Involvement of Cyclic Adenosine Monophosphate in the Interleukin 4 Inhibitory Effect on Interleukin 2–induced Lymphokine-activated Killer Generation

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Abstract

In previous studies, IL-4 has been reported to interfere with IL-2–driven generation of lymphokine-activated killer (LAK) activity. In this investigation, we have demonstrated that IL-4 inhibited the IL-2–induced differentiation of large granular lymphocytes (LGL) into LAK effectors by a mechanism involving, at least in part, an increase in LGL intracellular cAMP levels. In contrast, with its capacity to induce cAMP accumulation in resting LGL, IL-4 had a very negligible effect on LAK activity induction, and cAMP levels increase in LGL that had been preincubated with IL-2. Furthermore, the inhibitory effect of IL-4 on LAK activity generation also correlated with a marked decrease in *N*-CBZ-L-lysine thiobenzylester esterase activity, with an inhibition of tumor necrosis factor (TNF) mRNA expression and TNF production by IL-2–stimulated LGL. These results strongly suggest that complex signaling processes could be ascribed to the dual activities of cytokines and their interplay in LAK promotion. (*J. Clin. Invest.* 1990. 85:1909–1913.) cyclic adenosine monophosphate • cytokine interplay • lymphokine-activated killer

Introduction

Cytokines form a network of regulatory signals and several lines of evidence suggest the existence of a considerable overlap in their activities which leads to unexpected patterns of synergism or antagonism. First reported as a B cell stimulatory factor, interleukin 4 (IL-4) has been found to exhibit a wide range of cell activation properties on various types of immunocompetent cells in a species specific manner (1–4). IL-4 is reported to exert both positive and negative regulatory effects on the generation of non-MHC–restricted cytotoxicity (5–10). It has been demonstrated that addition of recombinant human IL-4 strongly inhibits the interleukin 2 (IL-2)–mediated proliferation and differentiation of human resting peripheral blood lymphocytes (PBL) into lymphokine-activated killer (LAK)[1] effectors (5, 9, 10). However, recent reports suggest that the immunoregulatory properties of IL-4 on non-MHC–restricted cytotoxicity are dependent on the cell activation state (5, 9). While having no effect alone on non-MHC–restricted lytic capacity of resting human PBL (5, 7–10), IL-4 by itself is able to selectively enhance LAK activity of IL-2–prestimulated PBL and large granular lymphocytes (LGL) (9, 10). The precise molecular mechanism underlying the antagonistic effect of IL-2 and IL-4 has not yet been established. Moreover, the signal transduction pathway of human IL-4 is not yet known. The inhibitory properties of cyclic AMP (cAMP) and cAMP-inducing agents for several immune responses (11–14) such as IL-2–induced cell activation have been well documented (15, 16). Although there are several important questions regarding the role of *N*-CBZ-L-lysine thiobenzylester esterase (BLT-E) in cell-mediated cytotoxicity (17, 18), there is a prevailing view that an elevated BLT-E activity seems to correlate with cytotoxic activity (19, 20). We questioned whether or not the IL-4 inhibitory effect on IL-2–driven LGL differentiation and the subsequent acquisition of their lytic potential involves the cAMP pathway. In this report, we demonstrate that heightened cAMP levels resulting from IL-2/IL-4 costimulation of LGL interfere with the transmission of the IL-2 signal leading to LAK promotion by inhibiting the serine esterase increase and TNF mRNA expression in LGL.

Methods

Cell preparation. Fresh human large granular lymphocytes were purified as previously described (21). Briefly, human peripheral blood mononuclear cells were obtained by leukapheresis of normal blood donors (Blood Bank, Hôpital St-Louis, Paris) and were separated on Ficoll/Hypaque. After a 1-h adherence to plastic at 37°C in 5% CO_2, nonadherent cells were loaded over a discontinuous Percoll gradient (Pharmacia Fine Chemicals, Uppsala, Sweden) and centrifuged 30 min at 500 *g*. LGL were recovered from the low-density fraction. The resulting LGL preparations (5% of the initial peripheral blood mononuclear cell population) contained > 90% LGL. Immunofluorescence analysis of CD14 (a monocyte/granulocyte-specific antigen) expression on LGL showed < 0.5% of positive cells, indicating the absence of contaminating monocytes.

LAK activation and cytotoxicity assay. LGL (10^6/ml) were cultured for 3 d in complete medium (RPMI 1640/10% normal human serum) in presence of the indicated lymphokines, dibutyryl-cAMP (Sigma Chemical Co., Strasbourg, France) or forskolin (Sigma Chemical Co.). The cells were then harvested, washed three times, and used as effector cells after serial dilutions in complete medium in round bottom microtiter plate. The natural killer (NK)-resistant B lymphoblastoid cell line Daudi, the myelomonocytic cell line U937, and the epidermoid carcinoma cell line ORL were used as targets. Target cells (2×10^6 cells in 0.2 ml) were labeled with 200 μCi Na2CrO (5 mCi/ml, Commissariat à l'énergie atomique, Saclay, France) for 1 h at 37°C followed by three washes. 0.1 ml of a suspension of ^{51}Cr-labeled target cells (10^5 cells/ml) were dispended into the wells containing dilutions of effector cells. After a 4-h incubation at 37°C, the supernatants were harvested using a harvesting device (Skatron, Lier, Norway). Spontaneous release

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1. *Abbreviations used in this paper:* BLT-E, *N*-CBZ-L-lysine thiobenzylester esterase; LAK, lymphokine-activated killers; LGL, large granular lymphocytes; NK, natural killer; TNF, tumor necrosis factor.

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was determined by incubating target cells with medium alone. Maximum release was determined by adding 100 μl of HCl 1N to 100 μl of the target suspension. Percent of specific lysis was calculated as follows: (experimental ^{51}Cr release [cpm] − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release [cpm] − spontaneous ^{51}Cr release) × 100. Data are reported as the number of lytic units per 10^6 cells as previously described (21). A lytic unit is the reciprocal of the number of effector cells that causes 30% lysis of 10^{-4} ^{51}Cr-labeled target cells.

[^3H]Thymidine incorporation assay. LGL (1 × 10^6 cells/ml) resuspended in RPMI 1640 supplemented with 10% normal human serum were distributed in flat bottomed microtiter plates (Costar Data Packaging Corp., Cambridge, MA) in the presence or the absence of the indicated concentration of lymphokines. After a 72-h incubation at 37°C in 5% CO_2 humidified atmosphere, the culture were pulse-labeled 12 h with 1 μCi of [^{3}H]thymidine (TdR) (TMM 48B, 5 mCi/mmol, CEA) and harvested onto filter paper with a harvesting apparatus (Skatron). Thymidine incorporation was then measured in a scintillation counter (LKB Produkter, Bromma, Sweden) (22). The results are expressed as mean incorporation of triplicate wells.

Measurement of intracellular cAMP. LGL were incubated 10 or 30 min in complete medium in presence of the indicated lymphokines or forskolin, then centrifuged 5 min at 2,000 rpm, resuspended in a lysis buffer (sodium acetate 50 mM) and placed 5 min in liquid nitrogen and 2 min in a boiling water bath. cAMP was measured in cell lysates using a cAMP kit assay (TRK432, Amersham, les Ulis, France) according to the kit procedure. Briefly, samples and standards were incubated 3 h at 4°C in presence of tritiated cAMP before addition of charcoal, which binds cAMP with a high affinity. Charcoal and supernatant were separated by centrifugation 5 min at 2000 *g*. Supernatants containing nonadsorbed cAMP were collected and tritiated cAMP was measured in triplicates in a scintillation counter (LKB Produkter).

BLT-E assay. After 3 d of incubation in complete medium with or without IL-2 and/or IL-4, LGL were lysed by incubating 10^7 cells/ml in PBS containing 0.5% Nonidet P40 (Sigma Chemical Co.) at 4°C for 30 min. Serine esterase activity in 50 μl of cell lysates was determined by measuring the clivage of *N*-CBZ-L-Lys-thiobenzyl ester (BLT, Sigma Chemical Co.) as reported (17). Each assay was performed in triplicate. The absorbance at 420 nm was measured after 2 h at room temperature using a microplate ELISA reader (Titertek Multiscan, MC, Helsinki, Finland). In all cases, the OD of a blank well containing sample but lacking BLT was substracted from that with substrate. An absorbance of 1.0 U of O.D. after 2 h was defined as 1 U of BLT-E activity.

Evaluation of TNF-α mRNA expression. LGL (10^6 cells/ml) were incubated for 6 h in complete medium in the presence of the indicated lymphokines. Cytoplasmic RNA were obtained according to the procedure described (23). Dilutions of denatured RNA of 2 × 10^6 cells were applied with suction to nylon sheet using a 96-well Minifold apparatus. After an 18-h hybridization at 65°C with either an [α-^{32}P]-dCTP (Amersham)-labeled TNF DNA probe or a β-actin DNA probe, nylon sheets were washed 30 min in 2× SSC and 0.2× SSC at 65°C and then exposed to x-ray films at −70°C for 3 d as described (24). TNF-α and β-actin DNA probes were labeled using the Multiprime DNA labeling systems (RPN1601Y, Amersham) according to the kit procedure.

Measurement of immunoreactive TNF-α. LGL (10^6 cells/ml) were incubated for 24 h in complete medium in presence of the indicated lymphokines. Supernatants were then collected and stored frozen at −20°C until the assay. Quantification of immunoreactive TNF-α was performed using an immunoradiometric method (TNF α-IRMA, Medgenix Diagnostics, Fleurus, Belgium) according to the kit procedure. The TNF-α IRMA does not cross-react with TNF-β.

Sources of lymphokines. Highly purified recombinant human IL-2 (specific activity = 23.3 × 10^6 U/mg of protein) used in this study was kindly provided by Sanofi (Bio Recherches, Labege, France). Highly purified recombinant human IL-4 (specific activity = 10^7 U/mg of protein) was kindly provided by Dr. J. Banchereau (Unicet, Dardilly, France).

Results

IL-4 induces an increase of intracellular cAMP in IL-2 activated LGL. Because cAMP has been reported as a negative signal for several immune responses (11–14), we investigated its possible involvement in the inhibitory effect of IL-4 on the IL-2–driven LAK generation. Intracellular cAMP was measured in LGL after a 10- or 30-min stimulation with IL-2 in the presence or absence of IL-4. Results presented in Table I indicate that costimulation of LGL with IL-2 (2 ng/ml) and an inhibitory concentration of IL-4 (100 U/ml) for LAK generation resulted in a marked increase of intracellular cAMP (fourfold) compared with the basal level. Incubation of LGL with the same concentrations of IL-4 alone leads to a less important increase of cAMP levels (2.5-fold) compared with what is observed with the combination of both cytokines whereas IL-2 alone did not significantly affect cAMP level. Our results indicate that cAMP increased to a maximal level

Table I. IL-4 Increases cAMP Levels in LGL

Culture conditions		cAMP level		Lytic units			HTdR uptake
IL-2	IL-4	10 min	30 min	Daudi	U937	ORL	
ng/ml	*U/ml*	*fmol/10^6 cells*					*cpm*
0	0	ND	4,200±110	4	3	<1	2,700
2	0	5,900±450	2,800±140	195	133	35	53,000
0	0.1	5,500±360	5,750±420	4	2	<1	3,000
0	1	8,000±220	7,500±530	3	2	<1	2,900
0	10	8,900±70	7,500±130	4	3	<1	2,100
0	100	9,200±360	11,500±470	9	5	<1	2,500
2	0.1	6,300±75	6,250±230	187	142	ND	ND
2	1	9,500±430	11,500±460	133	115	29	27,000
2	10	14,500±370	17,500±90	65	42	ND	17,000
2	100	15,250±280	19,000±170	6	2	2	12,000
2	0 + FK*	24,000±480	12,000±230	<1	<1	<1	1,500

cAMP levels in purified LGL were measured after 10 and 30 min of incubation in the presence of the indicated lymphokines. In parallel, LGL of the same donor were incubated 3 d in the same conditions and then tested for proliferation and cytotoxic activity. Data shown here are representative of five different experiments. * FK, forskolin (10^{-4}M).

after 10 and 30 min of incubation with both lymphokines then decreased after 60 and 120 min of incubation (data not shown). It is also shown in Table I that the IL-2/IL-4–mediated increase in cAMP was clearly associated with an inhibition of both LGL proliferation and their capacity to differentiate into LAK effectors as revealed by the killing of the NK-resistant targets (Daudi, U937, ORL). When forskolin (10^{-4} M) was added at the initiation of LGL culture, a significant increase of intracellular cAMP occurred. Such an increase also resulted in a marked inhibition of LAK activity and LGL proliferation. To examine whether cAMP increases are directly involved in the IL-4 inhibitory effect, experiments were performed to test cAMP levels in LGL treated with IL-4 after IL-2 prestimulation. Table II depicts the results observed when LGL were simultaneously treated with IL-2 (2 ng/ml) in the presence of IL-4 (100 U/ml) or preincubated 3 d in the presence of IL-2 and then treated with IL-4. It is shown in this table that IL-4 induced an inhibition of LAK activity and an increase of cAMP accumulation within resting LGL. In contrast, addition of IL-4 (100 U/ml) on IL-2–prestimulated LGL failed to inhibit LAK activity generation and did not affect the intracellular levels of cAMP. This indicates a differential effect of IL-4 on the cAMP levels increase by resting and IL-2–prestimulated LGL.

IL-4 inhibits BLT-E activity in LAK effectors. To gain more insight into the inhibitory effect of IL-4 on IL-2–driven LAK generation, we asked whether this lymphokine could affect the serine esterase activity in LGL, such as the BLT-E, suggested to be a marker of NK and CTL lytic capacity (17, 18). We investigated then the effect of IL-4 on the intracellular BLT-E activity in IL-2–activated LGL. Results presented in Fig. 1 indicate that the acquisition of the lytic competence of LAK effectors towards Daudi target was accompanied by a 2.5-fold increase in the intracellular BLT-E activity. Addition of inhibitory concentrations of IL-4 at the initiation of the culture markedly reduced the intracellular content of BLT-E in a dose-dependent manner. This decrease directly correlated

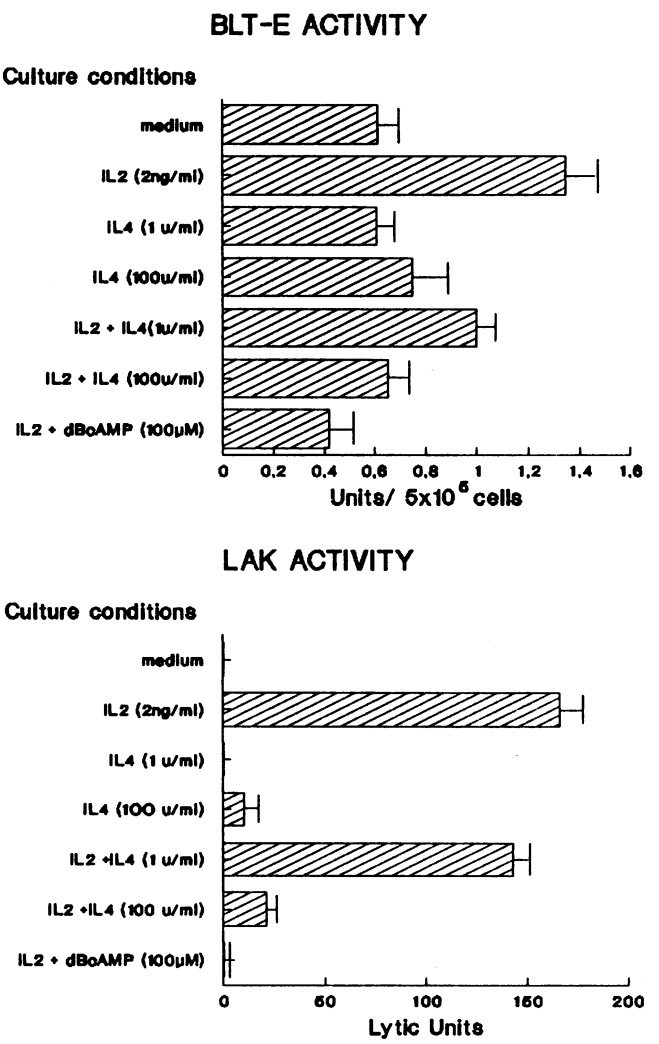

Figure 1. Effect of IL-4 on BLT-E activity in IL-2–activated LGL. (*Top*) Lysate of 5×10^5 LGL stimulated by the indicated lymphokines were tested in triplicates for BLT-E activity as indicated in Methods. (*Bottom*) LAK activity was measured against Daudi target in a 4-h ^{51}Cr-release assay. Bars indicate the mean and SD of a representative experiment out of five.

Table II. cAMP Levels in Resting and IL-2–prestimulated LGL after Stimulation with IL-4

Cells	Culture conditions			cAMP levels		Lytic units
	IL-2	IL-4	FK*	10 min	30 min	
	ng/ml	*U/ml*		*fmol/10^6 cells*		
Resting	0	0	0	NT	2,200±130	2
LGL	0	0	+‡	14,500±110	9,800±230	NT
	2	0	0	3,700±80	1,400±90	84
	0	100	0	3,900±120	5,100±80	8
	2	100	0	5,000±150	7,600±140	16
Preactivated	0	0	0	NT	1,400±25	8
LGL	0	0	+	11,500±240	8,700±180	NT
	2	0	0	1,300±100	1,250±230	192
	0	100	0	1,200±50	1,490±120	48
	2	100	0	1,350±200	1,480±130	166

cAMP levels were measured in resting and IL-2–preactivated (3 d) LGL after a 10- and 30-min stimulation by the indicated cytokines. LGL were incubated 3 d in the same conditions and tested for LAK activity against Daudi cell line. Cytotoxic activity is expressed in lytic units. * FK, forskolin (10^{-4} M). ‡ In the presence of forskolin.

with the level of cytotoxicity measured against Daudi target. Addition of dBcAMP (10^{-4} M) at the initiation of the culture similarly resulted in a significant decrease of BLT-E activity, concomitantly with an inhibition of LAK cytotoxic function of IL-2–activated LGL.

IL-4 inhibits TNF mRNA expression and TNF production by IL-2 activated LGL. IL-2 has been shown to induce TNF mRNA expression in LGL and TNF biological activity release in the culture medium, a phenomenon partially associated with acquisition of LAK activity by LGL (12, 25). To examine whether the inhibitory effect of IL-4 on IL-2–induced LAK generation interferes with TNF release, we have first investigated the effect of IL-4 on TNF mRNA expression in IL-2–activated LGL. Dot blot hybridization using a cDNA TNF probe (Fig. 2) shows that addition of a concentration of IL-4 (100 U/ml) able to inhibit LAK generation strongly reduces TNF mRNA expression in IL-2–stimulated LGL. It is also shown in this figure that addition of dBcAMP (10^{-4} M) also resulted in a marked inhibition of TNF mRNA expression in IL-2–stimu-

lated LGL. Rehybridization of the same filter with a beta actin cDNA probe confirmed that all samples contained similar amounts of RNA (not shown). To further investigate the effect of IL-4 on TNF release by IL-2–activated LGL, we measured TNF in LGL supernatants after 24 h of incubation in the same conditions (Fig. 2). Data presented here show that IL-2 induces the production of detectable concentrations of TNF by LGL (190 pg/ml). In contrast, when a concentration of IL-4 (100 U/ml) able to increase intracellular cAMP was added at the beginning of the LGL culture in the presence of IL-2, an important decrease (88% inhibition) of TNF production was observed (22 pg/ml). Addition of dBcAMP (10^{-4} M) at the initiation of the culture also consistently inhibited (92% inhibition) TNF production (15 pg/ml) by IL-2–stimulated LGL as compared to the control culture.

Discussion

The data presented in this report confirm earlier observations showing that IL-4 inhibits the capacity of human LGL to be activated by IL-2 and to differentiate into LAK effectors (5, 7–9). Although some reports suggested that IL-4 acts at the induction phase of cytolytic activity (8, 9), no conclusive explanation has been proposed to account for this inhibition. In the current study, we attempted to gain more detailed understanding of the mechanism by which IL-4 downregulates the capacity of IL-2 to generate LAK effectors.

Our findings clearly indicate that, when IL-4 is used in costimulation with IL-2, the marked suppression on LAK activity occurs concomitantly with a significant increase in intracellular cAMP levels in LGL. Our results support the hypothesis that cAMP could be involved in signal transduction of human IL-4 and provide additional insights on the inhibitory function of the cAMP pathway on the amplification and effector phase of the immune effector function (11–14). It has recently been shown that IL-4 alone is able to trigger the differentiation of IL-2–prestimulated PBL into LAK effectors. In addition, our results indicate that IL-4 induces a nonsignificant increase of intracellular cAMP in IL-2–prestimulated LGL, that already display LAK activity. These observations provide further support of the association of cAMP elevation with the inhibitory effect of IL-4 on LAK generation by resting LGL and also point to the possible existence of distinct controls of transduction pathways according to the activation state of the cells.

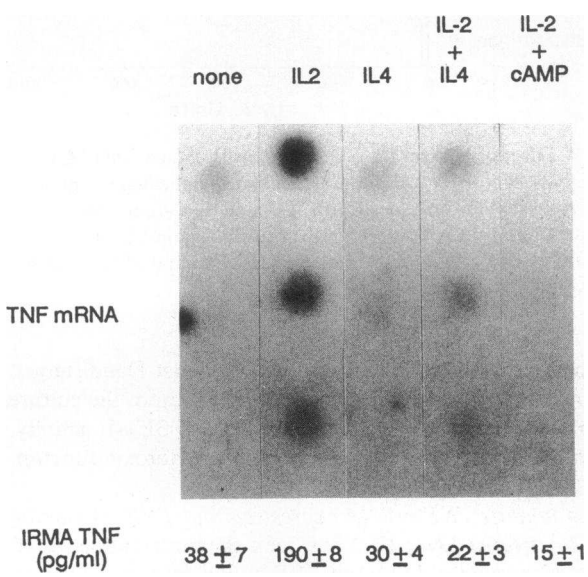

Figure 2. Effect of IL-4 on TNF-α production and TNF-α mRNA expression by IL-2–stimulated LGL. Dots represent serial twofold dilutions of RNA extracted from 2×10^6 LGL after a 6-h incubation with medium, IL-2 (2 ng/ml) alone or in conjunction with IL-4 (100 U/ml), IL-4 (100 U/ml) alone, and IL-2 (2 ng/ml) with dBcAMP (10^{-4} M). Rehybridization was performed with a β-actin probe and showed comparable amounts of RNA in each condition. Blots were exposed to x-ray films for 5 d. In parallel, LGL of the same donor were incubated 24 h in the same conditions. Supernatants were then collected and tested for TNF content by means of an IRM assay as described in Methods. Data presented are representative of three different experiments.

It is well established that the elevation of intracellular cAMP generally causes the activation of a cAMP-dependent protein kinase A (13). This enzyme phosphorylates substrate proteins that may regulate the initiation process including activation of a receptor coupling signal transduction system and gene transcription. It has also been reported that elevation of cAMP levels antagonizes the IL-2–stimulated T cell progression (16). It is conceivable therefore that the suppressive influence of cAMP through a cAMP-dependent protein kinase interferes with some intermediate biochemical events after IL-2 receptor triggering on LGL. For instance, cAMP may affect the protein kinase C activation and phosphorylation of some proteins associated with the development of functional expression by LAK effectors. It has been recently reported that down-modulation of protein kinase C correlates with a loss of LAK activity (26). Furthermore, it has been shown that BSF-1 interaction with its receptor activates a membrane associated protein kinase other than protein kinase C in resting B cells (27). In this respect, the elucidation of the mechanisms by which IL-4 interferes with the adenylate cyclase system and the subsequent protein kinase stimulation could be of considerable importance.

Multiple putative roles have been advanced for serine esterases in cell mediated cytotoxicity (17, 18). Our results indicate that IL-4 interferes with the IL-2–induced BLT-E activity in LGL. A similar inhibition of BLT-E activity also occured when LGL cultures were treated with dBcAMP further suggesting an involvement of this cyclic nucleotide in the IL-4–mediated inhibition of BLT-E activity. It should be stressed, however, that the inhibitory effect of IL-4 on LAK activity was more pronounced than on BLT-E activity. This is consistent with a lack of association between the intensity of LGL cytotoxic function and the BLT-E enzymatic activity and in agreement with the concept that the killing pathway and the BLT-E induction may be under a distinct control. Our findings also indicate that IL-4 interferes with BLT-E activity in LGL but does not affect the basal cellular content of the enzyme in unstimulated LGL indicating that IL-4 mainly affects the IL-2–dependent part of the lytic potential of IL-2–activated LGL. These results are in contrast with a previous report in the murine model (8) indicating that stimulation of PBL with the combination of IL-2 and IL-4 resulted in both marked increase of LAK cytotoxic capacity and BLT-E activity (8).

Our studies also show another site of IL-4 action on LAK generation by its capacity to inhibit TNF gene expression and TNF production by IL-2–stimulated LGL. This confirms previous observations suggesting that LAK development involves, at least in part, TNF production (22, 25). It should be noted

that dBcAMP and other cAMP inducing agents such as forskolin (not shown) also inhibited both TNF mRNA expression and TNF production by IL-2–activated LGL, confirming the involvement of cAMP in the IL-4–induced inhibition of LGL function. Although a recent report has demonstrated that human IL-4 impaired TNF-α gene expression by monocytes (28), the inhibitory effect of IL-4 on the IL-2–induced TNF production under our experimental conditions is not due to contaminating monocytes in LGL preparation since Leu M3-positive cells represented $< 0.5\%$ of LGL preparation (not shown).

In conclusion, our data suggest that IL-4, presumably through the cAMP pathway, is able to affect gene transcription or posttranscriptional events involved in the acquisition of the LAK lytic competence by the LGL. Further studies will be required to clarify this issue. The understanding of IL-4 signaling pathway and of the cytokines induced cross-talk between cellular signaling pathways during LAK differentiation are of considerable importance and may help to more precisely delineate the complex interplay of cytokines in cell-mediated cytotoxicity and their use in combination in antitumor therapy.

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