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Generation of Antigen-Specific Th2 Cells from Unprimed Mice In Vitro: Effects of Dexamethasone and Anti-IL-10 Antibody[1]

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We describe a system for the in vitro production of Ag-specific mouse CD4 cell lines from unprimed mice. Purified $CD4^+CD45RB^{high}$ T cells were exposed to Ag-pulsed accessory cells in serum-free medium for 24 h; cultured in the absence of Ag and in the presence of serum, IL-2, dexamethasone, and Abs to IL-10 for an additional 4 days; and then re-exposed to the original sensitizing Ag. The presence of dexamethasone and Abs to IL-10 during the initial expansion stage appeared to be critical for the ability of the stimulated and expanded T cells to respond to restimulation with the same Ag. Repeated cycles of in vitro stimulation led to increased specificity for the sensitizing Ag (in the current case, pigeon cytochrome *c*), a decline in production of IL-2 and IFN-γ, and increased production of IL-4, IL-5, and IL-10. This culture protocol provides a test system for exploration of factors that regulate the conversion of naïve cells to memory cells and the development of specific immune responses to protein Ags. The data are consistent with models that implicate glucocorticoids as regulators of immune response specificity. ***The Journal of Immunology*****, 1998, 160: 2700–2705.**

Attempts to generate Ag-specific CD4 cells from unprimed responders in vitro have met with only sporadic success (1–4), and indeed many investigators attribute the difficulties of such an endeavor to the rarity of encounter, in cultures of unprimed cells, between T cells with the proper receptors and accessory cells bearing the cognate peptide Ags. The few successes have typically not emerged from a systematic attempt to reproduce in culture some of the factors that might contribute to the success of primary sensitization in vivo. These previous investigations have also focused principally on the earliest aspects of functional response, such as proliferation and IL-2 production, rather than on later phases, including cell maturation and alterations in cytokine production profiles.

Previous efforts to develop culture methods for primary sensitization in vitro have examined the effects of manipulations of cell concentration and interactions between T cells and accessory cells, but have not considered the potential effects of other physiologic agents, such as endocrine hormones, that are ordinarily produced during the course of an immune reaction and that could play a role in regulating its course. Increased glucocorticoid blood levels have been observed to occur at or near the time of peak immune responses to several Ags. This phenomenon was first described many years ago (5, 6) and was confirmed in recent publications (7–9). The association between immune responsiveness and increased glucocorticoid levels suggested that these hormones might modulate the strength of the response and in particular might contribute to its specificity (5, 10). In vitro studies using polyclonal activators have also suggested a role for corticosteroids in immune regulation, and have shown that these agents, which are well known for their immunosuppressive activity, can paradoxically promote Th2 cytokine responses by $CD4^+$ T cells in vitro (11, 12).

Based on these earlier observations, we have conducted a series of experiments to see whether appropriately timed exposure to glucocorticoids can promote Ag-specific sensitization of naïve T cells in culture. We describe an experimental system in which $CD4^+$ T cells are first exposed to an Ag (typically pigeon cytochrome *c*, PCC[3]) in serum-free culture, grown in IL-2-containing medium to permit clonal expansion, and then tested for Ag-dependent proliferation and cytokine production. The presence of dexamethasone (Dex) and Abs to IL-10 at the expansion stage following primary antigenic stimulation appeared to be critical for the ability of the stimulated and expanded T cells to respond to restimulation with the same Ag. The appearance of a strong, stable response of T lymphocytes to restimulation makes it possible to follow the maturation of the response after a series of restimulations with the same Ag. We report that this series of culture steps permits the routine development, from unprimed T cells, of Ag-specific cell lines that produce IL-4, IL-5, and IL-10.

Materials and Methods

Mice

Specific pathogen-free (BALB/cJ × C57BL/6)F_1 male mice were purchased from The Jackson Laboratories (Bar Harbor, ME) and housed in a specific pathogen-free facility. Sentinel mice from this colony were checked quarterly for serologic evidence of viral infection and for parasites; all such examinations were negative throughout the course of these studies. Mice were used in experiments when 8 to 12 wk old.

Reagents

Ascites fluid was generated from hybridoma cells secreting rat IgG2a Ab to mouse CD8 (clone 53-6.72) and mouse IgM anti-mouse Thy-1.2 (clone HO-13-4) obtained from the American Type Culture Collection (Rockville, MD). FITC anti-CD4 was obtained from PharMingen (San Diego, CA). Anti-IL-10 Abs were from R&D Systems (Minneapolis, MN). Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem (La Jolla,

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[2] Address correspondence and reprint requests to Dr. Richard A. Miller, University of Michigan, Room 5316 CCGCB, Box 0940, 1500 East Medical Center Drive, Ann Arbor, MI 48109. E-mail address: millerr@umich.edu

[3] Abbreviations used in this paper: PCC, pigeon cytochrome *c*; KLH, keyhole limpet hemocyanin; PLL, polyL-lysine; Dex, dexamethasone; αIL-10, Abs to IL-10.

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CA). PCC, mitomycin C, polyL-lysine (PLL; m.w. = 80,000), and Dex were obtained from Sigma Chemical Co. (St. Louis, MO).

Culture medium

RPMI 1640 supplemented with 10% FCS, penicillin G (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), and 2-ME (5×10^{-5} M) was used for all cultures except the cultures for primary Ag stimulation, where serum-free culture medium was used to prevent interfering presentation of any serum protein Ags.

Cell preparation

Splenic single cell suspensions were isolated and mononuclear cells were enriched by centrifugation over a cushion of Lympholyte-M (Cedarlane, Hornby, Canada). T cells were then enriched by depletion of surface Ig-positive cells on petri dishes coated with anti-mouse IgM. This procedure typically yielded more than 90% pure T cells (i.e., $CD3^+$) with fewer than 5% contaminating B cells. Collected cells were incubated on ice with rat anti-mouse CD8 mAbs (1:200) either 1) alone, or 2) together with anti-CD44 (1:200) followed by incubation with goat anti-rat IgG-coated magnetic beads (PerSeptive Diagnostics, Cambridge, MA) on ice. The bead-adherent cells were then removed using a magnetic separator. The purity of the $CD44^{low}$ $CD4^+$ T (CD4 virgin) cells was 80 to 90%, when analyzed by flow cytometry.

T cell-depleted resident peritoneal cells were prepared by treating mouse peritoneal cells with anti-Thy-1.2 Abs and complement as previously described (13), except that mitomycin C (50 μg/ml added to 5×10^7 cells/ml for 30 min at 37°C) was added along with the complement treatment to diminish proliferative activity. They were used as APC mixed with a 20-fold excess of similarly treated spleen cells.

Preparation of APC monolayers for T cell stimulation

First, culture plates were coated with PLL (50 μg/ml in PBS), using 1 ml for 6-well plates (no. 3506; Costar, Cambridge, MA), or 0.2 ml for 24-well plates (no. 3524; Costar). After 60 min, plates were washed three times in PBS to remove unbound PLL. Next, APCs were resuspended at 7×10^6 cells/ml in serum-free medium, and 2.5 ml of this suspension were added to wells of 6-well plates (Costar) or 0.5 ml to wells of 24-well plates. Ag (PCC or KLH, 100 μg/ml) was added at this step if indicated by the protocol. Plates were incubated on a stationary platform for 90 min in a CO_2 incubator. The wells were washed to remove unattached cells and free Ag by flushing the bottom three times with 37°C serum-free culture medium. Free Ag was removed together with nonadherent cells because of published reports (14) and our own experiments showing that the presence of free Ag in culture decreases stimulation efficiency. These APC monolayers were then covered with fresh culture medium (serum free in case of cultures for the primary antigenic stimulation) and were either used immediately (primary antigenic stimulation) or used the following day (as supporting cells for the primed T lymphocyte expansion).

Stimulation and expansion of unprimed T cells

Our method is based on the previous work of several authors (12, 15, 16), and has been described elsewhere in more detail (17). In brief, T cells from unprimed mice are stimulated with Ag in serum-free medium; expanded in the presence of IL-2 plus Dex and Abs to IL-10 (αIL-10); and then tested for proliferation and cytokine production induced by the original Ag (typically PCC), an alternate nominal Ag (typically KLH), or no Ag. In the stimulation phase, responding $CD4^+$ T cells ($6–8 \times 10^6$ cells in 4 ml) were cultivated for 24 h on Ag-bearing APC monolayers in serum-free medium, and then transferred to new culture wells containing fresh (unpulsed) APC. In preliminary experiments (not shown) we noted that the yield of cells at the end of the expansion phase was decreased threefold when this transfer to fresh APCs was omitted. In the expansion phase, the cells were cultured for an additional 4 to 5 days, in 6- or 24-well plates, in the presence of rIL-2 (20 U/ml) with Dex and αIL-10. Typically, each 4-ml initiation culture yielded ~6 to 9×10^6 viable cells, which were divided into three expansion cultures in 6-well plates, or into 15 expansion cultures in 24-well plates. Concentrations of Dex and αIL-10 were, respectively, 10^{-7} M and 4 μg/ml unless otherwise indicated in the text. In the testing phase, expanded cells were harvested; depleted of dead cells using a Lympholyte step gradient; and then recultured with new APC monolayers bearing either the original Ag, a distinct Ag, or no Ag. In most experiments these test cultures used 24-well plates containing 3×10^6 APC pulsed with 100 μg/ml of Ag and 3.5×10^5 harvested T cells. In experiments designed to test a range of Ag concentrations, we used 96-well plates containing 5×10^5 APC/well and 5×10^4 harvested T cells. Proliferation was assessed in duplicate using 200-μl aliquots in 96-well plates on day 4 by a 12-h pulse of [^{3}H]thymidine. For tests of cytokine production, 50-μl aliquots of medium were harvested at intervals (1–3 days) indicated in the text. IL-2 was measured by bioassay using CTLL indicator cells, and expressed as U/ml using a standard curve. IFN-γ, IL-4, IL-5, and IL-10 were assayed using two-site ELISA assays using standard curves with known amounts of recombinant cytokines. Anti-IFN-γ, anti-IL-4, anti-IL-5, and anti IL-10 Abs (PharMingen) were used as capture reagents, and biotinylated Abs (PharMingen) as the detecting Abs. The biotinylated Abs were then detected using peroxidase-conjugated streptavidin and *O*-phenylenediamine (Zymed Laboratories, San Francisco, CA). The recombinant standards were IFN-γ, IL-4, and IL-2 from Genzyme; IL-5 (PharMingen); and IL-10 (R&D Systems).

Multiple restimulations

To assess the effect of multiple stimulation events on T cell differentiation and maturation of the response, expanded cells were in some experiments cultivated on fresh Ag-pulsed monolayers and then transferred every 2 days to new cultures with fresh, pulsed APCs. After every cycle, a portion of the cells were removed for functional analysis by proliferation and/or cytokine production. Cells to be tested were transferred into three new culture wells containing, respectively, APCs with the original Ag, a distinct Ag, or no Ag. Supernatants from cultures were taken after 24 and 48 h, frozen, and then later tested for cytokine content.

Results

Dex and anti-IL-10 Abs promote response of unprimed T lymphocytes to restimulation with Ag

We have previously described (17) a culture system in which T cells from unprimed mice are cultivated together with a foreign Ag (typically PCC), IL-2, Dex, and αIL-10, and shown that with appropriate concentrations and timed additions of these components, the cultures develop Ag-specific responses to the Ag used for in vitro priming, measured either as proliferation or as production of IL-2. Figure 1 shows the results of an experiment of this kind, in which preparations enriched for naive CD4 (i.e., $CD8^-$, $CD44^{low}$, Ig^-) T cells were used as responders. The combination of an initial 24-h encounter with PCC in serum-free medium, together with the inclusion of Dex and αIL-10 during a period of IL-2-driven clonal expansion, leads to a dramatic increase in the ability of the cultured cells to proliferate and secrete IL-2 even in the absence of restimulation with PCC, although addition of PCC to the test cultures leads to a significant increase in response level above that obtained in cultures free of nominal Ag. The response after this single cycle of Ag exposure and expansion is not Ag specific, in that similar levels of response are seen in the test cultures whether PCC is omitted (as in the *leftmost* bars of Fig. 1) or replaced by equal concentrations of KLH (not shown). Similar results can be obtained in cultures initially stimulated by KLH (17) or by OVA (not shown). Although typical experiments used preparations in which 80 to 90% of the responder cells were $CD4^+$ and $CD44^{low}$, similar results were obtained in a few experiments in which the responder cell purity exceeded 98%. Nonetheless, further work would be needed to determine whether cells with the $CD44^{high}$ phenotype contribute to these responses of cells from unprimed mice.

Generation of responsive cells is, however, to some extent dependent on the presence of a foreign Ag in the initial culture. Figure 2 shows an experiment, one of three performed with similar outcomes, in which naive CD4 T cells from unprimed mice were initially cultured in serum-free medium with or without 50 μg/ml of PCC; expanded in the presence of Dex, αIL-10, and IL-2 for 4 days; and then tested for IL-2 production by culture for up to 3 days with PCC at 20 μg/ml. The data show that inclusion of PCC in the initial 24-h culture does promote responsiveness of the cells expanded in Dex/αIL-10.

Figure 3 shows the results of a "checkerboard" titration experiment, one of three performed to determine the optimal amounts of Dex and αIL-10 to be used in the cell expansion phase. In the

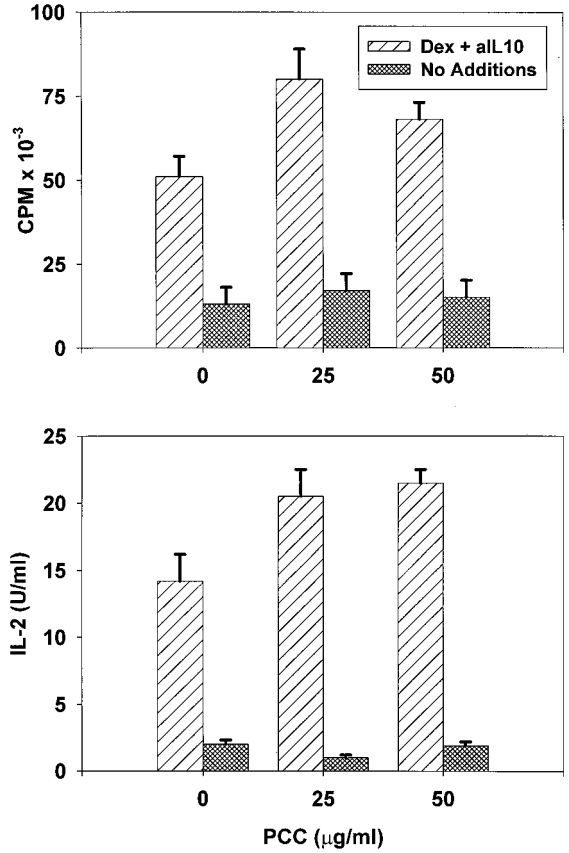

FIGURE 1. Effect of Dex and αIL-10 on the response of CD4 naïve cells sensitized to PCC in vitro. Cells from unprimed mice were exposed to PCC-pulsed accessory cells in serum-free medium, cultured for an additional 4 days on unpulsed accessory cells with IL-2 either with or without Dex (10^{-7} M) and αIL-10 (4 μg/ml), and then tested for proliferation (12-h pulse with [^{3}H]thymidine on day 4 of test culture; *top*) or IL-2 production (day 3 of test culture; *bottom*). Values shown are means and SEs of duplicate cultures from a representative experiment, one of three performed.

absence of Dex, αIL-10 had no effect on proliferation (*top*) or IL-2 production (*bottom*) after cell expansion. In the absence of αIL-10 (open symbols), Dex led to a small but significant increase in PCC-stimulated proliferation and a modest increase in IL-2 production by expanded cells. For Dex doses between 10^{-8} M and 10^{-7} M, the addition of αIL-10 led to synergistic increases in both proliferation and IL-2 production. The optimal Dex concentration varied somewhat from experiment to experiment, but was always between 10^{-8} M and 10^{-7} M. The αIL-10 titration curve was also biphasic, with optimal Ab concentrations typically at 4 μg/ml.

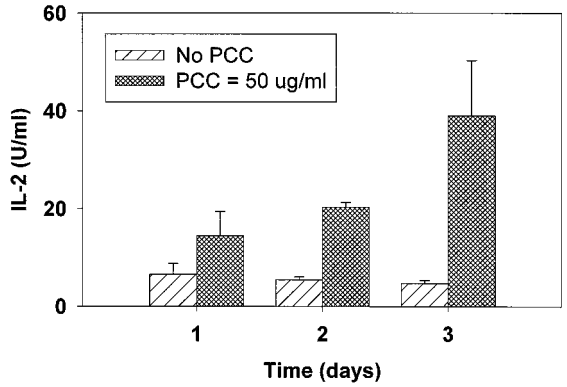

FIGURE 2. Proliferation of expanded T cells after initial stimulation with or without PCC. Cultures of T cells from unprimed mice were initially cultured either in the absence of PCC (light bars) or in the presence of PCC at 50 μg/ml (dark bars). After 24 h, they were expanded in the presence of IL-2, Dex (10^{-8} M), and αIL-10 (4 μg/ml); washed; and then tested for IL-2 production in the presence of PCC at 20 μg/ml. IL-2 accumulation was measured at 1, 2, or 3 days of test culture. Values shown are means and SEMs of duplicate wells.

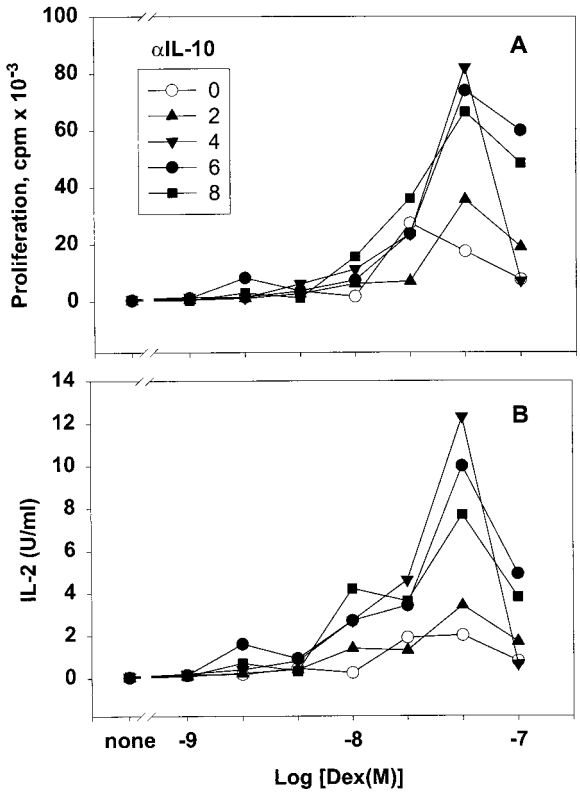

FIGURE 3. Dex and αIL-10 act in synergy to promote proliferation and IL-2 production by expanded T cells. T cells from unprimed mice were initially cultured for 24 h in the presence of PCC, then washed and expanded for 4 days in the presence (or absence, as indicated) of Dex, αIL-10, or both at the indicated concentrations before testing. The *top panel* shows PCC-stimulated proliferation (day 3.5). The *bottom panel* shows IL-2 production at day 2.

Cell yield from the expansion cultures decreases progressively over the range of Dex concentrations from 10^{-10} M through 10^{-7} M (Fig. 4, *top*). Thus, the data shown in Figure 3, which present T cell responses on a "per culture" basis, fail to convey the dramatic effect of Dex and αIL-10 on the "per cell" responsiveness. The *middle* and *bottom panels* of Figure 4 show proliferation and IL-2 production, per recovered cell, for cells expanded in the presence of various doses of Dex and αIL-10 at 4 μg/ml. It is clear that Dex doses up to at least 10^{-7} M lead to progressive increases in responses among those few cells that are recovered at the end of the 4 days of expansion. Cell recovery at Dex concentrations higher than 10^{-7} M was typically too low to permit detailed study of function (not shown).

Repeated cycles of stimulation improve Ag specificity and alter cytokine production profile

We conducted a series of experiments to determine whether repeated cycles of Ag exposure would alter the specificity and/or quality of the response obtained. In these experiments, naive CD4 cells from unprimed mice were cultured for 24 h in the presence of PCC-bearing APC, then expanded in the presence of IL-2, Dex

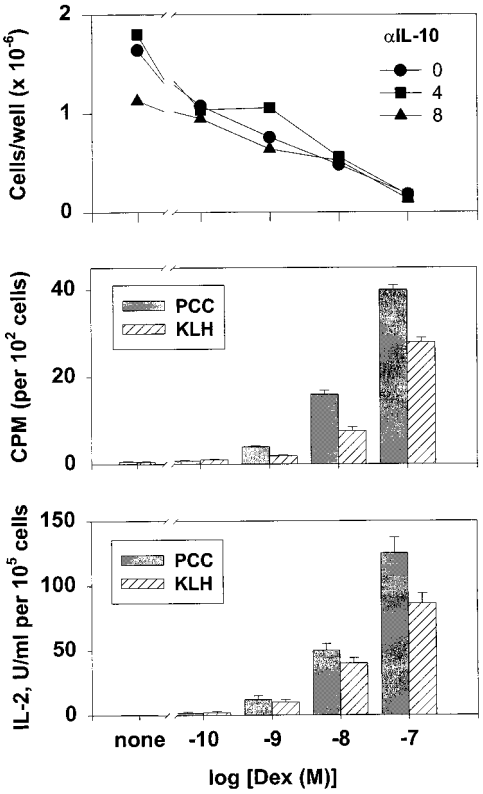

FIGURE 4. Increased proliferation and IL-2 production after expansion in Dex: adjustment for diminished yield. The *top panel* shows cell yields from cultures expanded at the indicated concentrations of Dex and αIL-10. The *middle panel* shows proliferation values from this experiment, presented as cpm of [^{3}H]thymidine incorporation per 100 cells recovered. Results are shown for αIL-10 at 4 μg/ml, and for cultures tested with either PCC or KLH. The *bottom panel* shows IL-2 production (day 2) from cultures set up as replicates of those shown in the *middle panel*. Differences between PCC-stimulated and KLH-stimulated cultures are statistically significant (at $p < 0.05$) for both proliferation and IL-2 at Dex concentrations of 10^{-8} and 10^{-7} M.

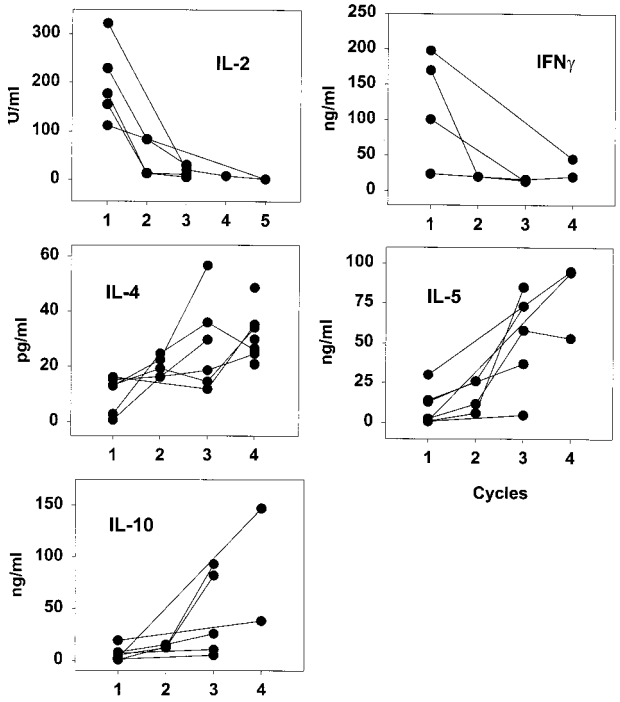

FIGURE 5. Transformation of cytokine secretion profile from Th1 to Th2 type after repetitive stimulations with PCC. Naïve CD4 T lymphocytes were stimulated on PCC-pulsed monolayers; expanded in the presence of IL-2, αIL-10, and Dex; and restimulated several times on fresh Ag-pulsed monolayers without added cytokines or Abs. Cytokine production was tested on the third day after the first restimulation (cycle 1), and on the second day after subsequent restimulations. Each line connects a series of cultures set up at the same time from the same starting cell population.

(10^{-7} M), and αIL-10 (4 μg/ml). Aliquots of these cultures (cycle 1) were tested for production of IL-2, IFN-γ, IL-4, IL-5, and IL-10 in responses to PCC, to the unrelated Ag KLH, or in medium that contained no nominal Ag (but did contain 10% FBS). Other aliquots from the same cultures were restimulated by re-exposure to fresh PCC-pulsed monolayers in the absence of added IL-2, Dex, or αIL-10. After each 48-h interval, these cells were harvested (cycles 2, 3, etc.) and divided into aliquots for functional testing or for further culture on PCC-pulsed APC.

Figure 5 shows that these cycles of repeated Ag exposure led to consistent alterations in the pattern of cytokines produced in the PCC-stimulated test cultures. Production of IL-2 and IFN-γ declined greatly by the third and later cycles, while production of IL-4, IL-5, and IL-10 increased in parallel.

Figure 6 shows that by the third cycle of PCC exposure, the remaining cells were almost entirely specific for PCC. At this stage, only IL-4, IL-5, and IL-10 are produced at significant levels, and in most experiments the responses in PCC-containing cultures are substantially higher than the responses to KLH-bearing APC or to APC that had not been pulsed with any nominal Ag. In some experiments, IL-10 production was low even in the PCC-challenged test cultures (see Fig. 5); we suspect that this inconsistency reflects the combined effects of nonlinearity in the dose curves for Dex, Ag, and αIL-10.

Discussion

This work clearly demonstrates that primary responses of CD4$^+$CD45RB^{high} T lymphocytes to novel Ags can be generated in vitro. This result, together with other several successful attempts using a diverse sample of approaches (1–4, 14, 18–22) contradicts the prevailing opinion that the low frequency of Ag-reactive precursors within naïve CD4 T cell populations should prevent primary immunization in vitro to Ags other than alloantigens and superantigens. Our data also provide new insights into the possible role of IL-10 and steroid hormones in regulating early immune responses.

Several factors may contribute to the absence of Ag-specific T cell response to new Ags under ordinary culture conditions. Virgin T cells may well have special costimulatory requirements (23–25). Costimulation through the CD45 Ag, for example, has been suggested to lead to an increase in Ag-specific Th cell frequencies (26). Inhibitory interactions between different types of T cells may also interfere with activation of naïve T cells in primary cultures. Previously, using a limiting dilution approach (27), we have shown that IL-10 production by memory T cells can abrogate proliferation by naïve cells in shared cultures stimulated by plant mitogens, and that this inhibition could be blocked by Ab to IL-10. We speculated that T memory cells activated either cross-reactively or through bystander effects might be able to inhibit activation of virgin T cells in response to Ag though an IL-10-dependent mechanism, and thus examined in the current program the effect of including anti-IL-10 Abs during the interval immediately following the initial exposure to Ag. The speculation (5) that glucocorticoids produced during the course of in vivo immune reactions

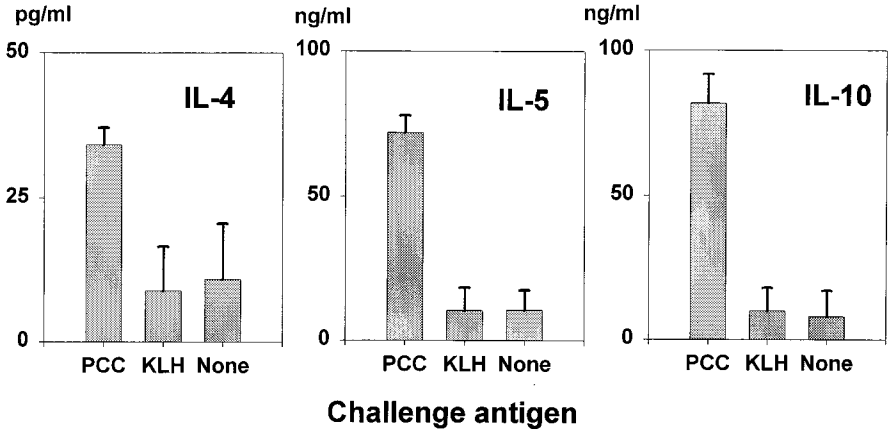

FIGURE 6. Ag specificity of CD4 naïve T cells sensitized in vitro to PCC. IL-5 and IL-10 production were measured on the second day after the third cycle of restimulation as in Figure 5; IL-4 was measured after the fourth restimulation. Results are means and SEMs of replicate cultures and are representative of two similar experiments performed.

might improve specificity by impeding the growth of T cells with poor Ag specificity prompted us to examine the role of Dex in the primary cultures. Our results show clearly (Figs. 1 and 3; and Ref. 17) that optimal proliferation and IL-2 production by expanded cells requires both Dex and αIL-10 during the expansion phase. The dose-response curves for both Dex and αIL-10 provide only a narrow window for positive effects. High doses of Dex, in particular, diminish cell recovery while continuing to increase response per recovered cell (Figs. 3 and 4).

Survival and clonal expansion at successive phases of the sensitization process may be modulated in part by changes in susceptibility to apoptosis. Although virgin T cells appear to be very sensitive to the inhibitory effects of Dex during the Ag-priming phase, they become increasingly resistant to corticosteroid inhibition once they have entered the proliferative phase (28, 29). Although both TCR hyperstimulation and glucocorticoids can induce cell death individually, previous work has shown that TCR-mediated programmed cell death can, paradoxically, be inhibited by glucocorticoids (30, 31). Removal of memory T cells from human PBMC makes the residual population more resistant to Dex (32). One effect of Dex that might interfere with expansion of Ag-stimulated naïve cells, i.e., its ability to block IL-2 production (33), was mitigated in our culture system by the addition of exogenous IL-2. The ability of IL-2 to rescue Ag-specific T cells from apoptosis may also contribute to positive selection during the expansion phase, because T cells that do not get antigenic stimulation cannot be rescued by IL-2 from apoptosis (34). The use of Dex and αIL-10 during the interval immediately following Ag exposure permits the rapid expansion of the Ag-primed cells, but does not by itself achieve antigenic specificity. Normal in vivo immune responses are also often accompanied by bystander proliferation of cells that have been activated nonspecifically (35). The maturation of naïve CD4 cells into effector cells able to generate Th2 cytokines has in other laboratories also depended upon addition of growth factors (16, 36) or corticosteroids (12, 37), or on repetitive in vitro restimulation (38). In our protocol, the combination of Dex-supported clonal expansion followed by several cycles of Ag exposure leads routinely to Ag-specific T cell lines. The observation that these lines gradually acquire specialization for secretion of IL-4, IL-5, and IL-10 rather than IL-2 and IFN-γ is consistent with other reports that glucocorticoids promote Th2 cell maturation (11, 29, 39).

Further exploitation of this experimental system may provide insights into the factors that promote the activation, growth, and differentiation of Ag-responsive naïve T cells. Studies of factors, such as IL-12, that could channel the differentiation process toward the development of IL-2 and IFN-γ-secreting effector cells will be of particular interest. The data presented here are consistent with models that implicate endocrine factors not merely as inhibitory modulators of response strength, but as required participants in early activation events. This experimental system presents opportunities for the investigation of other neuroendocrine influences that might modulate immune responses in intact organisms. It will also be of interest to determine whether analogous approaches allow the routine production of Ag-specific T cell lines from human blood.

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