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Effects of tumour necrosis factor-alpha (TNF-α), IL-1β and monocytes on lymphokine-activated killer (LAK) induction from natural killer (NK) cells and T lymphocytes

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SUMMARY

Roles of monocytes and cytokines were investigated on LAK induction from T and NK cells. Monocytes augmented more T-LAK induction than did NK-LAK. Expression of IL-1β, TNF-α and interferon-gamma (IFN-γ)-mRNA and their cytokine production were superior in NK cells compared with T cells in parallel with their LAK activities. An increase of TNF-α, IL-1β and IFN-γ production was induced by co-culturing NK or T cells with autologous monocytes. The augmentation of T cell cytokine production and T-LAK activity by monocytes was more prominent than that of NK cells. TNF-α and IL-1β were generated 24 h after IL-2 stimulation, and these cytokines were able to almost substitute for monocytes in LAK induction. Conversely, LAK induction was almost completely suppressed by both anti-IL-1β and anti-TNF-α antibodies, if they were added within 24 h after the start of the LAK induction. IFN-γ, which was produced at a later stage, scarcely affected LAK induction in spite of the cooperation with TNF-α. The results obtained indicate conclusively that the superiority of NK-LAK depends on their superior productivity of both IL-1β and TNF-α, and that the up-regulation of LAK induction by monocytes is largely due to the enhanced generation of both cytokines.

Keywords LAK induction T and NK cells monocytes IL-1β TNF-α

INTRODUCTION

Development of recombinant DNA technology has facilitated cytokine mass-production and the wide clinical application of cytokines on malignant tumours [1–6]. Among these cytokines, IL-2 is most frequently utilized for cytotoxic lymphocyte induction. Many trials of LAK transfusion and *in vivo* LAK induction have been applied [1,2]. From multiple basic and clinical studies on LAK, regulation of LAK induction by monocytes and macrophages has largely been clarified [7–11].

Action of monocytes in LAK induction is not yet fully understood, with some contradictory investigations. Monocytes and macrophages regulate LAK in both positive and negative manners, e.g. peritoneal macrophages up-regulate LAK activity, but alveolar macrophages down-regulate cytotoxicity of IL-2-induced LAK cells [9,10]. Monocytes can be activated not only by lipopolysaccharide or mitogens, but also by many cytokines such as IL-1, TNF, interferon-gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which are generated by monocytes, lymphocytes and many other cells in an autocrine-paracrine fashion [11,12]. When cooperative cytokine production takes place between monocytes and lymphocytes, more cytotoxic killers may be induced. Among various cytokines, IL-1β, TNF-α and IFN-γ have been recognized as factors with IL-2 in LAK induction [13–17]. Further, it is well known that activated NK cells are able to produce large amounts of these cytokines [18]. However, little is known about the role of monocytes in the cytokine generation of LAK cells.

LAK cells can be induced from many different precursors, and the manner of LAK induction seems to differ depending on the precursors. It has been reported that neither monocytes nor human serum are necessary for inducing sufficient cytotoxic LAK from NK cells, while both are requisite for T cells [19]. It is therefore necessary to examine the accessory cell actions on subpopulated lymphocytes in LAK induction. In the present study, we investigated influences of monocytes on LAK induction from T and NK cells. Our results indicate that LAK induction from T cells was more stimulated than that from NK cells by monocytes, corresponding with enhanced cytokine production, and that TNF-α is the strongest LAK enhancer, followed by IL-1β, while IFN-γ only slightly enhances LAK induction.

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MATERIALS AND METHODS

Reagents

Human recombinant (r)IL-2 and rIFN-γ were supplied by Shionogi Pharmaceutical Co. (Osaka, Japan). The units of rIL-2 were determined by ^{3}H-thymidine incorporation using CTLL-2 cell line according to the method of Gillis *et al.* [20], and were expressed in accordance with the titre of Jurkat IL-2 standard received from Biological Response Modifiers Programme (NCI). The rIL-2 preparation had a specific activity of $1{\cdot}0 \times 10^7$ U/mg of protein, and rIFN-γ, which was standardized by the NIH reference unit, had a specific activity of $5{\cdot}0 \times 10^6$ U/mg of protein. rIL-1β and rTNF-α, having specific activity of 1×10^7 and 1×10^8 U/mg, respectively, were obtained from Boehringer Mannheim (Mannheim, Germany). MoAbs such as anti-IFN-γ (Genzyme Co., Boston, MA), anti-IL-1β (Collaborative Research Inc., Bedford, MA) and anti-TNF-α (Hayashibara Biochemical Labs Inc., Okayama, Japan) were used after appropriate dilution.

IL-1β cDNA probe used was purchased from Oncogene Science (Manhasset, NY). IFN-γ probe (developed by Dr T. Taniguchi *et al.*) was kindly provided by Japanese Cancer Research Resources Bank (Tokyo, Japan). TNF-α probe was a generous gift from Dr T. Nishida (Otsuka Pharmaceutical Co., Tokushima, Japan). IL-1β probe was 5′-end labelled with γ-^{32}P-ATP (Amersham International plc, Amersham, UK) using T4 polynucleotide kinase. Both IFN-γ and TNF-α probe were labelled with α-^{32}P-dCTP (Amersham) by the random priming method of Feinberg & Vogelstein [21].

Cell preparations

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll–Paque (Pharmacia Fine Chemicals, Piscataway, NJ) density separation. Non-adherent lymphocytes were separated from adherent cells by culturing in plastic dishes for 1 h and passing through nylon-wool columns. Adherent cells were removed by gentle scraping with a cell scraper after the addition of cold PBS. Contamination of less than 2% lymphocytes in the adherent cells recovered by this manner was ascertained by non-specific esterase staining. Complement-mediated cytolysis was applied to further separation of lymphocytes into CD3$^-$-NK enriched and CD16$^-$-T enriched cells. After incubation in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 5 μg/ml anti-CD3 (OKT3, Ortho Diagnostic Systems Inc., Raritan, NJ) or 20 μg/ml anti-CD16 antibody (Leu-11b, Becton Dickinson Immunocytometry Systems, San Jose, CA) at 4°C for 30 min, lymphocytes were treated with five-fold-diluted rabbit complement (Cedarlane Laboratories, Hornby, Canada) at 37°C for 1 h. CD4$^+$ cell-enriched and CD8$^+$ cell-enriched populations were also obtained by the same manner as above, using complement and MoAbs of 20 μg/ml anti-CD16 and 20 μg/ml anti-CD8 (OKT8, Ortho) or 20 μg/ml anti-CD16 and 10 μg/ml anti-CD4 (OKT4A, Ortho), respectively. Each of the subpopulated cells was examined by flow cytometry, and more than 98% and 95% purity were obtained in T cell subsets and NK cells, respectively. Monocyte contamination in these lymphocyte subsets was ascertained to be less than 0·5% by non-specific esterase staining.

LAK activity assay

LAK activity was assessed by measuring released ^{51}Cr as described elsewhere in detail [22]. In brief, all subpopulated peripheral blood lymphocytes from healthy young adult donors (4×10^5/ml) were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum, and 0·25 ml of the suspension (1×10^5 cells) was poured into each microwell of 96-well flat-bottomed culture plates (Corning, Iwaki Glass, Tokyo, Japan), and incubated in the medium containing 10 U/ml rIL-2 and other indicated reagents with or without monocytes in a 5% CO_2-humidified incubator at 37°C. After 5 days, the non-adherent cells were washed twice and cocultured with ^{51}Cr-labelled Daudi cells (a cell line of Burkitt's lymphoma) at various effector-to-target (E/T) ratios. Incubating for 4 h, the plates were centrifuged and the radioactivity in the supernatants was measured by an autogamma counter. The percentage of specific tumour cell lysis was calculated by the following formula:

$$\text{Per cent specific lysis} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$

where spontaneous isotope release was determined by the activity in the supernatants of the target cell culture without the effectors. Maximum isotope release was obtained after cell treatment with 1% Triton X-100. Each assay was performed in triplicate. In some experiments, the results were also expressed by lytic units (LU). According to the method of Pross *et al.* [23], one LU was defined as the number of effector cells required for 30% lysis of 1×10^4 target cells, and the number of LU present in 1×10^6 effector cells was calculated.

Quantification of cytokines

Cytokine levels in the culture supernatants were measured using ELISA kits for IL-1β (Cistron Biotechnology, Pine Brook, NJ) and TNF-α (T Cell Sciences, Cambridge, MA), and also a radioimmunoassay kit for IFN-γ (Centocor, Malvern, PA). To ascertain biological activities of IL-1β and TNF-α in culture supernatants, the methods described by Lachman *et al.* [24] and Abe *et al.* [25] were used. One unit is defined as the amount of IL-1β to support half-maximal ^{3}H-thymidine incorporation into mouse thymocytes co-stimulated with low amounts of phytohaemagglutinin (PHA) [24], and as the amount of TNF-α sufficient to cause half-maximal cytotoxicity against L929 cells in the presence of actinomycin D [25]. The titres detected by ELISA and bioassay were then standardized by rIL-1β and rTNF-α (Boehringer Mannheim).

Northern blot analysis

T or NK cells (2×10^6 cells/ml) were stimulated for the indicated times with 10 U/ml rIL-2 in the presence or absence of monocytes (2×10^5 cells/ml). In coculture of T or NK cells with monocytes, LAK cells were collected by gentle washing of the culture dish surface with warmed RPMI 1640 medium, and monocytes were then recovered by scraping. Total RNA from 10^7 cells of NK, T or monocytes was isolated by the guanidinium-caesium chloride procedure. RNA (5 μg/lane) was electrophoresed on a 1·2% agarose gel containing 2·2 M formaldehyde, and transferred onto Hybond N$^+$ nylon membranes (Amersham), which were prehybridized at 42°C for 24 h in a prehybridization solution containing 50% (v/v) deionized for-

mamide, 1 × Denhardt's solution (Sigma Chemical Co., St Louis, MO), 1% SDS, 1 mM NaCl, 5 mM Tris-HCl (pH 7·4) and 10% dextran sulphate, and the membranes were then hybridized with ^{32}P-dCTP (Amersham)-labelled cDNA probes. After washing in 2 × standard saline citrate (SSC)/0·1% SDS at 55°C for 1 h and further washing in 0·16 × SSC/0·1% SDS at 55°C for 30 min, the membranes were exposed to Kodak x-ray film (Eastman Kodak, Rochester, NY) at −70°C for 24–72 h.

Statistical analysis

Statistical analysis of the difference between the test groups was performed using the Mann–Whitney *U*-test, and results were considered to be significant when $P < 0.05$.

RESULTS

Monocyte effects on LAK induction from NK and T cells

LAK cells were induced from CD3$^-$ (NK-enriched: NK) cells, CD8$^-$CD16$^-$ (CD4-enriched: CD4) and CD4$^-$CD16$^-$ (CD8-enriched: CD8) cells by incubation with 10 U/ml rIL-2 for 5 days (Fig. 1). Among the three subsets, the cytotoxic activity was highest in LAK from NK cells, followed by those from CD8 cells and CD4 cells (66±8%, 43±10% and 38±9%, respectively). Monocytes, which were not cytotoxic to Daudi cells and did not affect proliferation of the LAK precursors (data not shown), enhanced LAK induction in all subsets. Although their up-regulatory action was not so prominent in NK cells, LAK activities of CD4 and CD8 cells were amplified with an increase of coculturing monocytes. The activities reached the peak levels with 30% of monocytes to the effectors where CD4- and CD8-LAK activity were 56±11% and 64±6%, respectively. However, those activities were even lower than the activity of NK-LAK induced without monocytes.

Cytokine production of CD4, CD8 and NK cells in LAK induction

IL-1β, IFN-γ and TNF-α were generated from all subpopulations of cells during LAK induction (Table 1). Appreciable amounts of these cytokines could be detected in the supernatants of monocyte cultures. NK cells produced the highest level of IL-1β (12·6±6·4 pg/ml), IFN-γ (46·2±29·6 U/ml) and TNF-α (74·8±25·3 pg/ml). Compared with CD4 cells, about two-fold volume of these cytokines were produced from CD8 cells. By coculturing T or NK cells with monocytes, the amount of cytokines produced was synergistically increased in all subsets, except for TNF-α from NK cells.

Biological activities of cytokines detected were ascertained by the method described in Materials and Methods. In IL-1β, each biological activity was nearly equal to the immunochemically detected amount. However, bioassay units in TNF-α were about one seventh of ELISA units (data not shown), which probably depended on TNF-α binding to its receptors released into the culture medium from LAK cells.

Kinetics of cytokine generation in LAK induction and the influence of monocytes

A moderate amount (45±10 pg/ml) of IL-1β was generated during the first 24 h by monocytes in the presence of 10 U/ml

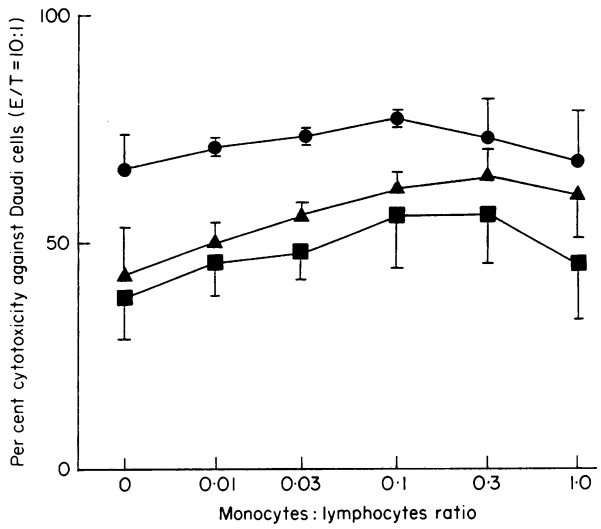

Fig. 1. Up-regulatory effect of monocytes on LAK induction. Subpopulated lymphocytes (4 × 10^5/ml) were cocultured with indicated numbers of monocytes in the presence of 10 U/ml rIL-2 for 5 days, and cytotoxicity against Daudi cells was examined. Values represent mean ± 1 s.d. of triplicates of three healthy donors. ●, NK cells; ▲, CD8 cells; ■, CD4 cells.

Table 1. Cytokine productivity of T, NK cells and monocytes, and cooperation of lymphocytes and monocytes on cytokine production

	Cytokines generated in the medium		
Cells cultured with rIL-2	IL-1β (pg/ml)	IFN-γ (U/ml)	TNF-α (pg/ml)
Monocytes	10·0±7·5	11·6±5·4	4·5±2·5
CD4 cells	4·2±1·4	10·4±8·4	16·8±10·2
CD4 cells + monocytes	28·4±7·4	23·6±9·4	38·5±13·7
CD8 cells	8·0±4·0	15·4±8·6	37·9±18·5
CD8 cells + monocytes	35·2±10·2	42·8±27·8	74·6±30·0
NK cells	12·6±6·4	46·2±29·6	74·8±25·3
NK cells + monocytes	63·2±26·4	101·8±35·4	105·8±29·9

Monocytes (4 × 10^4/ml), subpopulated LAK precursors (4 × 10^5/ml) or both cells were cultured with rIL-2 (10 U/ml) for 5 days, and each cytokine generated and released in the medium was measured. Each test was duplicated, and values represent mean ± 1 s.d. of six healthy donors.

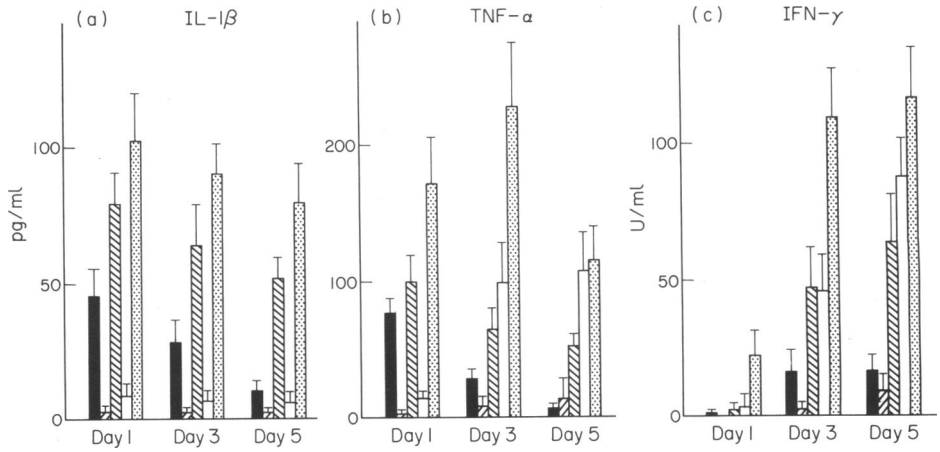

Fig. 2. Kinetics of cytokine production in LAK induction and the influence of monocytes. Monocytes/subpopulated LAK precursors were cultured with 10 U/ml rIL-2 for 5 days, and cytokine levels in the culture supernatants were estimated at the indicated times. Each test was triplicated, and bars represent mean ± 1 s.d. of five healthy donors. ■, Monocytes; ■, T cells; ▧, T cells + monocytes; □, NK cells; ▨, NK cells + monocytes.

rIL-2. The IL-1β level in the supernatants gradually decreased to minimum amount at day 5 (Fig. 2a). Only a marginal level of IL-1β was measured in the culture supernatants of T or NK cells alone. However, synergistic IL-1β generation was observed in both T cell-monocyte and NK cell-monocyte coculture.

TNF-α generation in monocytes was similar to that of IL-1β (Fig. 2b). During the first 24 h, 74 ± 5 pg/ml of TNF-α was generated from monocytes in the presence of rIL-2, and the amount of TNF-α decreased to 28 ± 6 pg/ml and 5 ± 3 pg/ml at day 3 and day 5, respectively. Kinetics of TNF-α level in the culture supernatants of $CD16^-$ (T-enriched: T) cells and NK cells was the reverse to that of monocytes; however, TNF-α generation ability of T cells was distinctly inferior to that of NK cells. When NK cells were cocultured with monocytes, TNF-α generation was synergistically increased. Over 200 pg/ml TNF-α were detected in the coculture supernatants of NK cells and monocytes at day 3, though both monocytes and NK cells individually generated about 40 pg/ml TNF-α at the time. TNF-α generation in the coculture system of T cells and monocytes, however, was not synergistic but additive. By coculturing monocytes with T cells or NK cells, high levels of TNF-α were maintained in the supernatants throughout the LAK induction.

IFN-γ was more slowly generated than IL-1β and TNF-α (Fig. 2c). IFN-γ was scarcely detected at day 1, but after that an amount of IFN-γ began to be generated. At day 3, IFN-γ level in NK cell culture supernatants was near 50 U/ml, and the level increased to 87 ± 14 U/ml at day 5.

NK cells were clearly superior in cytokine productivity to T cells, especially in TNF-α production. The coculture of LAK precursors with monocytes induced a synergistic cytokine production.

Cytokine-mRNA expression

The Northern blotting experiments provided further proof of the different cytokine productions among T, NK cells and monocytes (Fig. 3). When subpopulated monocytes, T and NK cells were individually incubated with rIL-2, the expressions of IL-1β, TNF-α and IFN-γ-mRNA were only slight even after 6 h incubation, while, by coculturing T or NK cells with monocytes, IL-1β and TNF-α mRNA were expressed in all cocultured cells 2 h after rIL-2 stimulation. IL-1β mRNA disappeared from the monocyte-cocultured T and NK cells after 6 h, but the monocytes still expressed a wide band of the message even after 24 h. In coculture of T cells with monocytes, TNF-α mRNA was clearly detected in both cells after 2 h incubation, but both cells lost the expression of the message after 6 h. When NK cells were cocultured with monocytes, TNF-α mRNA was expressed in monocytes up to 24 h, and a weak expression of the message was observed in NK cells at 24 h. Compared with the band in monocyte-cocultured NK cells, the band in cocultured T cells was narrower. In coculture of T cells with monocytes, IFN-γ-mRNA expression was not detected in any of the cells. In NK cells and NK cell-cocultured monocytes a detectable IFN-γ message was expressed after 24 h. However, the message was barely detected in T cells and cocultured monocytes.

Effect of the cytokines on LAK induction

rIL-1β and rTNF-α equally enhanced LAK induction from both T cell subsets (Table 2). One ng/ml of rIL-1β and rTNF-α increased LAK activities of both NK and T cell subsets up to the levels induced in the presence of monocytes, while rIFN-γ did not affect LAK induction. When 1 ng/ml of both rTNF-α and rIL-1β or 1 ng/ml rTNF-α plus 10 U/ml rIFN-γ were added, LAK induction was additively augmented, and cytotoxicity of CD4 and CD8 cells became about 2–2·5-fold of the activities induced by rIL-2 alone. However, NK cell-LAK induction was not affected by rTNF-α or rIFN-γ.

Suppression of LAK induction by antibodies against the cytokines

Although anti-IFN-γ antibody did not affect T or NK cells in LAK induction in the absence of monocytes, anti-IL-1β and anti-TNF-α antibodies significantly suppressed LAK induction in all cells examined ($P < 0{\cdot}05$). A synergistic suppression was observed by a combination of both antibodies (Table 3). When T or NK cells were cocultured with monocytes, cytotoxicities of subpopulated cells were decreased by antibodies to IL-1β, IFN-γ and TNF-α. Among these, the anti-TNF-α antibody most strongly inhibited LAK induction. The cytotoxicity of CD4, CD8 and NK cells was reduced from 21·1 ± 1·2 LU, 32·6 ± 2·3 LU and 67·6 ± 3·8 LU (without the antibody) to 11·3 ± 1·6 LU,

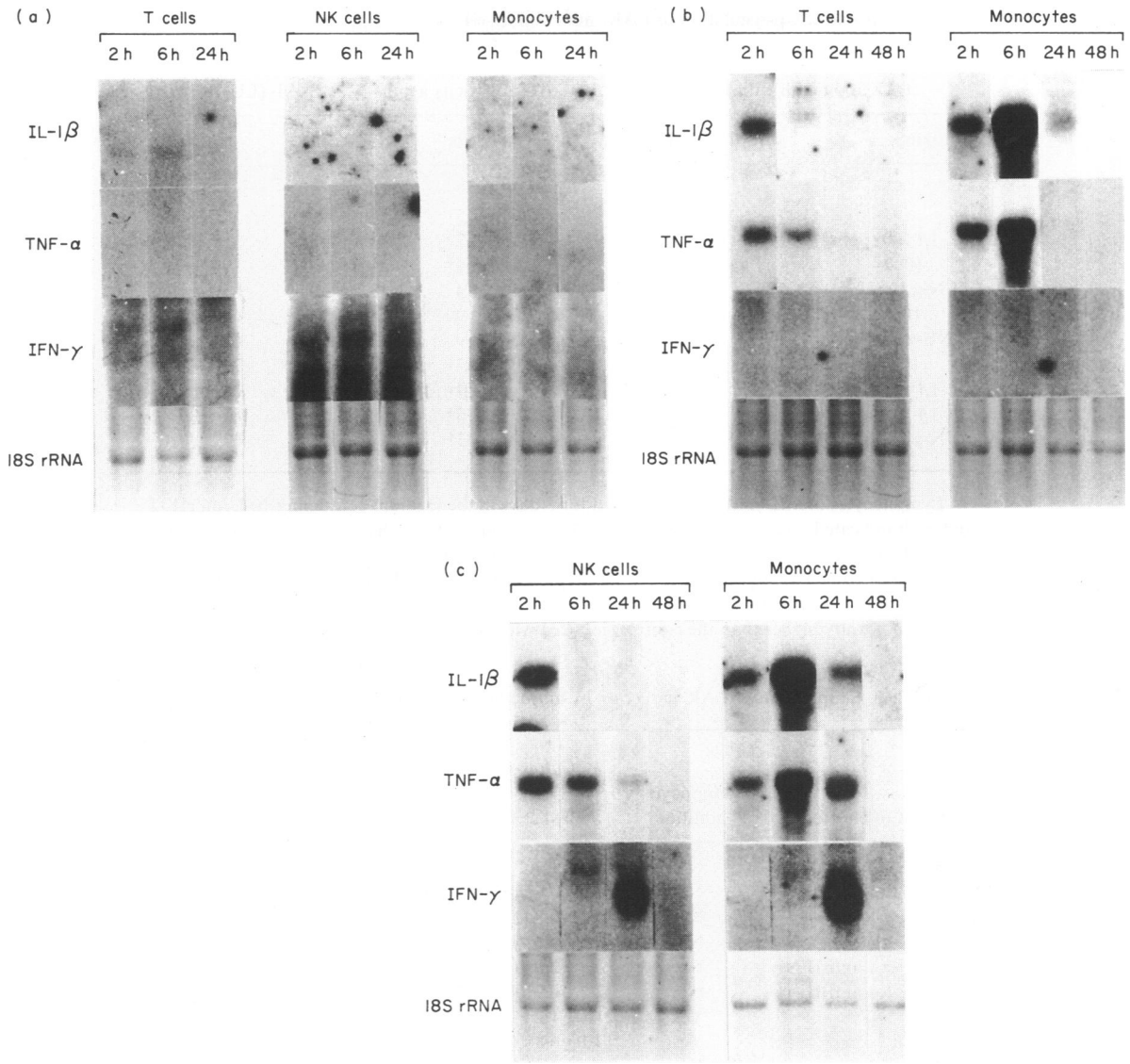

Fig. 3. Expression of IL-1β, TNF-α and IFN-γ mRNA in individually cultured T, NK cells and monocytes (a), cocultured T cells and monocytes (b), and cocultured NK cells and monocytes (c). Monocytes (2×10^5/ml)/subpopulated LAK precursors (2×10^6/ml) were cultured with 10 U/ml rIL-2 for indicated hours. Total RNA was extracted from each of the cells, electrophoresed, and transferred to nylon membranes. RNA was then hybridized to each ^{32}P-labelled cDNA probe.

15·0 ± 1·8 LU and 20·7 ± 9·4 LU, respectively. Further, cooperative suppression was induced by a combination of anti-TNF-α and anti-IFN-γ antibody (approximately 70% inhibition of LAK activity of each subset). The greatest inhibition (more than 90%) was obtained by a combination of anti-TNF-α and anti-IL-1β antibody.

When the anti-IL-1β or anti-TNF-α antibody was added at day 0 (just before LAK induction), more than 50% suppression of LAK activity was observed (Fig. 4a). By adding each antibody at day 1 and day 3, about 30–40% and 10–15% inhibition was observed in NK and T cells, respectively. Strength of LAK inhibition by the anti-TNF-α antibody was near to that of the anti-IL-1β antibody. Concomitant addition of both antibodies resulted in almost complete LAK inhibition. Both T and NK cells hardly became cytotoxic to Daudi cell targets in the presence of both antibodies. However, the additive inhibitory effect of the antibodies was not exhibited when they were added at day 3 (3 days after the start of the LAK induction). Compared with the effect of the anti-TNF-α and anti-IL-1β antibody combination, the inhibitory cooperation of the anti-TNF-α with anti-IFN-γ antibody was small, and resulted in mild LAK suppression (Fig. 4b).

DISCUSSION

It has been clarified that LAK cells can be induced from various lymphocyte subpopulations, and that the manner of LAK induction is somewhat different among the subsets [18,19,26]. In the present study, NK cells were found to be the best LAK precursors, and LAK from CD8 and CD4 cells, in that order, followed, correlating with their TNF-α-generating abilities. Interestingly, CD4 or CD8-LAK activity could not reach NK-LAK activity even if the subpopulated T cells were cultured in the presence of a high dose (100 U/ml) rIL-2 plus high titre

Table 2. Up-regulation of LAK induction by rIL-1β, rTNF-α and rIFN-γ

LAK induction with IL-2 plus		Cytotoxicity against Daudi cells (LU/10^6 cells)		
Cytokine	Monocytes	CD4 cells	CD8 cells	NK cells
—	−	12·8±1·7	16·7±1·7	69·0±10·9
—	+*	20·4±2·5†	25·6±2·0†	78·2±8·1
rIL-1β 100 (pg/ml)	−	19·5±4·2†	23·8±4·8†	75·3±6·2
1000	−	22·0±5·4†	26·6±5·7†	79·4±7·3
rIFN-γ 10 (U/ml)	−	11·6±1·4	18·2±2·0	63·1±10·5
100	−	10·5±0·9	14·5±0·6	66·0±11·8
rTNF-α 100 (pg/ml)	−	17·3±3·9	22·4±3·5†	67·8±7·4
1000	−	20·1±4·7†	24·1±4·5†	73·1±9·1
rIL-1β 100+rTNF-α 1000	−	26·0±4·4†	27·1±4·9†	68·0±7·1
rIL-1β 1000+rTNF-α 1000	−	32·3±3·8†	36·4±5·6†	85·8±7·8†
rIFN-γ 10+rTNF-α 1000	−	33·3±4·3†	32·2±4·8†	67·2±7·1

Lymphocytes (4×10^5/ml) were incubated for 5 days in the medium containing 10 U/ml rIL-2 and each indicated volume of the cytokines. The cells were then washed, and their cytotoxicities against Daudi cells were tested by a ^{51}Cr release assay at E/T ratios of 10:1, 5:1, 2·5:1 and 1·25:1. Each examination was triplicated, and values represent mean±1 s.d. of four healthy donors.

* Monocytes to effectors = 1:10.

† Significantly higher than the controls induced with rIL-2 alone ($P<0·05$).

Table 3. Suppression of LAK induction by antibodies against IL-1β, IFN-γ and TNF-α

Treatment of effectors	Monocyte addition	Cytotoxicity against Daudi cells (LU/10^6 cells)		
		CD4 cells	CD8 cells	NK cells
None	No	15·4±1·7	16·5±2·7	46·7±5·2
Anti-IL-1β	No	7·2±0·8† (53)*	9·1±3·2† (45)	21·2±9·4† (55)
Anti-IFN-γ	No	15·1±0·8 (2)	17·0±4·3 (−3)	46·0±16·8 (1)
Anti-TNF-α	No	7·0±4·5† (55)	9·3±3·6† (44)	28·5±17·5† (39)
Anti-TNF-α+anti-IFN-γ	No	6·4±2·0† (58)	9.3±2.1† (44)	22.2±14.1† (52)
Anti-TNF-α+anti-IL-1β	No	2·2±0·9† (86)	1·2±1·1† (93)	4·2±3·0† (91)
None	Yes	21·1±1·2	32·6±2·3	67·6±3·8
Anti-IL-1β	Yes	14·1±6·8† (33)	14·9±8·5† (54)	38·4±22·7† (43)
Anti-IFN-γ	Yes	18·8±3·0 (11)	24·6±5·6 (25)	51·9±14·4 (23)
Anti-TNF-α	Yes	11·3±1·6† (46)	15·0±1·8† (54)	20·7±9·4† (69)
Anti-TNF-α+anti-IFN-γ	Yes	7·2±1·3† (66)	8·6±2·1† (74)	14·7±9·0† (78)
Anti-TNF-α+anti-IL-1β	Yes	1·9±1·4† (91)	2·5±1·4† (92)	4·8±4·4† (93)

CD4, CD8 or NK cells (4×10^5/ml) were cultured alone or cocultured with 4×10^4/ml monocytes in the presence of 10 U/ml rIL-2 with or without antibody to IL-1β (10 neutralizing units/ml), IFN-γ (1000 neutralizing units/ml) and TNF-α (200 neutralizing units/ml). After 5 day culturing, the effectors were washed and tested in 4-h ^{51}Cr release assay against Daudi cells at E/T ratios of 10:1, 5:1, 2·5:1 and 1·25:1, and lytic units (LU) were calculated. Each test was triplicated, and values represent mean±1 s.d. of four healthy donors.

* Values in parentheses indicate per cent inhibition.

† Significantly lower than the controls, in which effectors were induced in the absence of the antibodies ($P<0·05$).

rIL-1β and rTNF-α. These results indicate that the inferiority of T-LAK is due not only to its inferior cytokine productivity, but also to its inferior response to the cytokines.

NK cells possessed high cytokine productivity compared with T cells. In the coculture system, LAK activities were correlated with the yielded IL-1β and TNF-α, and both cytokines could almost substitute for the LAK-enhancing ability of monocytes. However, the influence of cell–cell contact should be considered, because LAK induction was suppressed when contact between lymphocytes and monocytes was inhibited by a pored filter (data not shown). Therefore, it seems likely that by contact with monocytes, LAK precursors are stimulated to transduct signals for activation.

IL-1β and TNF-α were rapidly generated, and the antibodies to these cytokines greatly suppressed LAK induction if they were added in the early phase of LAK induction. Crump *et al.*

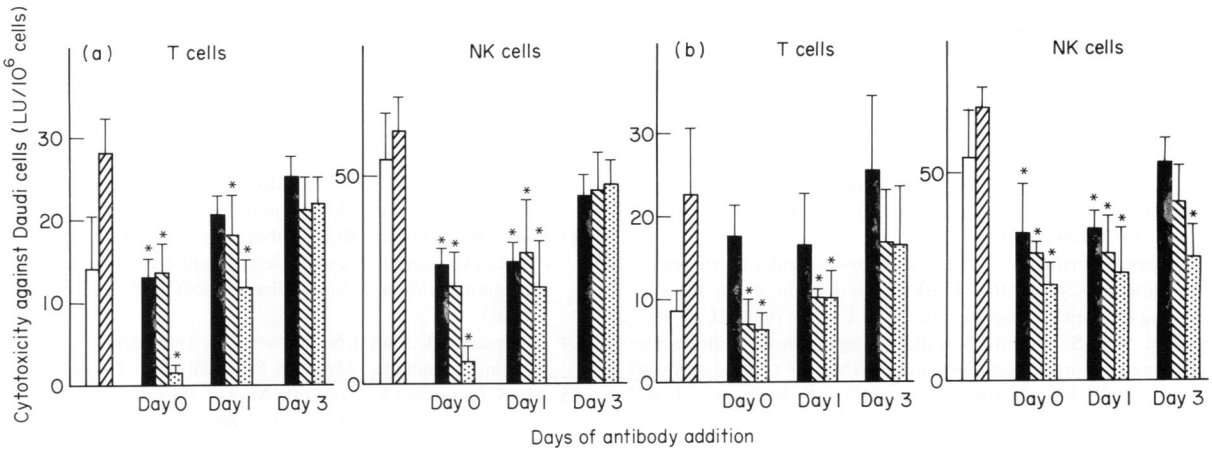

Fig. 4. Suppression of LAK induction by the antibodies against TNF-α, IL-1β and IFN-γ—a kinetic analysis. Sufficient dose of each antibody (indicated in Table 3) against TNF-α, IL-1β or IFN-γ was added in the culture medium at the indicated time, and the induced LAK activity after 5-day cultivation with 10 U/ml rIL-2 was measured. Each test was triplicated, and values represent mean ± 1 s.d. of three healthy donors. * Significantly lower than controls (without antibody) ($P < 0.05$). (a) □, Without monocytes; ■, with monocytes; ■, with monocytes and anti-IL-1β antibody; ▧, with monocytes and anti-TNF-α antibody; ▨, with monocytes and both antibodies. (b) □, Without monocytes; ■, with monocytes; ■, with monocytes and anti-IFN-γ antibody; ▧ with monocytes and anti-TNF-α antibody; ▨ with monocytes and both antibodies.

[13] reported that for sufficient LAK induction, IL-1β must be added with IL-2 at the beginning of the induction. It is therefore likely that IL-1β and TNF-α, which augment IL-2 receptor expression [27,28] and up-regulate their own secretions as well as secretions of other cytokines in an autocrine and paracrine manner [14,29], act on LAK precursors during the early stage of LAK induction. It also seems likely that the synergistic cytokine production in the coculture systems was due to the autocrinal and paracrinal manners. The synergistic cytokine network is considered to be most possible in a combination of TNF-α and IFN-γ. IFN-γ was generated late after release of large amounts of TNF-α and IL-1β in the culture medium. A fair amount of TNF-α was at first generated from monocytes, after which NK cells started to generate a considerable amount of TNF-α. Therefore, both IFN-γ release from the cocultured cells and TNF-α release from NK cells might be enhanced by paracrinally produced IL-1β and TNF-α. However, enhancement of IL-1β production in a paracrine or autocrine fashion appears negligible, because IL-1β was released to the maximal level from monocytes, T and NK cells within 24 h after the initiation of LAK induction.

Though LAK induction was hardly enhanced by rIFN-γ and only a slight inhibition of LAK activity could be induced by anti-IFN-γ antibody alone, the antibody augmented the LAK suppression by anti-TNF-α antibody. From these results, IFN-γ, which was generated slowly in LAK induction, appears to play an accessory role, perhaps to maintain enough expression of cell surface TNF-α receptors, which might be down-regulated by TNF-α [30–32], without affecting IL-2 receptor expression.

The results of Northern blotting seem to indicate that in the coculture system most IL-1β was derived from monocytes, and TNF-α was largely generated by monocytes and NK cells, but only slightly by T cells. In fact, the IL-1β productivity of NK cells was only slightly superior to that of T cells, but was inferior to that of monocytes. TNF-α was sufficiently generated from NK cells and monocytes, but barely from T cells, in parallel with each mRNA expression. These results show the superiority of NK-LAK due to their superior TNF-α productivity. Using IL-1 receptor antagonist, Fujiwara & Grimm [33] examined LAK induction, and postulated that IL-1β was the initiator of LAK induction. However, the expression of IL-1β-mRNA synchronized with that of TNF-α-mRNA. Taken together with the influences of the antibodies to these cytokines, it can be considered that both TNF-α and IL-1β synchronously up-regulate LAK induction. Further investigation of, for example, the influence of both the cytokines on signal transduction, appears essential to clarify the mechanism of LAK induction.

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