Interleukin-7 Induces Differential Lymphokine-Activated Killer Cell Activity Against Human Melanoma Cells, Keratinocytes, and Endothelial Cells

Dirk Schadendorf, Markus Böhm, Peter Möller, Thomas Grünewald,* and Beate M. Czarnetzki
University Hospital Rudolf Virchow, Department of Dermatology and *II Department of Internal Medicine, FU Berlin, Germany

To assess the potential role of interleukin (IL)-7 in immunotherapy of human malignant melanoma, we have examined the lymphokine-activated killer (LAK) cell sensitivity of four human melanoma cell lines against LAK cells generated by IL-7 or IL-2. Lysis was determined by a 24-h cytotoxicity test using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). All melanoma cell lines were susceptible to IL-7- and IL-2-generated LAK cells. The sensitivity of melanoma cells to IL-2-induced LAK cells was higher compared to IL-7-induced LAK cells. At an effector target ratio of 20:1, the lysis by IL-7-induced LAK cells ranged between 41% and 52%, whereas IL-2-induced lysis ranged between 80% and 94% ($p < 0.01$). IL-7-induced LAK cells, however, showed almost no cytotoxicity towards HaCat keratinocytes and human umbilical vein endothelial cells (HUVECs). Immunophenotyping revealed a higher expression of the tac antigen (CD 25) on IL-7-generated LAK cells, particularly those cells that were CD 56 negative or CD 3 positive compared to IL-2-induced LAK cells. In contrast, IL-2-generated LAK cells killed 62% of the HaCat keratinocytes and 60% of the HUVECs. Secretion of tumor necrosis factor-alpha into culture supernatants was significantly higher in IL-2-generated LAK cells compared to IL-7-stimulated LAK cells ($p < 0.01$), whereas TNF-alpha levels of IL-7-induced LAK cells were in the range of unstimulated lymphocytes. Because nonspecific cytotoxicity against other normal cells such as keratinocytes and endothelial cells contributes to the dose-limiting side effects of immunotherapy with IL-2, immunotherapy using IL-7 might be a better tolerated future alternative. *Key words: LAK cells/cytotoxicity/TNF-α/IL-2. J Invest Dermatol 102:838–842, 1994*

Immunotherapy with interleukin (IL)-2, or combinations of IL-2 and lymphokine-activated killer (LAK) cells, has been particularly successful in certain tumors such as malignant melanoma and renal cell carcinoma [1–3]. IL-2 treatment has, however, marked limitations because of severe clinical toxicity including fluid retention, pulmonary edema, hypotension, and anuria. These adverse effects are thought to be related to damage of the vascular endothelium, known as vascular leakage syndrome [4–7].

IL-7 is a stroma-derived cytokine that has a number of biologic effects on lymphocytes. IL-7 was originally identified because of its ability to support the growth of pre-B cells in Whitlock-Witte bone marrow cultures [8]. In addition to its growth-promoting effect on pre-B cells, IL-7 has been shown to play an important role in the regulation of human T-lymphocyte development [9–12]. Recently, IL-7 has been shown to enhance the generation of cytotoxic T lymphocytes and LAK cells [13–15]. These studies were conducted predominantly with the natural killer (NK)-sensitive myeloid leukemia cell line K562 and the NK-resistant lymphoma cell line Daudi [16,17]. Little is known about the susceptibility of human melanoma cells to LAK cells generated by IL-7. We here report on the sensitivity of four phenotypically different human melanoma cell lines as well as HaCat keratinocytes and human endothelial cells to LAK cells induced by IL-7, in comparison to IL-2.

Manuscript received January 5, 1993; accepted for publication January 20, 1994.

This work was presented in part at the meeting "Molecular Pharmacology of the Skin," February 7–9, 1992 in Berlin, at the Annual Meeting of the Society for Investigative Dermatology, April 29–May 2, 1992 in Baltimore, Maryland, and at the Annual Meeting of "Arbeitsgemeinschaft Dermatologische Forschung," November 13–15, 1992 in Mainz, Germany.

Reprint requests to: Dr. Dirk Schadendorf, University Hospital Rudolf Virchow, Department of Dermatology, FU Berlin, Augustenburger Platz 1, D-13344 Berlin, Germany.

Abbreviations: E:T, effector:target; LAK, lymphokine-activated killer; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PBL, peripheral blood lymphocytes.

MATERIALS AND METHODS

Cytokines Human recombinant IL-7 with a specific activity of 4.7×10^7 U/mg and 3.9 endotoxin-units (EU) per ng protein was provided by Immunex (Seattle, WA). The specific activity was determined with a bioassay that employs the IL-7-dependent IxN/Lb pre-B-cell line [8]. Human recombinant IL-2 with a specific activity of 2×10^6 U/mg and < 100 EU/mg was purchased from Boehringer, Mannheim, Germany.

Cell Culture Human melanoma cells were cultured as previously described [18]. The permanent melanoma cell lines SK-MEL-23, SK-MEL-37, SK-MEL-186, and MeWo were established several years ago at the Memorial Sloan Kettering Cancer Center from metastatic lesions of patients with malignant melanoma and have been characterized extensively regarding their differentiation markers [19]. Melanoma cell lines MeWo and SK-MEL-23 express melanosomal antigens and are negative for HLA-DR and epidermal growth factor (EGF)-receptor expression, whereas SK-MEL-37 and SK-MEL-186 exhibit the inverse phenotype. HaCat cells, kindly provided by Dr. N. Fusenig, Heidelberg, Germany, represent a permanent cell line that resembles normal keratinocytes in most features. The cells were maintained as described [20]. Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA) and maintained as recommended by the distributor.

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LAK Cell Generation Peripheral blood mononuclear cells (PBMCs), obtained from 10 healthy donors by Ficoll-Hypaque centrifugation of venous blood, were used as effector cells. The adherent cells (monocytes) were removed by incubating the PBMCs in plastic plates for 1 h at 37°C. The non-adherent cells (PBLs) were decanted and used for the generation of LAK cells, as previously described [21]. In short, PBLs were incubated at a density of 2×10^6 cells/ml in RPMI-1640 (Gibco, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS; Seromed, Berlin, Germany), 1% penicillin/streptomycin (Seromed), and 10% glutamine (Gibco) in the presence of IL-7 (10–1000 U/ml) or IL-2 (100 U/ml). One thousand units IL-7 represented 0.1 μg and 100 U IL-2 represented 0.033 μg of each agent, respectively. The PBLs were incubated in 25-cm^2 flasks (Greiner, Frickenhausen, Germany) in an upright position for 4 d in 5% CO_2 at 37°C. On day 4, LAK cell concentration was determined by the counting of cells; excluding 0.18% trypan blue dye.

Cytoxicity Assay LAK activity against melanoma cells, HaCat keratinocytes, and HUVECs was measured by a 24-h tetrazolium-based colorimetric cytotoxicity assay using 3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), as described by Mosman and Fong [22,23]. The principle of the test is based on the selective reduction of the tetrazolium salt, MTT, by mitochondrial dehydrogenases. The yellow form of the salt turns purple intracellularly. Solubilized formazan crystals can then be quantitated spectrophotometrically, and absorbance is proportional to the number of viable cells. Modified as a cytoxicity assay for the quantification of antitumor effects mediated by human LAK cells on tumor cell targets, this assay has been found to compare favorably with the ^{51}Cr release in terms of sensitivity and reproducibility [24]. The test was performed as follows. Melanoma cells, HaCat keratinocytes, or HUVECs were brought to single-cell suspension by incubation with 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Seromed). Cell concentration and viability were determined by hemocytometer counts of cells, excluding 0.18% trypan blue dye. Cells were plated at a density of 1×10^4 cells/well in 96-well flat-bottomed tissue culture microtiter plates (Greiner) 20 h prior to the addition of effector cells. LAK cells were added at different effector : target (E:T) ratios ranging from 20 : 1 to 2.5 : 1. Quintuplicates were used for each E:T ratio. After incubation for 24 h, non-adherent cells were removed by performing three consecutive washes with unsupplemented medium. Recultivation of detached target cells was performed to reassure that target cells were damaged lethally. Fifty microliters MTT (Sigma, Deisenhofen, Germany) solution at a concentration of 5 mg/ml, followed by 150 μl of complete medium, were added to each well. The plates were incubated in a humidified atmosphere in 5% CO_2 at 37°C. After 4 h, MTT solution was aspirated completely and formazan crystals formed in viable cells were solubilized by addition of 150 μl dimethylsulfoxide (DMSO) (Merk, Darmstadt, Germany) to each well and by repetitive aspiration. Absorbance was read within 1 h at a wavelength of 540 nm on a scanning multiwell spectrophotometer (Titertek Multiscan MCC/340, Meckenheim, Germany). The percentage of cell deaths (lysis) was determined as

$$\frac{A-(B-C)}{A} \times 100 = \text{ \% specific cell lysis,}$$

where A is defined as the mean optical density (A_{540}) of 1×10^4 target cells without addition of effector cells, B is the mean experimental absorbance A_{540} of adherent tumor cells remaining in the wells after washing, and C as the absorbance A_{540} of adherent effector cells remaining in the wells after washing. To determine nonspecific A_{540} activity due to adherent LAK cells, different numbers of LAK cells were plated into the wells of 96-well microtiter plates without any target cells. Intratest standard deviation was below 10%.

Tumor Necrosis Factor (TNF)-Alpha Secretion of LAK Cells To determine TNF-alpha levels secreted by LAK cells and unstimulated lymphocytes, peripheral blood lymphocytes (PBLs) (10^6 cells/ml) of seven patients were incubated as described for LAK cell generation. After 4 d of incubation, aliquots were removed and tested for TNF-alpha secretion by enzyme-linked immunosorbent assay (ELISA) (Quantikine, RD Systems, Minneapolis, MN), which had a detection range between 10 and 7500 pg/ml. All tests were performed in quadruplicate and standard deviation was below 10%.

Flow Cytometry After 4 d of culture, lymphocytes were adjusted to the concentration of 0.5×10^6 cells/ml in phosphate-buffered saline (PBS) and directly stained with the following fluorescein- or phycoerythrin-labeled monoclonal antibodies: anti-Leu 4 (CD3), Leu 2a (CD8), Leu 19 (CD56), Leu 12 (CD12), and tac (CD25). All monoclonal antibodies were purchased from Becton Dickinson, Heidelberg, Germany. Two-color flow cytometry analysis was performed on a FACScan (Becton Dickinson).

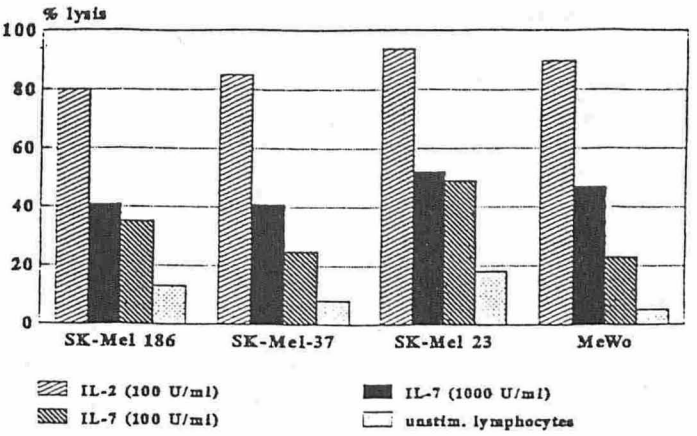

Figure 1. Specific lysis of four different human melanoma cell lines by IL-7– and IL-2–induced LAK cells and unstimulated lymphocytes at an E:T ratio of 20:1. Data are expressed as percent mean lysis of lymphocytes from 10 healthy donors by the MTT test. All experiments were performed in quintuplicate and have been repeated at least twice. Standard deviation of the calculated mean percent cytotoxicity for all donors was below 10%.

Statistical Analysis Statistical significance of the data was calculated using the modified Wilcoxon signed-rank test [25]. The procedure is to convert the observations to rank order and then calculate the t statistic by using ranks instead of the original observations.

RESULTS

Sensitivity of Human Melanoma Cells to IL-7–Induced LAK Cells from Healthy Donors IL-7–induced LAK cells of 10 healthy donors lysed all four allogeneic human melanoma cell lines tested (Fig 1). Cytotoxicity generated by IL-7 was dose dependent, reaching a plateau at an IL-7 concentration of 1000 U/ml. No additional melanoma cell lysis was seen at higher IL-7 concentrations (data not shown).

LAK cell activity was also proportional to the E:T ratio, with maximal lysis at an effector-target ratio of 20:1 (Fig 2). The mean cytotoxicity of IL-7–induced LAK cells varied from 41% (SK-MEL-37 and SK-MEL-186) to 47% (MeWo) and 52% (SK-MEL-23).

Comparison of IL-2– and IL-7–Induced LAK Cells LAK cell activity generated by IL-2 was higher against all melanoma cell lines tested compared to IL-7 stimulation ($p < 0.01$) (Fig 1). No statistically significant differences were observed between the susceptibility of the more differentiated melanoma cell lines SK-Mel 23 and MeWo and the less differentiated melanoma cell lines SK-Mel 186 and SK-Mel 37 cells regarding both IL-7– and IL-2–induced LAK cells.

Flow Cytometry Analysis of Effector Cell Population The patterns of various cell-surface markers of the LAK cells were studied using flow cytometry analysis (Table I). The most striking difference between IL-7– and IL-2–induced LAK cells was the higher percentage of IL-7–induced CD25-positive (tac antigen, low-affinity receptor of IL-2) LAK-cells. CD 25 was expressed on 35% of the CD3-positive cells, but on only 3% of the CD3-negative cells. Furthermore, 38% of the CD56-negative cells were CD25 positive whereas only 2% of CD56-positive cells were CD25 positive. These results are consistent with the possibility that IL-7–induced upregulation of CD25 occurs principally on CD3+CD56− cells; however, three-color flow cytometric analysis would be necessary to validate this possibility more conclusively (Table I) (Figs 3, 4).

LAK Cell Activity Against HaCat Keratinocytes To evaluate possible differences between IL-7– and IL-2–induced effector cells on keratinocytes, PBLs from seven healthy donors were stimulated with 100 U/ml IL-2 or 1000 U/ml IL-7 and then co-incubated

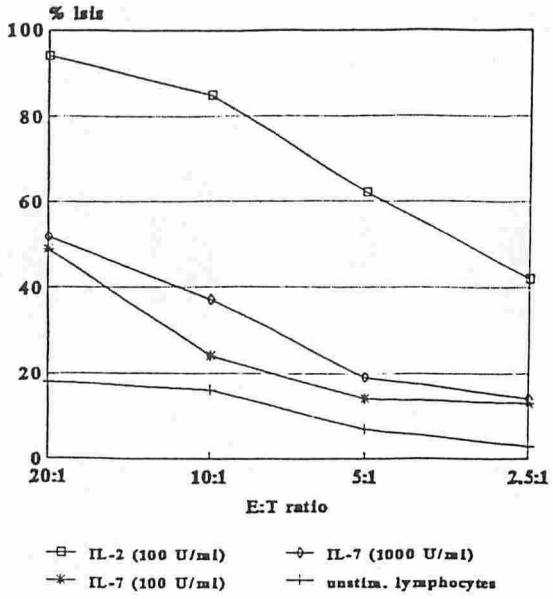

Figure 2. Specific lysis of SK-Mel 23 by IL-7- and IL-2-induced LAK cells and unstimulated lymphocytes at different E:T ratios. Percent mean lysis is shown for one representative donor determined by MTT test. Comparable results were obtained for SK-Mel 186, SK-Mel 37, and MeWo using the same lymphocytes. All experiments were performed in quintuplicate and have been repeated at least twice. Standard deviation was below 10% in all experiments.

with HaCat cells (Fig 5). At the highest E:T ratio of 20:1, IL-7-induced LAK activity was only 30% (range, 11-42%) and comparable to the cytotoxicity mediated by unstimulated PBLs (25%, range 9-36%; p = NS). In contrast, non-specific lysis of HaCat keratinocytes by LAK cells generated by IL-2 at the same E:T ratio was 62% (range 30-91%; $p < 0.01$).

LAK Cell Activity Against HUVECs To test the susceptibility of human endothelial cells to LAK cell-mediated cytotoxicity, PBLs of three donors were stimulated with 100 U/ml IL-2 or 1000 U/ml IL-7. At an E:T ratio of 10:1, the mean LAK cell activity of unstimulated lymphocytes against HUVECs was 8% (range, 0-17%). IL-7-generated LAK cell cytotoxicity was within the same range (0-14%; mean, 7%). In contrast, IL-2-induced LAK cells were highly effective in killing HUVECs (mean, 60%; range, 45-65%).

TNF-Alpha Secretion by Lymphokine-Activated PBLs To address one possible mechanism involved in the LAK activity, secretion of TNF-alpha into the culture supernatants of PBLs of seven donors was determined by enzyme-linked immunosorbent assay (ELISA). Aliquots of the same PBL preparation were tested without and after stimulation with IL-2 and IL-7. As shown in Table II, TNF-alpha levels secreted from IL-7-exposed lymphocytes were mostly in the same range of unstimulated lymphocytes, with one exception (number 2), whereas IL-2-stimulated LAK cells secreted more TNF-alpha compared to unstimulated and IL-7-generated LAK cells in all cases ($p < 0.01$). Using the Wilcoxon rank test for unpaired parameters, we statistically analyzed the data for TNF-α secretion upon stimulation individually for each patient. There was no statistical difference in TNF levels between PBLs and IL-7-stimulated LAK cells. However, comparison of TNF levels secreted upon IL-2 stimulation differed significantly from levels of unstimulated PBLs ($p < 0.01$) and IL-7-generated LAK cells ($p < 0.01$).

Table I. Characterization of IL-7- and IL-2-Induced LAK Cells from Eight Healthy Donors by Flow Cytometry Analysis[a]

Phenotype	Unstimulated	IL-2 (100 U/ml)	IL-7 (1000 U/ml)
CD3+	76% (66-86%)	82% (70-91%)	77% (63%-87%)
CD8+	41% (34-50%)	39% (22-50%)	45% (39-49%)
CD56+	15% (7-25%)	18% (8-22%)	16% (8-24%)
CD25+	13% (3-18%)	16% (12-24%)	41% (26-52%)
CD19+	3% (3-5%)	5% (4-8%)	5% (3-8%)
CD56+CD3+	2% (1-12%)	3% (1-7%)	2% (1-10%)
CD56+CD3-	13% (6-26%)	18% (12-20%)	17% (7-26%)
CD56+CD25+	1% (0-2%)	2% (0-2%)	2% (1-4%)
CD56-CD25+	12% (7-14%)	16% (12-25%)	38% (6-51%)
CD3+CD25+	8% (3-12%)	11% (9-15%)	35% (23-46%)
CD3-CD25+	2% (1-6%)	3% (1-8%)	3% (2-9%)
CD8+CD25+	1% (1-4%)	3% (1-6%)	9% (6-15%)

[a] Mean percent values are given, as are ranges (in parentheses).

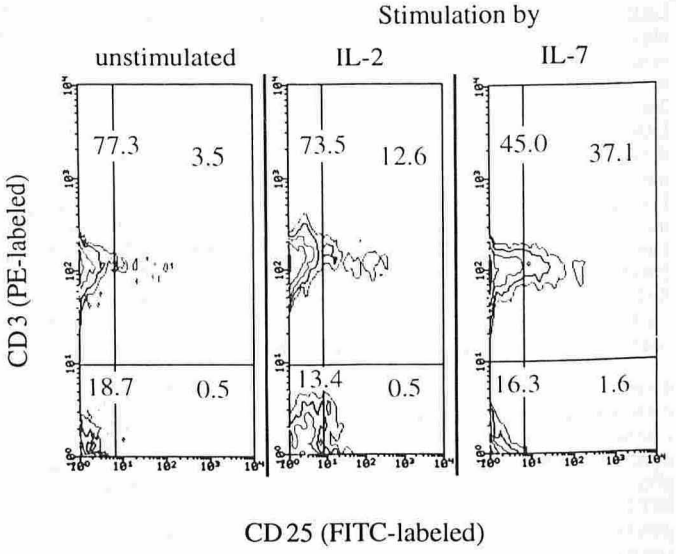

Figure 3. Representative flow cytometry analysis of CD56 and CD25 antigen expression of LAK cells stimulated with IL-7 (1000 U/ml) or IL-2 (100 U/ml) for 4 d. Unstimulated lymphocytes were used as controls. The percentage of gated cells is given for each quadrant.

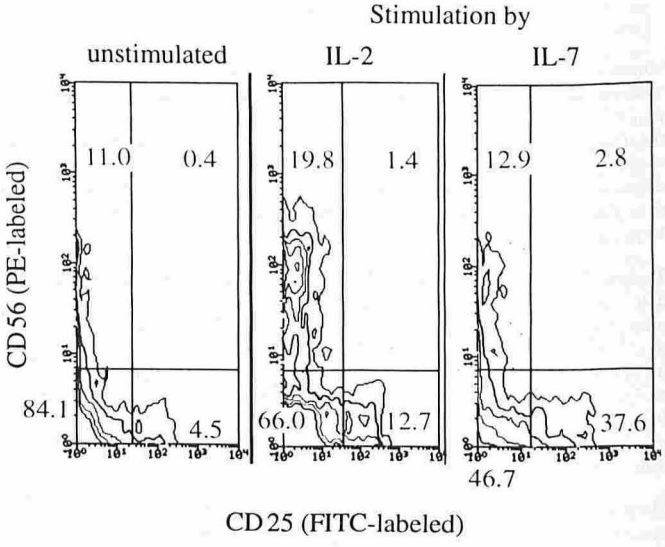

Figure 4. Representative flow cytometry analysis of CD3 and CD25 antigen expression of LAK cells stimulated with IL-7 (1000 U/ml) or IL-2 (100 U/ml) for 4 d. Unstimulated lymphocytes are shown for control. The percentage of gated cells is given for each quadrant.

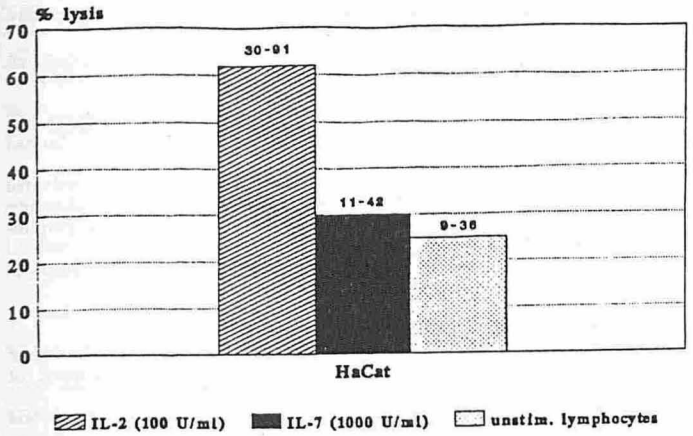

Figure 5. Percent mean lysis of HaCat keratinocytes by IL-7– and IL-2–induced LAK cells and by unstimulated lymphocytes as assessed by MTT test. LAK cells were generated by incubating PBLs from seven healthy donors with IL-7 (1000 U/ml) or IL-2 (100 U/ml) for 4 d. Ranges are given at the *top* of each bar graph. All experiments were performed in quintuplicate and have been repeated at least twice. Standard deviation of the calculated mean percent cytotoxicity for all donors was below 10%.

DISCUSSION

After autologous melanoma cells were found *in vitro* to be highly susceptible to LAK cells generated by IL-2, this cytokine gained clinical acceptance as an agent in immunotherapy of patients with advanced melanoma [1,3,26,27]. Severe toxicity, however, is a major obstacle to immunotherapy with IL-2. Recently, it has been demonstrated that IL-7 can induce LAK cell activity that is largely independent of IL-2 secretion [17]. In the present study, we have shown that IL-7–induced LAK cells lyse human melanoma cells in a dose-dependent manner. However, at the same E:T ratio, this LAK activity is only half as high as that of IL-2–induced LAK cells. This is in accordance with data from others who described a 5–10 times lower LAK cell activity of IL-7–induced LAK cells compared to IL-2–induced LAK cells when Daudi cells were used as target cells and cytotoxicity was measured with the ^{51}Cr release assay [16]. No major differences in the susceptibility of phenotypically different melanoma cell lines to LAK cells induced by IL-7 or IL-2 were obvious.

Natural killer (NK) cells are suggested to be the predominant precursor cell population of LAK cells generated by either IL-2 or IL-7 [17]. The phenotypic characterization of surface markers of LAK cells generated by IL-7 or IL-2 revealed differences in the expression of cell-surface molecules (Table I), particularly of CD25 (Tac antigen, low-affinity form of the IL-2 receptor), which was expressed more markedly on LAK cells stimulated by IL-7 expressing the phenotypic characteristics CD56– or CD3+. As no three-color fluorescence-activated cell sorter (FACS) analysis has been performed, the conclusion that CD25 upregulation occurred on LAK cells with a CD56– CD3+ phenotype remains indirect. Increased expression of CD25 on LAK cells upon stimulation with IL-7 was observed previously by others to be even more pronounced than after IL-2 stimulation [16,17,33], in line with a previously reported increased upregulation of CD25 by IL-7 on PBMCs and T cells [9,12]. Because the expression of the high-affinity IL-2 receptor has been shown to be involved in the proliferation of NK cells [32], it was suggested that the upregulation of the IL-2 receptor by IL-7 would make susceptible cells more sensitive to IL-2 [17]. Although IL-2–induced LAK activity is commonly associated with a CD56+ CD3– phenotype, it is clear that at least some of the large granular lymphocytes exhibiting CD3 expression also have the potential for mediating cytotoxic actions [34]. It remains to be clarified whether and how the increase in the CD25+/CD3+ but CD56– subpopulation in IL-7–generated LAK cell cultures contributes directly or indirectly to the total LAK cell activity generated by IL-7.

Table II. Secretion of TNF-Alpha (in pg/ml) by PBLs upon Stimulation with 100 IU/ml IL-2 and 1000 IU/ml IL-7 in Seven Normal Donors, Determined in Quadruplicate by ELISA[a]

Donor	Unstimulated PBL	IL-2	IL-7
1	392	475	320
2	50	1750	1055
3	15	70	15
4	150	172	138
5	155	785	205
6	85	176	90
7	100	340	220

[a] Standard deviation was below 10%.

Recently, it has been shown that IL-2–induced LAK cells possess an indiscriminative lytic potential that does not seem to be specific for tumor targets [29–31]. Numerous significant side effects have been reported during high-dose immunotherapy with IL-2 and are thought to be related in part to the cytotoxic effects of IL-2–induced LAK cells towards normal cells, including endothelial cells and keratinocytes. The present comparative studies of IL-7– and IL-2–induced LAK cells demonstrate that IL-7–induced LAK cells are far less cytotoxic against normal HaCat keratinocytes and human endothelial cells than are IL-2–induced LAK cells. LAK cell activity against HaCat keratinocytes and HUVECs was in the range of unstimulated PBLs, in contrast to a lysis of more than 60% mediated by IL-2–induced LAK cells. Whether this interesting finding reflects the overall weaker cytotoxicity of IL-7–induced LAK cells compared to IL-2–induced LAK cells or a more discriminative tumor-cell–killing behavior of LAK cells generated by IL-7 needs further elucidation.

Because TNF-alpha is incriminated as one of the cytokines induced secondarily during immunotherapy with IL-2 and because it appears to be responsible in part for the vascular leakage syndrome, we determined the TNF-alpha secretion from PBLs with and without stimulation. Although one of seven patients analyzed showed a substantial TNF response to IL-7 — albeit weaker compared to IL-2 — TNF-alpha levels of IL-7–induced LAK cells were in most cases in the range of unstimulated lymphocytes, whereas they were significantly increased after IL-2 stimulation. This is in accordance with the observation that immunomagnetically purified CD56+ lymphocytes secrete 50 times less TNF-alpha after stimulation with IL-7 compared to IL-2, although they are almost as cytotoxic as IL-2–induced LAK cells [33]. IL-7 is thus likely to have a higher therapeutic index than IL-2 in immunotherapy of malignant melanoma. The present findings therefore warrant further studies with IL-7, including *in vitro* experiments with autologous melanoma cells and preclinical pilot studies in patients with advanced malignant melanoma.

This work was supported by the DFG (Scha 422/2-1). The authors are grateful to L. J. Old (Ludwig Institute for Cancer Research, New York) for providing the human melanoma cell lines and monoclonal antibodies.

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