vaders or pathogens until hatching is probably related to the enzymatic properties of the FE outermost layer, which are acquired through the cortical reaction, in addition to the function of the FE as a physical barrier. FE extracts have already been shown to exert a bactericidal activity [11, 13]. Further experiments are necessary to verify the presence of any antiviral defense mechanism. This information may lead to new insights into the function of the FE and the cortical alveoli.

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Roles for interleukin-2 (IL-2) and IL-4 in the generation of allocytotoxic T cells in the primary and secondary responses in vitro

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Abstract. Roles for interleukin-2 (IL-2) and IL-4 in the generation of murine allocytotoxine T lymphocytes (allo-CTL) in the primary and secondary responses were studied in vitro. The generation of allo-CTL in the primary response was inhibited by anti-IL-2 monoclonal antibody (mAb), but was not inhibited by anti-IL-4 mAb. On the other hand, the generation of allo-CTL in the secondary response was partially inhibited by either anti-IL-2 or anti-IL-4 mAb, and it was almost completely inhibited by the combination of two mAbs. $CD8^+$ cell-depleted splenocytes produced IL-2, but not IL-4, in response to alloantigens in the primary response, and these cells produced both IL-2 and IL-4 in the secondary response. Both exogenous IL-2 and IL-4 induced functionally active allo-CTL in the primary response from $CD4^+$ cell-depleted splenocytes when these cells were stimulated with T cell-depleted allogeneic cells. These results suggest that the allo-CTL induction in the primary response is IL:-2-dependent and secondary allo-CTL induction is both IL-2 and IL-4-dependent, because unprimed $CD4^+$ T cells produce IL-2, but not IL-4, whereas primed cells produce both IL-2 and IL-4 in response to alloantigens.
Key words. Interleukin-2; interleukin-4; cytotoxic T lymphocytes.

The growth and differentiation of cytotoxic cells are regulated by soluble factors, of which interleukin-2 (IL-2), a lymphokine produced by activated helper T cells, has been shown to play a major role in the generation of cytotoxic cells, such as allospecific cytotoxic T lymphocytes (CTL)[1] and lymphokine activated killer (LAK) cells[2]. On the other hand, IL-4, which is also produced by helper T cells, is demonstrated to have potency in the generation of cytotoxic cells, in addition to its effect on B cells. Murine IL-4 induces alloreactive CTL[3] and LAK cells[4] in in vitro cultures. In human systems, it was reported that IL-4 augmented the induction of allo-CTL in IL-2-supplemented cultures, whereas IL-2-mediated induction of LAK cells was inhibited by IL-4[5].

Although activated helper T cells produce both IL-2 and IL-4, the roles of these two lymphokines for allo-CTL induction in the primary and secondary responses are not well characterized. Recent studies have shown that murine helper T cells are composed of at least two subsets that can be distinguished on the basis of their patterns of lymphokine production[6]. One subset, termed Th1, pro-

duces IL-2 and INF-r, and the other subset, termed Th2, produces IL-4 and IL-5 in response to antigenic stimuli. The purpose of the present study was to analyze the patterns of IL-2 and IL-4 production and their roles for allo-CTL induction in the primary and secondary responses.

Materials and methods

Mice. Female BALB/c and C3H mice were purchased from Shizuoka Experimental Animal Center, Japan.

Media. Cytotoxic assays were carried out in RPMI 1640 medium containing 10% heat-inactivated FCS. RPMI 1640 supplemented with 2 mM L-glutamine, 12.5 mM Hepes, 0.05 mM 2-mercaptoethanol, 100 U/ml penicillin, 30 µg/ml gentamicin, and 10% heat-inactivated FCS was used for cell cultures.

Reagents. Human recombinant IL-2 was supplied by Shionogi Pharmaceutical Co., Osaka, Japan. IL-2 activity was defined by the supplier. Murine recombinant IL-4 was supplied by Ono Pharmaceutical Co., Tokyo, Japan. IL-4 activity was determined by a thymocyte proliferation assay [7]. The monoclonal antibody (mAb) producing hybridomas: anti-IL-2 (S4B6) originally obtained from Dr T. Mossman (DNAX, Palo Alto, CA) and anti-CD8 (3.155) originally obtained from Dr F. W. Fith (University of Chicago), and the culture supernatant of anti-CD-4 (Hybridoma RL 174-4), were gifts of Dr J. Quintans, University of Chicago. The mAb producing hybridomas, anti-IL-4 (11B11) and anti-Thyl.2 (Jlj), were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The specificities of anti-IL-2 and anti-IL-4 mAbs were previously described [8, 9]. Culture supernatants of these hybridomas were used in this study.

Preparation of splenocytes depleted of $CD4^+$ cells, $CD8^+$ cells, or $Thyl.2^+$ cells. To prepare $CD4^+$ cell- or $CD8^+$ cell-depleted splenocytes, BALB/c splenocytes were treated with 1:5 diluted anti-CD4 or anti-CD8 hybridoma supernatant and 1:8 diluted rabbit complement (Low Tox-M rabbit C, Cedarlane Laboratories) for 1 h at 37 °C. $Thyl.2^+$ cell-depleted C3H splenocytes were prepared by treatment with anti-Thyl.2 mAb and complement. These treatments routinely led to less than 5% positive cells, as determined by immunofluorescence examination.

Mixed lymphocyte cultures. 1) Primary allo-CTL induction: BALB/c splenocytes (5×10^6 cells/well) were cultured with an equal number of irradiated (3000 rad) C3H splenocytes in 1.5 ml RPMI 1640 medium in macrowells for 4 days. Thereafter, viable cells were separated using Ficoll-Hypaque, and were used as effector cells. In some experiments, $CD4^+$ cell-depleted BALB/c splenocytes (2×10^6 cells/well) were cultured with irradiated $Thyl.2^+$ cell-depleted C3H splenocytes (3×10^6 cells/well) in the presence of 5 or 10 U/ml of either IL-2 or IL-4 for 4 days. Viable cells were separated using Ficoll-Hypaque, and were used as effector cells.

2) Secondary allo-CTL induction: BALB/c splenocytes (5×10^5 cells/well) cultured with irradiated C3H splenocytes for 10 days were recultured with C3H splenocytes (5×10^6 cells/well). After 3 days, the cytotoxic activity of these cultures was measured.

3) Culture supernatants: $CD8^+$ cell-depleted BALB/c splenocytes were cultured with irradiated $Thyl.2^+$ cell-depleted C3H splenocytes (3×10^6 cells/well) for 48 h, then the supernatant of the culture was collected (primary culture supernatant). These cultures were recultured with irradiated $Thyl.2^+$ cell-depleted C3H splenocytes 10 days after the primary culture at 10^6 cells/well. The supernatant of the culture was collected after 48 h (secondary culture supernatant). IL-2 or IL-4 activities in these culture supernatants were determined using CTLL-2 indicator cells. CTLL-2 cells (10^4 cells/well in microwells) were cultured for 24 h (0.2 ml) in the presence or absence of anti-IL-2 and/or anti-IL-4 mAbs. Cultures were pulsed for the final 6 h with 0.5 µCi[^{3}H] thymidine before harvest.

Target cell preparation. C3H splenocytes (4×10^6 cells/well) were cultured with 2 µg/ml Concanavalin A (ConA) for 2 days. The cells were separated Ficoll-Hypaque and used as targets.

Cytotoxic assays. C3H ConA blasts were labeled with 100 µCi ^{51}Cr during 1 h incubation at 37 °C in 5% CO_2. Effector cells in 80 µl medium were serially diluted in V-bottom 96-well microtiter plates (Costor, Cambridge, MA) and mixed with 5×10^3 ^{51}Cr-labeled target cells in 80 µl medium. The plates were spun at 650 g for 3 min. After a 4-h incubation, the radioactivity in 80 µl of each supernatant was measured with a gamma counter. The percent specific ^{51}Cr release was calculated using the following formula:

$$\text{Specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

Proliferation assays. The proliferative responses of splenocytes in primary and secondary responses were determined from triplicate cultures in microwells. Cultures were incubated in the presence or absence of anti-IL-2 and/or anti-IL-4 mAbs for 2–3 days at 37 °C in 5% CO_2, and pulsed for the final 6 h with 0.5 µCi [^{3}H] thymidine before harvest.

Results

We first investigated the inhibitory effects of anti-IL-2 and anti-IL-4 mAb on the generation of allo-CTL in the primary and secondary responses. BALB/c splenocytes or recovered cells from the primary culture were cultured with irradiated C3H splenocytes in the presence or absence of anti-IL-2 and/or anti-IL-4 mAbs. The cytotoxic activities of these cultures were assessed against C3H ConA blasts. The results shown in table 1 demonstrated that the addition of anti-IL-2 mAb, but not of anti-IL-4 mAb, to the primary culture inhibited the generation of

Table 1. Inhibition of allo-CTL induction by anti-IL-2 and/or anti-IL-4 mAbs in the primary and secondary responses[a]

Addition to culture	Effector: target ratio 12:1	6:1	3:1	1:1
Primary				
(−)	91	70	42	25
anti-IL-2	24	10	5	0
anti-IL-4	85	73	59	26
anti-IL-2 + anti-IL-4	29	13	6	0
	5:1	2:1	1:1	0.5:1
Secondary				
(−)	79	70	54	34
anti-IL-2	21	10	5	4
anti-IL-4	49	40	17	10
anti-IL-2 + anti-IL-4	4	2	6	3

[a] In the primary response, BALB/c splenocytes (5×10^6 cells/well) were cultured with an equal number of irradiated C3H splenocytes in the presence or absence of anti-IL-2 (20%, vol/vol) and/or anti-IL-4 (20%) mAbs for 4 days, then cytotoxic activities of these cultures were measured against C3H ConA-blasts. In the secondary response, BALB/c splenocytes (5×10^5 cells/well), cultured with allogenic cells for 10 days, were recultured with allogenic cells (5×10^6 cells/well) for 3 days. Thereafter cytotoxic activities of these cultures were measured.

Table 2. Effects of exogenous IL-2 and IL-4 on the generation of allo-CTL in the primary response[a]

Addition to culture	Effector: target ratio 20	10	5	2
(−)	1	0	4	2
IL-2 5 u/ml	49	41	32	21
20 u/ml	49	42	37	24
IL-4 5 u/ml	45	37	29	17
20 u/ml	75	69	65	52

[a] $CD4^+$ cell-depleted BALB/c splenocytes (2×10^6 cells/well) were cultured with irradiated T cell-depleted C3H splenocytes (3×10^6 cells/well) in the presence or absence of IL-2 or IL-4 for 4 days. Thereafter the cytotoxic activities of these cultures were measured against C3H ConA-blasts.

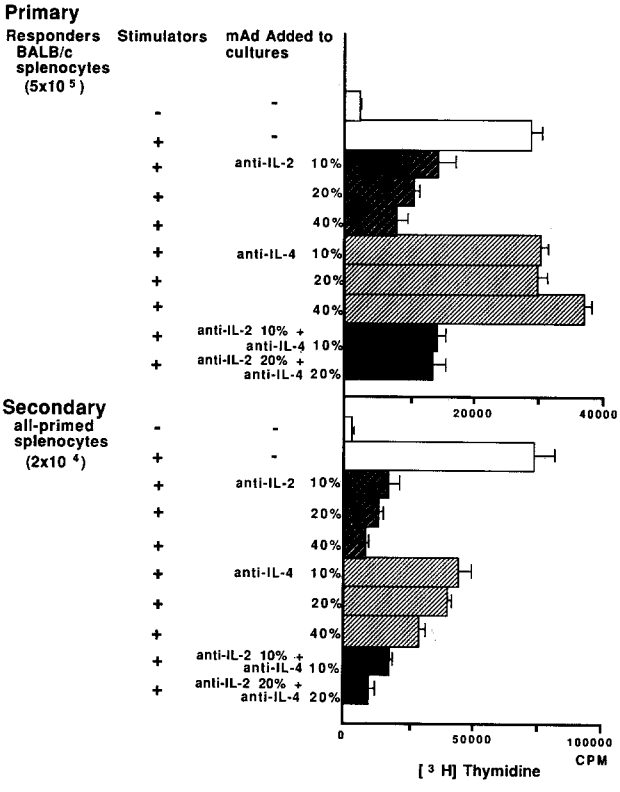

Figure 1. Inhibitory effects of anti-IL-2 and anti-IL-4 mAbs on proliferative responses in the primary and secondary responses. In the primary response, unprimed BALB/c splenocytes (5×10^5 cells/well) were cultured with irradiated C3H splenocytes (5×10^5 cells/well) in the presence or absence of increasing concentrations of anti-IL-2 and/or anti-IL-4 mAbs (vol/vol). The cultures were incubated for 3 days, and pulsed for the final 6 h with 0.5 μCi [^{3}H] thymidine before harvest. In the secondary response, BALB/c splenocytes, (2×10^4 cells/well), cultured with allogeneic cells for 10 days, were recultured with allogeneic cells (5×10^5 cells/well) for 2 days, and pulsed for the final 6 h with [^{3}H] thymidine. The results are expressed as means ± SD of triplicate cultures.

cytotoxic activity. In the secondary response, the generation of cytotoxicity was inhibited by either mAb, and the combination of these two mAbs further suppressed the activity. These results suggest that the primary allo-CTL induction can be inhibited by anti-IL-2 Ab, and the secondary allo-CTL induction can be inhibited by both anti-IL-2 and anti-IL-4 Ab.

We next examined the effects of anti-IL-2 and anti-IL-4 mAbs on proliferative response during the primary and secondary responses. As shown in figure 1, the proliferation of unprimed cells was inhibited by the addition of anti-IL-2 mAb to the cultures. In contrast, anti-IL-4 mAb showed no inhibitory effect on the primary response. On the other hand, the secondary response was partially inhibited by either mAb, and an additive effect with anti-IL-2 and anti-IL-4 mAbs was observed. However, these inhibitory effects by mAbs were not complete even at high concentrations of these mAbs. This suggests that factors other than IL-2 and IL-4 might participate in proliferative responses against allogeneic cells during both the primary and secondary responses, or that the amounts of mAbs added to cultures were insufficient.

The results described above suggest that unprimed cells produce IL-2, but not IL-4, and primed cells produce both IL-2 and IL-4 in response to alloantigens. To confirm this, enriched populations of helper T cells were cultured with T cell-depleted allogeneic cells, and the IL-2 or IL-4 activities of these primary and secondary cultures were determined using lymphokine-dependent indicator cells. As shown in figure 2, the supernatant from the primary culture supported CTLL-2 cell growth which was inhibited by anti-IL-2 mAb but not by anti-IL-4 mAb. The supernatant from the secondary culture supported lymphokine-dependent cell growth which was inhibited by both anti-IL-2 and anti-IL-4 mAbs, with profound inhibition by the combination of two mAbs. These results suggest that IL-2 was contained in the primary culture supernatant and both IL-2 and IL-4 were contained in the secondary culture supernatant.

To test whether naive $CD8^+$ cells, when stimulated with alloantigens, could differentiate to functionally active allo-CTL in response to both IL-2 and IL-4, $CD4^+$ cell-depleted BALB/c splenocytes were cultured with T cell-depleted allogeneic cells in the presence of exogenous IL-2 or IL-4, and their allocytotoxic activity was measured 4 days later (table 2). Both lymphokines induced function-

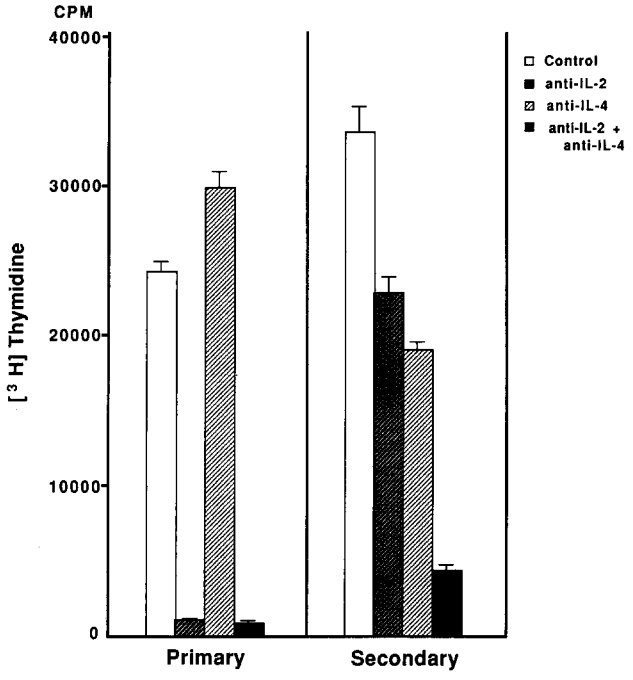

Figure 2. CTLL-2 assay of supernatants from the primary and secondary anti-allogenic cultures. CTLL-2 cells were cultured with 1:4 diluted culture supernatants in the presence or absence of anti-IL-2 and/or anti-IL-4 mAbs (25% vol/vol) for 24 h. The cultures were pulsed with 0.5 μCi [^{3}H] thymidine for the final 6 h.

ally matured CTL in the primary culture. This suggests that naive CD8$^+$ cells can differentiate to functional allo-CTL in response to either alloantigens plus IL-2 or alloantigens plus IL-4.

Discussion

The present study has demonstrated that IL-2 play a major role in the generation of allo-CTL induction in the primary response, and both IL-2 and IL-4 play a role in the secondary response. The production by CD4$^+$ cells of IL-2, but not IL-4, was detected in the primary anti-alloantigenic response, and the production of both IL-2 and IL-4 was detected in the secondary response. Both IL-2 and IL-4 induced the functional maturation of allo-CTL from naive CD8$^+$ cells, therefore we concluded that the findings described above were due to functional alteration of helper T cells to produce lymphokines after priming with alloantigens.

Recent studies have demonstrated phenotypic and functional heterogeneity among CD4$^+$ cells[6, 8, 10]. Murine helper T cell clones are separated into at least two distinct subsets according to their lymphokine production and utilization. One subset, named Th1, produces IL-2 and IFN-r, while the other, named Th2, secretes IL-4 and IL-5. The former proliferates in response to exogenous IL-2, whereas the latter requires IL-1 as an accessory molecule for the maximal response to IL-2 or IL-4[11]. In a recent study[12], we further divided Th2 cell clones into two subsets according to their function of producing IL-2 and IFN-r. Freshly isolated murine splenic T cells, when stimulated with mitogen or alloantigens, were found to secrete IL-2 but not significant amounts of IL-4[13]. However, these primed cells produced IL-4 after mitogenic or antigenic restimulation in in vitro cultures. Hayakawa and Hardy[10] have suggested that cells secreting IL-2 switch to IL-4-secretion after priming with antigens on cell maturation. In vivo studies in mice have also demonstrated the generation of IL-4-producing cells after injection of anti-IgD[14]. IL-4-producing cells were found among large- and intermediate-sized T cells. Mohler and Butler[15] have reported the expression of IL-4 mRNA after antigenic stimulation in vivo. These observations, together with our present findings, suggest that IL-4 may play a role in the secondary response.

IL-4, first described as B cell stimulatory factor-1, facilitates humoral immunity by its effects on B lymphocyte proliferation and maturation[16, 17]. However, IL-4 is now known to act on T cells[18]. IL-4 has been shown to be active in the development of cytotoxic effector cells. Widmer and Grabstein[3] have demonstrated that IL-4 acts as a potent helper factor for the generation of allo-CTL in primary and secondary mixed lymphocyte cultures. In agreement with this finding, our present results have shown that naive CD8$^+$ cells can mature to functionally active allo-CTL in response to both exogenous IL-2 and IL-4. Therefore the failure of inhibition by anti-IL-4 mAb in the primary response was not due to unresponsiveness of naive CD8$^+$ cells to IL-4. Our present results suggest that allo-primed helper T cells produce IL-4 in response to rechallenge of alloantigens, which play a role in the generation of allo-CTL in the secondary response.

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