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Generation of lymphokine-activated killer cells: Synergy between tumor necrosis factor and interleukin 2

(large granular lymphocytes/lymphokine receptors)

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ABSTRACT **Large granular lymphocytes (LGL) can be activated by interleukin 2 (IL-2) to lymphokine-activated killers (LAK). The effect of tumor necrosis factor (TNF) on LAK generation was investigated. TNF was found to act synergistically with low concentrations of IL-2 (0.10–0.25 ng/ml), which were ineffective by themselves in inducing LAK activity, to promote the differentiation of LGL into non-major histocompatibility complex-restricted killers. When IL-2 was used at concentrations optimal for LAK generation, TNF did not further enhance this phenomenon. Specific binding of ^{125}I-labeled TNF to LGL was increased by IL-2 stimulation. Scatchard analysis of TNF binding revealed the existence of two classes of binding sites with markedly different affinities (K_d values of 57 and 600 pM). We also demonstrated that the IL-2/TNF synergistic induction of LAK activity did not involve either IL-1 or interferon-γ. This IL-2/TNF synergistic effect was blocked by anti-Tac antibodies. Immunofluorescence analysis revealed that IL-2/TNF selectively up-regulated Tac antigen expression on LAK precursors. Our results suggest a functional interaction between IL-2 and TNF on LAK precursors, which results in a reduction of the IL-2 concentration required for differentiation of LGL into LAK killers.**

Tumor necrosis factor (TNF) is a cytokine produced by activated mononuclear phagocytes (1, 2). In addition to its direct cytotoxic/cytostatic effects *in vitro* and its antitumoral activity *in vivo* (2, 3), TNF has been shown to possess pleiotropic effects not only on neoplastic cells but also on normal cells. Recently, several studies have shown that TNF has a broad range of biological activities, including modulatory effects on growth (4), proliferation (5), and differentiation (6). Like other cytokines, the action of TNF requires specific binding to cell surface receptors, which are expressed not only on most malignant cells but also on normal cells (7, 8). Lymphokines 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.

The *in vitro* culturing of human peripheral blood lymphocytes with interleukin 2 (IL-2) results in the generation of lymphokine-activated killers (LAK) that are able to lyse a wide range of target cells in a non-major histocompatibility complex-restricted manner. These cells have been shown to be therapeutically effective in eliminating neoplastic cells *in vivo* (9, 10). The LAK precursor cells have been described predominantly as CD16$^+$ large granular lymphocytes (LGL) (11). The LAK activation by IL-2 most probably results from a ligand–receptor interaction. There are at least two subunits of the cellular receptors for IL-2. One is the Tac protein (p55) defined by the anti-Tac monoclonal antibody. Recently, a non-Tac IL-2 binding site (p75) has been reported by several groups, suggesting that the p75 antigen is associated with the p55 Tac to form the high-affinity receptor complex (12–14). Furthermore, it was demonstrated that the p75 receptor subunit but not the Tac subunit is expressed constitutively on freshly isolated LGL (15) and is suspected to explain LAK activation by IL-2.

In the present report, we investigated the possible interaction between IL-2 and TNF on LGL to generate LAK. We demonstrate that, despite their inability to induce LGL into LAK effectors, low doses of IL-2 are efficient for TNF receptor induction on LGL and appear to be sufficient for LAK activity development when used in conjunction with exogenous TNF. We therefore provide evidence for a functional interaction between TNF and IL-2 on LGL to generate LAK.

MATERIALS AND METHODS

Cell Preparations. Human peripheral blood mononuclear cells were obtained by leukapheresis of normal blood donors (Blood Bank, Hospital Saint Louis, Paris) and were separated on Ficoll/Hypaque. After a 1-hr adherence to plastic at 37°C in 5% CO_2, nonadherent cells were loaded over a discontinuous Percoll (Pharmacia Fine Chemicals, Bois d'Arcy, France) gradient consisting of 2-ml layers of 31, 34, 37, 41, 45, and 47% Percoll and were centrifuged 30 min at 1800 rpm (600 × *g*). LGL were recovered from the low-density fraction and were further purified by removal of contaminating T cells with anti-T3 antibody plus complement treatment. The resulting LGL preparations (5% of the initial peripheral blood mononuclear cell preparation) contained >90% LGL.

Source of Lymphokines and Antibodies. Highly purified recombinant IL-2 (rIL-2) (>95%) used in this study was kindly provided by Sanofi (Bio-recherches, Labege, France; specific activity = 23.3 × 10^6 units/mg of protein). Highly purified (>99%) recombinant human TNF (rTNF) (specific activity = 6.63 × 10^6 units/mg of protein) was kindly provided by Knoll (Ludwigshafen, F.R.G.). Recombinant interferon γ (IFN-γ), recombinant interleukin 1α (IL-1α), and IL-1β were supplied by Biogen (Geneva, Switzerland). Rabbit antisera directed against IL-1α and IL-1β were kindly provided by A. Shaw (Biogen). The monoclonal antibody against IFN-γ was kindly provided by M. A. Cousin (Roussel-Uclaf, Romainville, France).

Indirect Immunofluorescence and Flow Cytofluorometric Analysis. Cells were stained by incubation with the monoclonal antibodies at the appropriate dilution followed by the addition of fluorescein-labeled goat F(ab′)$_2$ anti-mouse im-

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Abbreviations: IL-1, -2, interleukin 1 and 2; TNF, tumor necrosis factor; LGL, large granular lymphocytes; LAK, lymphokine-activated killer; IFN-γ, interferon γ; ^{125}I-TNF, ^{125}I-labeled TNF; rIL-2 and rTNF, recombinant IL-2 and TNF.
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munoglobulin as previously described (16). The following antibodies were used in this study: The NKH1 monoclonal antibody, a mouse immunoglobulin (IgM) reactive with LGL (17), was a kind gift from T. Hercend (Institut Gustave Roussy). OKT3 antibody was purchased from Ortho-Diagnostic (Aubervilliers, France). M232, a monoclonal antibody that reacts with the β chain of the LFA-1 molecule, and BW209/2 (anti-CD16), which reacts with the Fc receptor for IgG expressed on natural killer cells, were a kind gift from A. Bernard (Institut Gustave Roussy). Anti-Tac antibody, which reacts with the human IL-2 receptor, was provided by T. Waldmann (National Institutes of Health, Bethesda, MD).

LAK Activation and Cytotoxicity Assay. LGL (10^6 per ml) were cultured at 37°C for 3 days in complete medium (RPMI 1640/10% normal human serum) supplemented with rIL-2. The cells were then harvested, washed three times, and used as effector cells. The ^{51}Cr release cytolysis assay was performed as previously described (18). The natural killer-resistant Burkitt lymphoma-derived B lymphoblastoid cell line, Daudi, was used as the target. Data are reported as either specific lysis or the number of lytic units. The percent specific cytotoxicity was calculated as follows:

$$\frac{\text{experimental cpm released} - \text{spontaneous cpm released}}{\text{maximum cpm released} - \text{spontaneous cpm released}} \times 100.$$

A lytic unit is the reciprocal of the number of effector cells that cause 30% lysis of 10^4 ^{51}Cr-labeled target cells.

Radiolabeling of TNF and Binding Assay. TNF was radiolabeled with ^{125}I by the method of Bolton and Hunter (19). Briefly, 10 μg of rTNF in 100 μl of borate buffer (pH 8.5) was incubated for 3–5 hr on ice with 1 mCi of diiodinated Bolton–Hunter reagent (New England Nuclear). Iodinated TNF was purified by gel filtration on a Sephadex G-25 column. A final recovery of >85% of the input bioactivity was routinely obtained following radiolabeling and gel filtration. The specific radioactivity of ^{125}I-labeled TNF (^{125}I-TNF) preparation ranged from 2.5 to 5 × 10^{17} cpm/mol. Binding assays were performed basically as previously described for IL-2 (20) with minor modifications. Briefly, cells (2 × 10^6 in 50 μl of binding medium) were incubated with increasing concentrations of ^{125}I-TNF (50 μl) for 4 hr at 4°C with occasional shaking. Bound and free radioactivity were separated by centrifugation over a phthalate oil cushion. Radioactivity was measured in a γ counter (Kontron 480, Trappes, France). Nonspecific binding was determined by incubating, in parallel, aliquots with a 100-fold excess of unlabeled TNF. The average number and affinity of binding sites were determined by the graphic analysis of Scatchard.

RESULTS

Effect of TNF on LAK Cell Generation Following LGL Stimulation with Various Doses of IL-2. We first observed (Table 1) that LAK generation was IL-2 dose dependent. Doses ≤0.25 ng/ml were ineffective in inducing a lytic activity, whereas a concentration of 5 ng/ml appeared to be optimal in generating LGL cytotoxicity against natural killer-resistant Daudi cells. TNF in combination with ineffective doses of IL-2 (0.25 ng/ml) promoted a marked induction of LAK cells. Early experiments with graded doses of TNF ranging from 50 to 1000 ng/ml indicated that the maximal synergistic effect was obtained with TNF at 500 ng/ml. This concentration was used in this study. It can also be seen (Table 1) that TNF significantly potentiated LAK generation at suboptimal doses of IL-2 (0.5 and 1.25 ng/ml) but had no effect at the optimal dose (5 ng/ml). When added alone to LGL cultures, TNF failed to support the development of LAK cells at all doses tested. The above results indicate that optimal LAK generation can be obtained in the presence of

Table 1. Synergistic effect of TNF with low doses of IL-2 on LAK generation

		Cytotoxic activity, lytic units per 10^6 effector cells					
	TNF,	IL-2, ng/ml					
Exp.	ng/ml	0	0.25	0.5	1.25	2.5	5
1	0	2	2	8	27	79	102
	250	3	36	42	66	92	106
	500	3	38	46	69	89	101
2	0	3	3	9	24	52	63
	250	2	22	33	43	59	62
	500	3	29	37	38	58	65
3	0	2	3	6	21	63	71
	250	3	18	24	31	68	74
	500	2	24	27	34	65	76

LGL were cultured with the indicated concentrations of rIL-2 and rTNF for 3 days and then were assayed for LAK-mediated cytotoxicity against Daudi cells in a 4-hr ^{51}Cr release assay.

otherwise ineffective doses of IL-2 when TNF is added to the culture.

TNF and IL-2 Act by Means of a Direct Mechanism on LGL for LAK Generation. Because TNF has been shown to induce the production of IL-1 in some cells and because IL-2 has been shown to induce the production of IFN-γ (21, 22), one possible indirect mechanism could be the induction of these two factors following IL-2/TNF treatment of LGL. To rule out this possibility, we examined the possible involvement of endogenously produced IL-1α, IL-1β, and IFN-γ in the process of LAK generation by LGL in response to IL-2/TNF stimulation.

LGL were cultured in the presence of neutralizing doses of rabbit anti-IL-1α or anti-IL-1β antibodies. As shown in Fig. 1, these antisera failed to prevent the LAK cell generation by the combination of IL-2 and TNF. Similarly, no effect was observed when anti-IFN-γ was added in the culture. These results indicate that neither IL-1α, IL-1β, nor IFN-γ secretion can explain the TNF/IL-2-mediated LAK activation.

Induction of TNF Receptor Expression on LGL by Low Doses of IL-2. Since TNF appeared to be active on LAK generation only in the presence of IL-2, we postulated a possible effect of IL-2 on TNF receptor expression. To address this question, purified LGL were incubated for 72 hr in the presence of various concentrations of IL-2 and were tested for their capacity to specifically bind TNF. Data shown in Table 2 indicate that, when incubated for 3 days in culture medium supplemented with IL-2 at a concentration of 0.25 ng/ml (16 pM), a 3- to 8-fold increase in TNF binding to LGL was observed relative to control cultures. TNF binding to purified T cells, under the same culture conditions, remained unaffected. Increasing the IL-2 concentration to 5 ng/ml (300 pM) or greater did not result in a further augmentation of TNF binding to LGL, whereas T cells were able to significantly bind TNF.

A typical curve of ^{125}I-TNF binding to LGL stimulated by IL-2 (0.25 ng/ml) is shown in Fig. 2. TNF binding appears to be dose dependent and saturable. Nonspecific binding never exceeded 5% of the total cpm. The Scatchard plot analysis of the binding data indicates that two classes of sites with markedly different affinities were detectable on IL-2-activated LGL. These cells express an average of 600 binding sites with a K_d of 57 × 10^{-12} M and about 1200 sites displaying an apparent K_d of 605 × 10^{-12} M. Interestingly, purified T cells can express TNF receptors when stimulated with high concentrations of IL-2 (Table 2), but only a single class with a K_d of 110 × 10^{-12} M is detectable (data not shown).

Selective Induction of Tac Antigen Expression on LGL by Low Doses of IL-2 and TNF. LGL were cultured with various

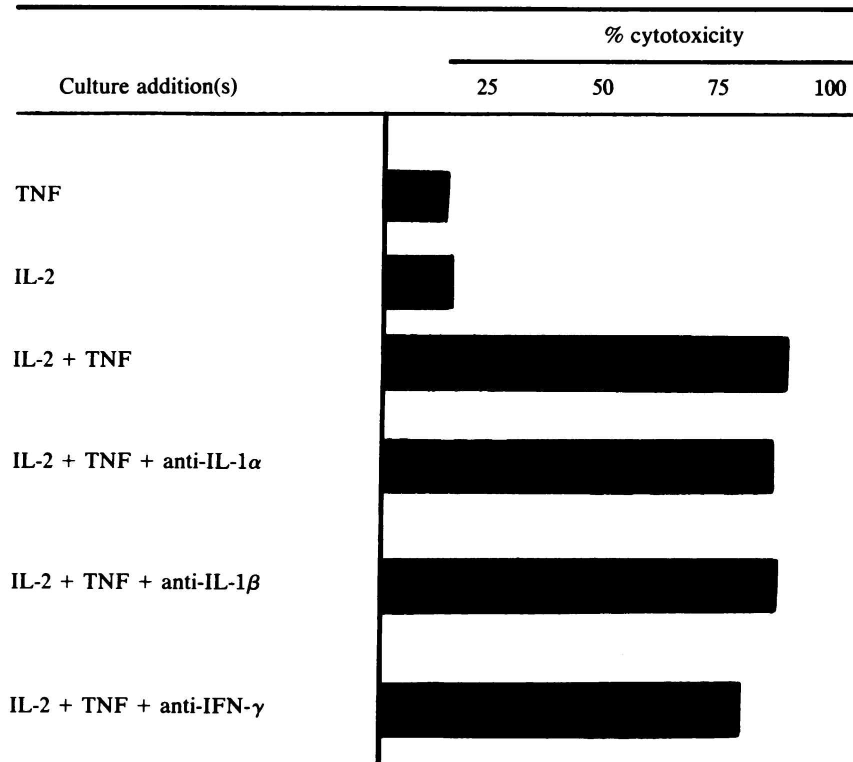

FIG. 1. IL-2 and TNF act by means of a direct mechanism on LGL for LAK generation. LGL were cultured in the presence of rIL-2 (0.25 ng/ml) and rTNF (500 ng/ ml) and where indicated rabbit anti-IL-1α, anti-IL-1β (1:200), or 1000 neutralizing units/ml of monoclonal anti-IFN-γ antibodies (RU 308.7.12). After 3 days of activation, the cells were washed and tested in a ^{51}Cr release assay against Daudi cells.

concentrations of IL-2 in the presence or absence of TNF (500 ng/ml). The cells were washed and analyzed for the expression of NKH1 and CD16, which are selectively expressed on lymphocytes with non-major histocompatibility complex-restricted cytotoxic activity, and for the expression of LFA-1, CD3, and Tac antigens. As previously reported, a small percentage of LGL cells spontaneously expressed the Tac antigen (6%). TNF alone had no apparent effect on the expression of the indicated markers (Table 3). However, after costimulation of LGL with TNF and low doses of IL-2, a significant induction of Tac antigen expression, to the level obtained with optimal doses of IL-2, was observed. The increase in Tac$^+$ LGL after IL-2/TNF costimulation could not be attributed to an expansion of Tac$^+$ contaminating T cells because the stimulation neither reduced the proportion of NKH1$^+$ cells nor increased the proportion of CD3$^+$ cells.

Effect of Anti-Tac Antibodies on IL-2/TNF Synergistic LAK Cell Generation. We next examined if induction of LAK cells upon costimulation with low doses of IL-2 and TNF involves the high-affinity IL-2 receptor (p55 plus p75). Experiments on LGL stimulation by optimal doses of IL-2 or IL-2/TNF were performed for 3 days with anti-Tac antibody or with an isotype-matched control antibody (M18, an anti-human β_2-microglobulin). When LGL were cultured in the presence of a low dose of IL-2 (0.25 ng/ml) and TNF (500 ng/ml) (Fig. 3 *Left*), anti-Tac antibody (25 μg/ml) inhibited LAK activation, whereas the control antibody had no effect (data not shown). In contrast, when stimulation of LGL was provided by optimal doses of IL-2 (5 ng/ml), anti-Tac antibody had no inhibitory effect on LAK activation (Fig. 3 *Right*). These observations suggest that the activation of LGL into effector killers after stimulation with low doses of IL-2 is most likely mediated by the high-affinity IL-2 receptor complex involving the Tac and the p75 subunits.

Table 2. Increased TNF receptor expression on LGL by IL-2

Cells	IL-2, ng/ml	TNF bound, cpm per 2 × 10^6 cells Exp. 1	Exp. 2
LGL	0	761	253
	0.25	1938	2218
	5	1791	1593
T cells	0	764	596
	0.25	825	855
	5	1374	1335

The data shown represent the average of duplicate determinations of ^{125}I-TNF binding at a concentration of 5×10^{-10} M to LGL. The background determined in the presence of a 100-fold excess of unlabeled TNF has been subtracted.

DISCUSSION

The *in vitro* culture of human peripheral lymphocytes with IL-2 results in the development of LAK cells that lyse tumor target cells without major histocompatibility complex restriction. The precursors of IL-2-induced killer cells are predominantly confined to LGL, which are known to play a central role in natural immunity (23). In these studies, we investigated the possible interaction between IL-2 and TNF on highly purified LGL for LAK generation.

Our results indicate that purified rTNF alone is not capable of inducing LAK. In contrast, when LGL were cultured in the presence of IL-2, TNF had a differential effect. TNF exhibited a synergistic inducing effect with low ineffective doses of IL-2 and potentiated the LAK induction with suboptimal concentrations of IL-2. At optimal doses of IL-2, exogenous TNF was not required for LAK generation. Preliminary experiments indicate that measurable levels of endogenously produced TNF are detectable in IL-2-stimu-

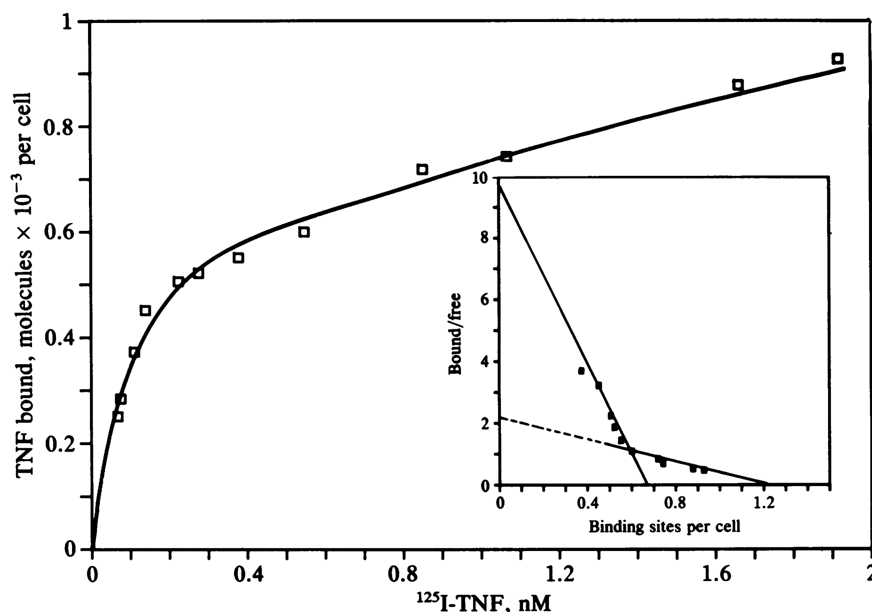

FIG. 2. Binding of ^{125}I-TNF to LGL at day 3 after IL-2 stimulation (0.25 ng/ml). (*Inset*) Scatchard plot of equilibrium binding shown as bound/free ($\times 10^{-12}$) vs. binding sites per cell ($\times 10^{-3}$).

lated (5 ng/ml) LGL cultures, suggesting a role for endogenous TNF in LAK generation.

The following experiments were performed to elucidate the mechanisms of IL-2/TNF synergistic action. We first examined if IL-2/TNF had a direct action on LGL during the LAK induction phenomenon. It is known that LGL can produce IL-1 and IFN-γ when stimulated by TNF and IL-2, respectively (21, 22). Our data clearly show that anti-IFN-γ and anti-IL-1α, as well as anti-IL-1β antibodies, failed to prevent LAK generation when LGL were stimulated by the combination of IL-2/TNF, which suggests a direct effect of TNF and IL-2 on LGL cells. We also examined whether the enhanced LAK function upon stimulation of LGL with IL-2/TNF was due to membrane-associated TNF in the effectors. No inhibitory or stimulatory effect of LAK function was observed following addition of either anti-TNF antibodies or exogenous TNF during the lytic phase (data not shown). In search for an explanation of the synergy between IL-2 and TNF, we investigated if IL-2 induced TNF receptor expression on LGL as previously reported for T cells (24). The experiments indicate that treatment of LGL with low doses of IL-2 significantly increases the expression of TNF receptors on LGL. Although low and optimal concentrations of IL-2 equally increased the number of TNF binding sites on LGL, exogenous TNF was only required for LAK generation when LGL were stimulated with low doses of IL-2. Binding studies with ^{125}I-TNF indicate that two classes of receptors with different affinities are induced. Since LGL are phenotypically heterogeneous cells, it is not clear if two classes of sites are expressed at the surface of each individual cell or if they represent binding sites on a heterogeneous population.

Table 3. TNF and low doses of IL-2 synergize to up-regulate Tac expression on LGL

Addition		% positive cells				
IL-2, ng/ml	TNF, 500 ng/ml	Tac	LFA-1	CD3	NKH1	CD16
0	–	6	92	4	55	77
	+	7	94	6	62	74
0.125	–	12	95	3	57	76
	+	31	97	4	59	83
0.25	–	17	93	4	60	81
	+	39	95	5	61	83
0.50	–	17	96	4	58	85
	+	41	98	5	63	81
2.5	–	36	97	5	59	82
	+	47	97	6	64	86

LGL were cultured for 3 days in medium alone or with the indicated doses of rIL-2 in the presence or absence of rTNF (500 ng/ml) and then were stained for fluorescence-activated cell sorter analysis by using the indicated antibodies.

Immunofluorescence analysis showed that the combination of low doses of IL-2 and TNF induced a marked increase in Tac antigen expression on LGL. Since TNF has been found to up-regulate the expression of several antigens (25, 26), we performed control experiments to examine the effect of IL-2/TNF on LFA-1, NKH1, and CD16 antigens. No increase in the expression of these markers was observed. The synergistic action of IL-2/TNF may contribute to the high-affinity IL-2 receptor formation by inducing Tac expression on LGL.

It has been reported that LGL in the circulating blood express predominantly or exclusively the p75 IL-2 receptor protein. The lack of an effect of anti-Tac antibodies on LAK generation by relatively high doses of IL-2 is in good agreement with the capacity of the p75 peptide to contribute to the initial triggering of LGL activation. However, our results provide evidence for an alternative pathway of LAK generation induced by low doses of IL-2 synergizing with TNF. Under these conditions, the Tac antigen is apparently associated with the initial triggering of LAK generation following IL-2/TNF stimulation. This interpretation is based on the observation that anti-Tac antibodies prevent the induction of LAK when added at the initiation of LGL culture with IL-2 and TNF.

In conclusion, our data are consistent with a model in which low doses of IL-2 bind high- and/or intermediate-affinity IL-2 receptors present on LGL. This interaction would be essential for TNF receptor induction but not sufficient for LAK development. TNF would in turn induce the expression of the Tac antigen on LGL and lead to the subsequent formation of the high-affinity IL-2 receptor complex (Tac plus p75), thus lowering the IL-2 concentration required for LGL differentiation into LAK cells. The description of this pathway for LAK generation may serve as a

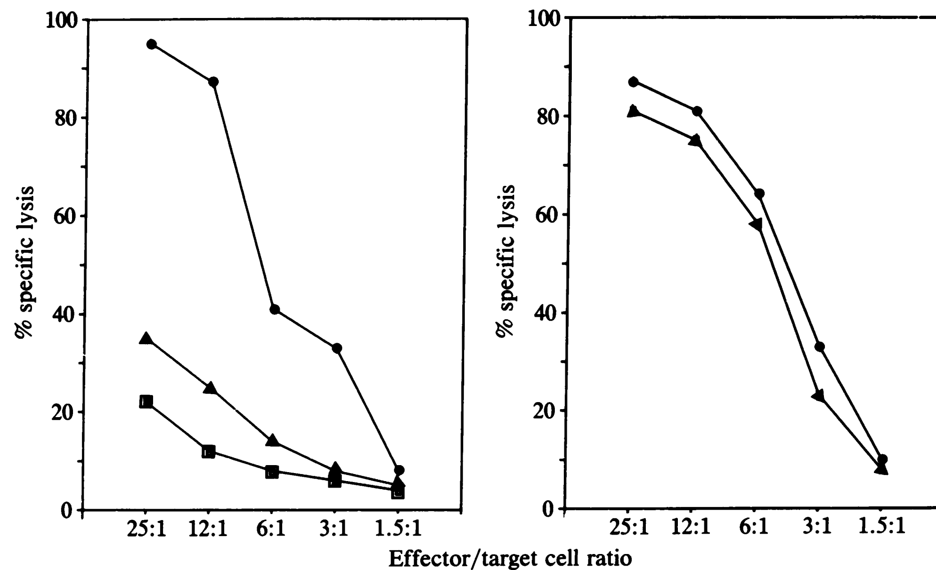

FIG. 3. Effect of anti-Tac antibodies on the development of LAK activity. (*Left*) LGL were cultured with IL-2 at 0.25 ng/ml (□) or with IL-2 at 0.25 ng/ml plus TNF at 500 ng/ml in the absence (●) or presence of anti-Tac antibodies at 25 μg/ml (▲). (*Right*) LGL were stimulated with IL-2 (5 ng/ml) in the absence (●) or presence of anti-Tac antibodies at 25 ng/ml (▲). After 3 days, cultured cells were examined for the cytolytic effect on Daudi target cells.

basis to propose an alternative protocol for LAK immunotherapy. The combination of low doses of IL-2 and TNF may be more efficient than high toxic doses of IL-2.

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