population did give rise to IL-4-producing T cells (data not shown). This is consistent with the capacity of high density T cells to produce IL-2 in response to culture with anti-CD3 and, as shown in Fig. 6 *A*, with the finding that relatively small amounts of exogenous IL-2 in primary cultures with anti-CD3 and IL-4 are sufficient to permit the appearance of IL-4-producing cells. Anti-CD3 was essential during the first culture. Cells cultured in IL-2 plus IL-4, or in IL-2 or IL-4 alone, failed to produce IL-4 upon secondary culture with anti-CD3 plus IL-2 (Fig. 5 *B*).

The requirement of anti-CD3 plus IL-2 and IL-4 could not be explained by improved cell yields, as the number of living cells were essentially the same in groups cultured with anti-CD3 plus IL-2 or IL-4 as they were in the group cultured with anti-CD3 plus IL-2 and IL-4 (data not shown). In addition, IL-4-producing capacity is expressed in terms of IL-4

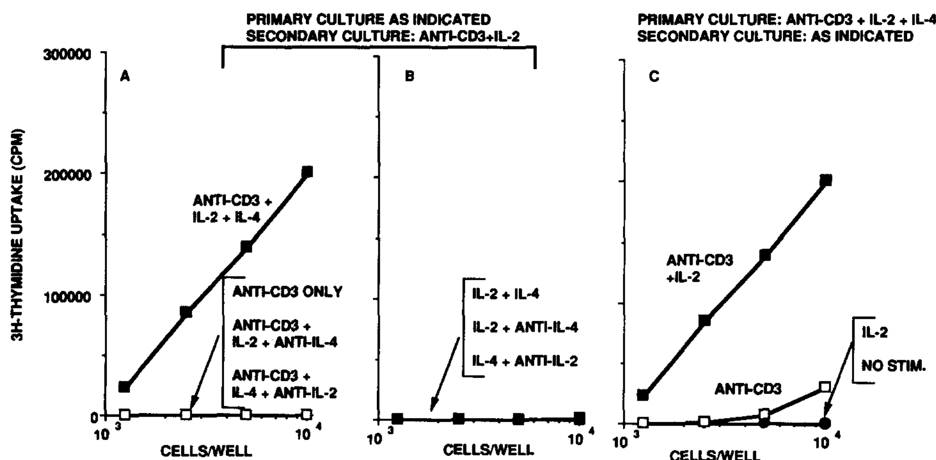

Figure 5. Small, dense T cells cultured with anti-CD3 plus IL-2 and IL-4 give rise to IL-4-producing cells. (*A*) Dense T cells from naive mice were cultured, as described in Fig. 4, for 5 d on dishes coated with anti-CD3 with the addition of no lymphokine, of IL-2 (50 U/ml) plus IL-4 (1,000 U/ml), of IL-2 plus anti-IL-4 (11B11; 10 μg/ml) or of IL-4 plus anti-IL-2 (S4.B6; 10 μg/ml). The cells were then harvested and tested for IL-4-producing capacity by culture, in the presence of IL-2, in 96-well plates coated with anti-CD3. (*B*) Dense T cells from naive mice were cultured as in *A*, except that the dishes had not been coated with anti-CD3. IL-4-producing capacity was measured by culture, in the presence of IL-2, in 96-well plates coated with anti-CD3. (*C*) Dense T cells that had been cultured for 5 d on anti-CD3-coated culture dishes in the presence of IL-2 and IL-4 were transferred to 96-well plates that had been coated with anti-CD3 or had not been coated. The cells were then cultured in the presence or absence of IL-2 (50 U/ml), and IL-4 production was measured.

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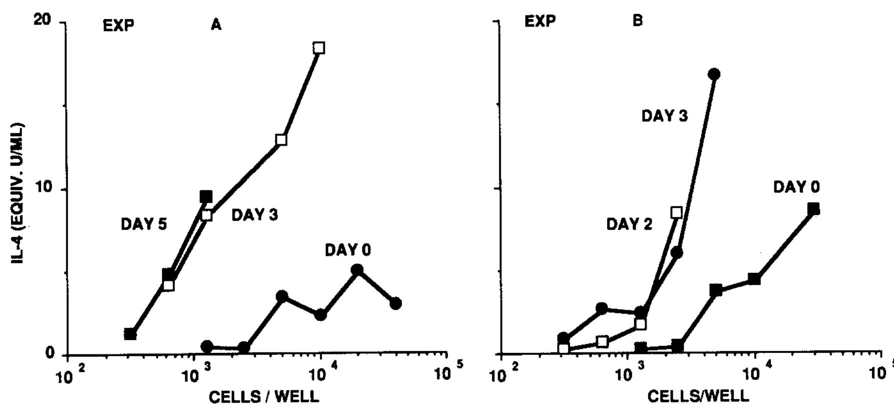

Figure 7. Anti-CD3 plus IL-2 and IL-4 increase IL-4-producing capacity of T cells within 2 d. Dense T cells from naive donors were cultured in 5-ml culture dishes coated with anti-CD3 in the presence of IL-2 (50 U/ml) and IL-4 (1,000 U/ml) for 3 or 5 d (Exp. A) or 2 or 3 d (Exp. B). At the end of the initial culture period, the cells were harvested and transferred to 96-well plates that had been sensitized with anti-CD3. Secondary cultures were carried out in the presence of IL-2. Results are expressed as equivalent units, since secondary assays were not carried out at the same time. Dose-response curves used for calculation of equivalent units were performed in parallel with the CT.4S coculture assay.

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produced per number of viable cells cultured in the secondary culture.

In the secondary culture, the IL-4-producing cells showed requirements for stimulation of lymphokine production similar to that of T cells from both naive and anti-IgD-injected donors. Anti-CD3 alone did elicit IL-4 production, but the addition of exogenous IL-2 markedly enhanced IL-4 production (Fig. 5 *C*). In other experiments (data not shown), monoclonal anti-IL-2 antibody diminished the production of IL-4 in response to anti-CD3 alone, indicating that the in vitro primed cells retain a substantial dependence on IL-2 for IL-4 production.

Relatively small amounts of IL-2 are required during the priming culture with anti-CD3 and IL-4. Thus, in the presence of anti-CD3 and 1,000 U/ml of IL-4, 0.4 U/ml (120 pg/ml) of human IL-2 gave a definite induction of cells capable of producing IL-4 upon subsequent culture with anti-CD3 and IL-2 (Fig. 6 *A*). In these experiments, endogenously produced murine IL-2 was neutralized with the monoclonal anti-mouse IL-2 antibody S4.B6. In the presence of 50 U/ml of human IL-2, 100 U/ml of IL-4 (~50 pg/ml) has some inducing activity, but there is a dose-dependent increase with 1,000 U/ml being much more effective.

Requirements for Induction of IL-4-producing Cells. Our experiments thus far have all utilized a protocol in which dense T cells from lymph nodes of naive donors are cultured on plates coated with anti-CD3 plus IL-2 and IL-4 for a period of 5 d, and are then removed from these plates and restimulated with anti-CD3 plus IL-2. We examined two additional aspects of this process: the time required for this induction and the capacity of IFN-γ to inhibit it. Fig. 7 presents two experiments in which cells were cultured with anti-CD3, IL-2, and IL-4 for periods of <5 d. Exp. A compares 3 d of priming with 5 d. In that experiment, the cells harvested at the end of a 3-d priming culture are quite similar to cells harvested at 5 d in their capacity to produce IL-4, and are considerably superior to the cells that had been tested without such preculture (day 0 cells) or to cells cultured without IL-2 or IL-4 (data not shown). Exp. B shows that induction of IL-4-producing cells can also be achieved within 2 d, indicating that the process of inducing IL-4-producing cells from a resting T cell population is a relatively rapid one if the stimulatory conditions are correct.

IFN-γ has been reported to inhibit the growth of clones of T cells that produce IL-4 in response to stimulation with antigen, APC, and IL-2 (15). To test the effect of IFN-γ during the in vitro priming of lymph node T cells with anti-CD3 plus IL-2 and IL-4, we added IFN-γ to these cultures and compared the IL-4 producing capacity of the cells that were obtained. Fig. 8 *A* shows that 100 U/ml of IFN-γ added to the priming culture has little or no effect on the appearance of cells that can produce IL-4 in response to subsequent culture with anti-CD3 plus IL-2. 500 U/ml has a modest inhibitory activity. Furthermore, addition of IFN-γ (250 U/ml) during the elicitation phase of the culture also failed to prevent IL-4 production (Fig. 8 *B*). These results indicate that IFN-γ does not have a profound effect on the appearance of IL-4-producing cells in the anti-CD3 plus IL-2 and IL-4 system described here.

Discussion

T cells from naive donors stimulated with anti-CD3 adsorbed onto culture wells produce little IL-4. In the presence of IL-2, IL-4 production is substantially enhanced, but this production is found mainly in cells of low and intermediate density. High density cells, which contain small resting T cells, produce very little IL-4, even in the presence of IL-2 and with supplementation by accessory cell populations (11). Swain et al. (9) had previously reported that resting normal T cells were poor IL-4-producers. They had concluded that IL-4-producing capacity was a property of primed T cells.

The poor IL-4-producing capacity of dense T cells from naive donor stimulated with anti-CD3 are somewhat at odds with a recent report by Carding et al. (30) using in situ hybridization to detect IL-4 mRNA. Those studies indicated that 30–80% of T cells, from naive donors, stimulated with PWM, Con A, or PHA are positive for IL-4 mRNA. On the other hand, Cardell and Sander (31) have reported a frequency of cells expressing IL-4 mRNA in response to anti-CD3 or to Con A of 0.5–1%. We have recently developed

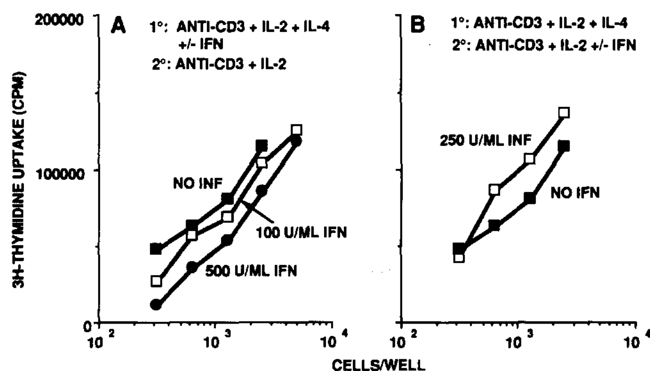

Figure 8. IFN-γ does not prevent the appearance of IL-4-producing cells, nor does it block their production of IL-4. (*A*) Dense T cells from naive donors were cultured for 5 d on dishes coated with anti-CD3 in the presence of IL-2 (50 U/ml) and IL-4 (1,000 U/ml). IFN-γ was added to a final concentration of 0, 500, or 1,000 U/ml. At the end of the 5-d culture period, the cells were harvested and cultured on 96-well plates that had been coated with anti-CD3. IL-2 was added to secondary cultures. (*B*) Cells harvested from initial cultures, described in *A*, that had been carried out in the presence of anti-CD3, IL-2, and IL-4 were recultured in anti-CD3-coated wells in the presence of IL-2 with or without IFN-γ (250 U/ml).

a limiting dilution assay to enumerate IL-4-producing cells (Seder et al., manuscript in preparation). Using this approach, we find that the frequency of T cells from lymph nodes of naive donors that produce IL-4 in response to anti-CD3 plus IL-2 is ~1:1000. It should also be pointed out that studies of recently primed mice have shown that among antigen-primed T cells, IL-4 producers are rare, although they increase in number upon repetitive stimulation in vitro (10).

In this manuscript, we demonstrate that in vivo polyclonal activation of mice by injection of anti-IgD antibodies strikingly increases the capacity of lymph node T cells to produce IL-4 in response to anti-CD3 plus IL-2. This is in keeping with the increase in serum IgE levels in such animals (12) and with the demonstration that treatment of these mice with mAbs to IL-4 (13) or to the IL-4R (32) completely inhibits this increase. Indeed, we have previously shown that anti-IL-4 antibody treatment is effective in inhibiting the IgE response to anti-IgD when administered as late as the fourth (but not fifth) day after injection (14). The observation that increased IL-4 production by T cells from anti-IgD-injected donors is first observed on day 4 is consistent with the time course of sensitivity to anti-IL-4. It indicates that the control of induction of IL-4-producing capacity is critical to the increase in IgE that occurs in certain forms of immunization. We have also observed that infection with *Nippostrongylus brasiliensis*, which causes a striking increase in serum IgE, causes a similar increase in T cell production of IL-4 (Le Gros et al., unpublished observations).

IL-4 production by T cells from anti-IgD-injected donors has many features in common with IL-4 production by T cells from naive donors. It is mainly a property of low and intermediate density T cells, and IL-2 plays a very important role in IL-4 production. When anti-CD3-treated T cells from naive donors are treated with monoclonal anti-IL-2 antibody (S4.B6), IL-4 production is not detectable (11). Treatment of T cells from anti-IgD-injected donors with S4.B6 causes a marked inhibition of IL-4 production, particularly when one compares IL-4 production in anti-CD3-treated cells that have received exogenous IL-2 with anti-CD3-treated cells to which anti-IL-2 has been added. Nonetheless, a portion of the IL-4 response by T cells from anti-IgD-primed donors is not inhibited by anti-IL-2. This may reflect an IL-2-independent component of IL-4 production that develops with time after priming. Indeed, the fact that T cell lines of the Th2 type produce IL-4 in response to anti-CD3, mitogens or antigens in the absence of exogenous IL-2 strongly suggests that certain cells can produce IL-4 without the need for IL-2 (33).

Although dense T cells from naive donors have very limited capacity to produce IL-4 in response to anti-CD3 plus IL-2, they contain cells that develop into IL-4 producers. Indeed, Swain et al. (9) have reported that stimulation of T cells from naive donors with mitogens and T cell supernatants leads to the appearance of cells capable of producing IL-4 upon subsequent culture. Our results demonstrate a striking appearance of cells that can produce IL-4 in response to anti-CD3 plus IL-2 as a result of culture for 2–5 d in wells coated with anti-CD3 to which both IL-2 and IL-4 have been added. Both lymphokines are required; culture with anti-CD3 plus IL-2 and anti-IL-4 antibody or culture with anti-CD3 plus IL-4 and anti-IL-2 antibody does not yield any increase in IL-4-producing capacity, although cell yields and viability are quite similar in all three groups. Direct comparison of IL-4-producing capacity of dense T cells from naive donors and T cells that have been primed in vitro by 5-d culture with anti-CD3 plus IL-2 and IL-4 shows that the latter cells are almost 100 times more active than the former. Seder et al. (manuscript in preparation) have recently shown that the frequency of IL-4-producing cells in these populations increases by a comparable degree.

This system provides an in vitro approach for the study of the events that regulate development of cells into IL-4 producers. One obvious question is whether stimulation with anti-CD3 plus IL-2 and IL-4 causes "uncommitted" cells to differentiate into cells that can produce IL-4, or whether it causes the proliferation of cells already capable of producing IL-4 in response to anti-CD3 plus IL-2. Although the evidence available is not decisive, we favor the idea that cells that can produce IL-4 in response to anti-CD3 plus IL-2 in the dense T cell population from naive donors are not the precursors (or the sole precursors) of the cells that produce IL-4 that appear after culture with anti-CD3 plus IL-2 and IL-4. Our major reason for this is that the IL-4-producing capacity of the dense T cell population from naive donors is very meager and, as our unpublished work has shown, IL-4-producing cells are very rare. Thus, these cells would have to proliferate in preference to other cell types that are being stimulated to proliferate by anti-CD3 plus lymphokines. While it is certainly possible that such proliferation could explain the appearance of IL-4 producers in a 5-d period, the fact that they can be observed in as few as 2 d suggests that it is more likely that the cells have differentiated from precursors that do not, themselves, produce IL-4 in response to anti-CD3

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plus IL-2. Whether those cells are committed to making IL-4 or can differentiate in other ways can best be addressed by limiting dilution progenitor analyses.

Finally, it is important to point out that crosslinking the TCR in the presence of IL-2 and IL-4 is unlikely to be the only means through which IL-4 producing cells may be induced. This is particularly so since IL-4-producing cells are rare among naive T cell populations. Thus, in initial priming for a response dominated by IL-4-producing cells, IL-4 may not be available in the location or in the amounts required for the induction we have observed. We have recently shown that mast cells can produce IL-4 (34, 35), and we have reported the existence of a population of splenic and bone marrow cells, lacking B and T cell markers, that express high affinity Fcε receptors and produce IL-4 in response to the crosslinkage of such receptors (25). These cells are a potential source of IL-4 for the priming system described here. However, IL-3 is required for substantial IL-4 production in response to Fcε receptor crosslinkage on non-B, non-T cells (36). Thus, in unstimulated animals, the non-B, non-T pool is not a strong candidate for a source of IL-4 for priming small T cells to develop into IL-4-producing cells. Indeed, non-B, non-T cells from naive donors are relatively poor producers of IL-4 when compared with non-B, non-T cells from anti-IgD-injected or *Nippostrongylus brasiliensis*-infected mice.

Another reason to suggest that the anti-CD3 plus IL-2 and IL-4 model is not the only way through which IL-4-producing cells can be induced is the finding that IFN-γ blocks the appearance of IL-4-producing cell lines in response to stimulation with antigen, APC, and IL-2 (15). By contrast, IFN-γ does not inhibit the generation of IL-4-producing cells in the anti-CD3 plus IL-2 and IL-4 system, nor does it inhibit IL-4 production by such T cells in response to anti-CD3 plus IL-2.

We would suggest that the induction of IL-4-producing cells from precursors in response to receptor crosslinkage plus IL-2 and IL-4 could be important in situations in which some IL-4-producing T cells had already been induced. Based on the characteristics of T cell clones isolated from chronically infected mice (7, 8), it appears that responses tend to be dominated by T cells that produce IL-2 or T cells that produce IL-4. This "polarization" of response at least in the direction of IL-4-producing cells could represent a positive "feedback" regulation resulting from the presence of substantial amounts of IL-4 at the time that newly emerging T cells initially encounter antigen, particularly an antigen to which the immune system was chronically exposed. Indeed, it has recently been reported that treatment of *Leishmania major*-infected BALB/c mice with anti-IL-4 antibody attenuates infection in these animals and diminishes levels of IL-4 mRNA in lymph nodes draining sites of infection (37). This suggests that IL-4 may be important, in vivo, in appearance or persistence of IL-4-producing cells.

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