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Interleukin-2 Bolus Therapy Induces Immediate and Selective Disappearance from Peripheral Blood of all Lymphocyte Subpopulations Displaying Natural Killer Activity: Role of Cell Adhesion to Endothelium

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As early as 10–15 min after the start of a 30 min interleukin-2 (IL-2) infusion, a rapid, virtually complete disappearance of all natural killer (NK) lymphocyte subpopulations (including both $CD3^-$ $CD56^+$ and $CD3^+$ $CD56^+$ cells with either alpha/beta or gamma/delta T-cell receptor) was observed from peripheral blood. In contrast, the number of T lymphocytes ($CD3^+$ $CD56^-$) was unmodified for at least 2 h after IL-2 injection. The IL-2-induced, rapid disappearance from peripheral blood of NK and NK-like lymphocytes may be related to their massive adherence to the activated endothelium. In this regard, IL-2 infusion caused a very rapid rise of tumour necrosis factor-alpha (TNF-α) plasma concentration, whereas other cytokines, such as interferon-gamma (IFN-γ), were induced only at later times. *In vitro* experiments indicated that IL-2, either alone or better combined with TNF-α, exerts a rapid and selective stimulatory effect on NK adhesion to endothelial cells. On the basis of these findings, we suggest that the activation of NK lymphocytes induced by IL-2, alone or combined with TNF-α, plays a key role in mediating the massive and selective adherence of NK and NK-like cells following IL-2 bolus infusion.
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INTRODUCTION

ADOPTIVE CANCER immunotherapy involves activation *in vitro* by recombinant interleukin-2 (rIL-2) of lymphocyte subpopulations (lymphokine-activated killer (LAK) cells) from a tumour-bearing patient and their subsequent administration to patients [1, 2]. LAK cells exhibit the *in vitro* capacity of lysing fresh tumour cells in a non-MHC restricted manner [2].

In the present study, we used the National Cancer Institute adoptive immunotherapy protocol [1, 2], which has been mainly applied in patients with metastatic malignant melanoma and renal cell carcinoma resistant to standard therapy [1, 2]. This protocol includes three phases: (i) high-dose IL-2 administration; (ii) autologous lymphocytes obtained by leukapheresis are collected and cultured with IL-2 to generate LAK cells; (iii) patients are reinfused with LAK cells together with high-dose IL-2.

In vitro infusion of IL-2 produces a variety of biological effects [3–5], ranging from induction of LAK cell activity markers (i.e. IL-2 receptors) [4] to alterations in the differential leukocyte count (i.e. eosinophilia) thought to be caused by the secretion of other cytokines [4]. Particularly, IL-2 infusion elicits dramatic fluctuations of the number of peripheral blood lymphocytes,

Table 1. Characteristics of the patients undergoing adoptive immunotherapy with IL-2 and LAK cells

Patient diagnosis	Age (sex)	IL-2 dosage (U/m^2/day)
A Renal carcinoma	60 (F)	4.5×10^6
B Melanoma	52 (M)	6.0×10^6
C Melanoma	51 (F)	6.0×10^6
D Melanoma	67 (M)	6.0×10^6
E Melanoma	69 (F)	6.0×10^6
F Renal carcinoma	39 (M)	4.5×10^6

with an early depletion of all lymphoid cells followed by their rebound after discontinuation of IL-2 therapy [6, 7]. These studies also showed a rapid decrease of peripheral blood NK cells occurring at 30 min–1 h after IL-2 administration [6, 7]: this phenomenon was tentatively attributed to a rapid adhesion of these cells to the vascular endothelium [8, 9].

Recent studies indicate that lymphoid cells exhibiting *in vitro* NK activity pertain to three different cell types, as evaluated by membrane phenotype and T-cell receptor (TCR) genes rearrangement and expression: (a) $CD56^+$/TCR alpha–$beta^-$/TCR gamma–$delta^-$/$CD3^-$; (b) $CD56^+$/TCR alpha–$beta^+$/gamma–$delta^-$/$CD3^+$; (c) $CD56^+$/TCR alpha–$beta^-$/gamma–$delta^+$/$CD3^+$ [10]. In this study we have investigated the kinetics of these three cell populations in peripheral blood at early times after IL-2 bolus infusion. Our results indicate that all three NK populations, regardless of their different cellular origin, membrane phenotype and *in vivo* distribution, almost totally disappear from peripheral blood within 5–15 min, with an identical kinetic pattern.

PATIENTS AND METHODS

Patients

The characteristics of the 6 patients submitted to adoptive immunotherapy are reported in Table 1: 4 patients had melanoma and 2 had renal cell carcinoma; their Karnofsky performance status ranged from 70 to 100. Their response to therapy will be reported separately.

Adoptive immunotherapy protocol

The adoptive immunotherapy protocol consisted of three phases. The first one (days 1–5) involved intravenous administration of human recombinant IL-2 (Roche) by means of a central catheter. The IL-2 dosage was 4.5×10^6/3 times/day/m^2 of body surface for patients A and F, and $6 \times 10^6/m^2$ for cases B, C, D and E.

In phase II, after a 48-h rest period, patients underwent daily leukapheresis (days 8–11). Harvested cells were centrifuged through a Ficoll-Hypaque density gradient. The resulting lymphocyte preparations were cultured in RPMI-1640 (Flow) containing 2% heat-inactivated AB serum, penicillin (10 U/ml), glutamine (2 mM) and gentamicin sulphate (5 mg/ml). rIL-2 was then added to the culture at a final concentration of 1500 U/ml.

Phase III (days 12–15), consisted of infusion of IL-2 activated (LAK) cells together with IL-2 every 8 h as in phase I or as tolerated.

Plasma specimens

Blood samples were collected in 5 ml vacuum-sealed tubes containing 10.5 mg EDTA and 3.5 units aprotinine (Sigma) and centrifuged at 750 *g* for 10 min. The plasma was carefully removed and centrifuged at 10000 *g* for 4 min to eliminate residual platelets and stored at −70°C until assayed for cytokines.

Lymphocyte analysis

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples obtained before and sequentially after the first 30-min IL-2 bolus infusion. Time 0 corresponds with the start of the injection. Venous blood, mixed with 12 U/ml sodium heparinate, was separated on Ficoll-Hypaque (Pharmacia) at 450 *g*/40 min. PBMC were washed three times with RPMI-1640 medium (Flow) counted and suspended (5×10^6 cell/ml) in the same medium containing 2 mg/ml bovine serum albumin (BSA, Sigma). 0.1 ml aliquots were then incubated for 60 min at 4°C with an appropriate dilution of two monoclonal antibodies (Mab) recognising different lymphocyte subsets and labelled with different fluorochromes (i.e. fluorescein or phycoerythrin).

The following Mabs were used: Leu 2 (anti-CD8), Leu 3 (anti-CD4), Leu 4 (anti-CD3), Leu 7 (anti-CD57), Leu 19 (anti-CD56) (Becton-Dickinson) and TCR-delta 1 (anti-gamma/delta T-cell receptor) (T Cell Sciences, Boston).

The cells were washed three times at 4°C in phosphate buffered saline, resuspended in the same solution containing 2% formaline and analysed using flow cytometry.

Flow cytometry analysis

Flow cytometry (FCM) analysis was performed using a FACScan (Becton-Dickinson) equipped with a CONSORT 30 computer (Hewlett-Packard). Data were analysed by the 'Lysis Program'. Fluorescence data are displayed as 'dot plots' in which logarithmically increasing green fluorescence intensity on the *X*-axis and red fluorescence on the *Y* axis are plotted, respectively.

Evaluation of NK activity

Cytotoxicity was determined in a 4 h ^{51}Cr-release assay. Human target NK sensitive cells K562 (free of mycoplasma) were labeled with sodium ^{51}Cr solution (New England Nuclear) in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS, Flow), 10 mmol/l Hepes, 100 U/ml penicillin, and 100 μg/ml streptomycin. The effector-to-target cell ratios varied from 50:1 to 1.5:1. All groups were tested in triplicate. The percentage of specific cytotoxicity was calculated as follows: % specific cytotoxicity = (experimental release–spontaneous release) × 100/(maximal release– spontaneous release). The mean spontaneous release from the medium control was 5% and never exceeded 10%. One lytic unit (LU) was defined as the number of effector cells required to produce 50% lysis of target cells.

Cytokine concentration

The level of IFN-γ in patients' plasma and culture supernatants was measured by a sensitive radioimmunoassay

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Revised 22 Oct. 1991; accepted 1 Nov. 1991.

(Centocor, Philadelphia), which can detect values as low as 0.1 U/ml.

TNF-α levels in plasma and culture supernatants were measured by an enzyme sandwich immunoassay (T Cell Sciences), which allows detection of 1 pg TNF-alpha/ml.

The level of IL-1-beta was assessed by a sensitive enzyme linked immunosorbant assay (ELISA) (Cistron Biotechnology, Pine Brook, New York), which can detect as little as 5 pg/ml.

Control experiments were performed to carefully assess the specificity of these immunoenzymatic assays: particularly addition of IL-2, even at very high dosage (10^4 U/ml) did not modify the assayed level of these cytokines.

In vitro *adherence of NK or PBMC to endothelium*

Endothelial cells (EC). Human EC were obtained from umbilical vein and cultured as described [11]. Routinely, we used confluent cells (10^5/2 cm^2 culture well) between the first and the fourth passage maintained in 199 medium (Biochrom KG, Berlin) with 20% newborn calf serum (Hyclone Lab, Logan, UK) supplemented with EC growth supplement (50 μg/ml; Collaborative Research Inc., Lexington) and heparin (100 μg/ml; Sigma). The purity of EC cultures was checked by expression of factor VIII antigen (>99% positive cells).

Adhesion assay. Adhesion of purified NK lymphocytes to EC was evaluated as described [12]. EC were grown to confluence in flat bottomed 96-well trays. For the assay of NK lymphocyte adherence, purified NK lymphocytes were resuspended in complete medium at (10^7 cells/ml) and labeled with 3.7 MBq ^{51}Cr (sodium chromate, Amersham International) at 37°C for 1 h. At the end of the incubation, the cells were washed with medium and resuspended at a density of 10^6 cells/ml. Thereafter, 0.1 ml was dispensed to each well and incubated for 30 min at 37°C. At the end of the incubation, the wells were carefully washed three times with phosphate buffered saline (PBS) + 1% FCS. Adherent cells were solubilised with 0.2 ml of 0.025 mol/l NaOH, 0.1% sodium dodecyl sulphate (SDS), and radioactivity was counted in a gamma counter. Results are presented as the percentage of adherent cells (S.D.) (3–5 replicates/group). The level of statistical significance was evaluated by *t* test.

The adherence of monocyte-depleted PBMC (PBL) was assayed by an identical assay except that: (i) EC were grown to confluence in flat-bottomed 6-well trays; (ii) PBMC were not labeled with ^{51}Cr, and 1 ml of cells at a density of 5 × 10^6 cells per ml was dispersed into each well; (iii) at the end of the incubation, the membrane phenotype of non-adherent cells was assessed by immunofluorescence.

Cytokine stimulation. In the adhesion assay IL-2 (10^3 U/ml) alone or combined with TNF-α (10^3 U/ml) was added during both the preincubation (5–20 min) of purified NK cells or monocyte-depleted PBMC and the adhesion assay. In other experiments, EC were preincubated for 30 min with IL-2 and/or TNF-α at the above dosages, and the adhesion assay was then performed as described.

Effect of IL-2 on the expression of cell adhesion molecules on the membrane of NK lymphocytes. NK lymphocytes were purified according to the procedure outlined above. The cells were resuspended in Iscove's medium containing 5% heat-inactivated human AB serum and then either IL-2 (10^3 U/ml) or TNF-α (10^3 U/ml) or IL-2 plus TNF-α (both at 10^3 U/ml) were added and aliquots of cells were removed from bulk cultures at different time intervals (10, 30, 60 min and 20 h) and processed for the evaluation of the expression of membrane cell adhesion molecules by indirect immunofluorescence using specific Mabs including: anti-CD11a and -VLA-4 Mabs (Immunotech, Marseille); anti-ICAM-1 (RR1/1, kindly provided by Dr T. Springer, Boston); anti-CD56 (Leu 19) and -CD16 (Leu 11) (Becton-Dickinson). The cells were then processed and analysed for immunofluorescence and flow cytometry as reported above.

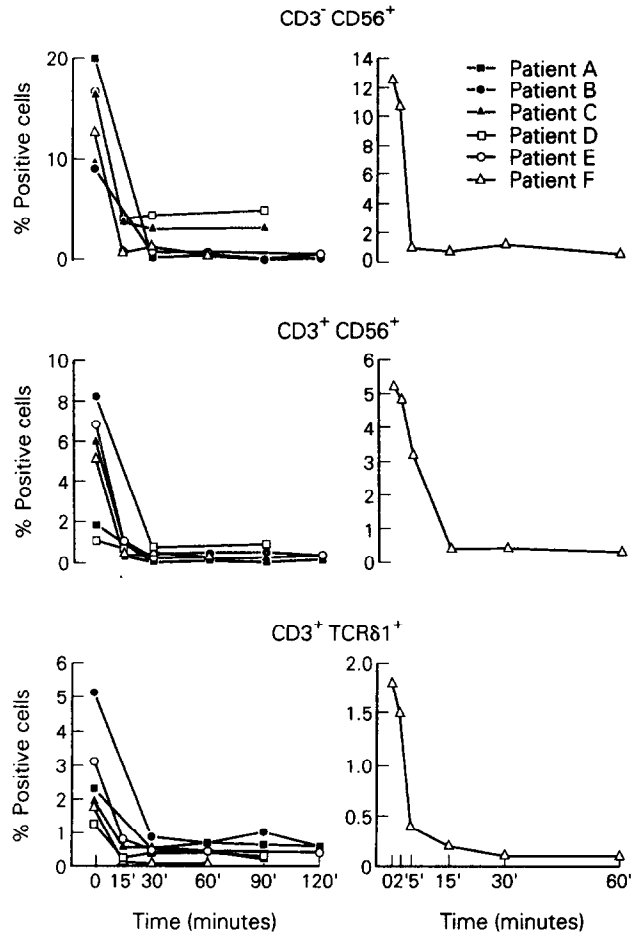

Fig. 1. Immunofluorescence labeling of NK lymphocyte subpopulations at different times after IL-2 bolus injection. The NK lymphocyte subpopulations were identified by double staining immunofluorescence using anti-CD56, anti-CD3 and anti-TCR gamma/delta Mabs.

RESULTS

Effect of IL-2 bolus infusion on NK lymphocyte subpopulations

Peripheral blood samples were obtained just before and after the start of bolus IL-2 infusion. The percentage of cells reacting with either anti-CD56 and anti-CD3, or anti-CD3 and TCR-delta-1 Mab were evaluated using a double labeling assay. This analysis showed that CD56$^+$CD3$^-$, CD56$^+$CD3$^+$ and CD3$^+$TCR-delta-1$^+$ lymphocytes rapidly disappear from peripheral blood as a consequence of IL-2 infusion, being virtually undetectable 15 min after the start of IL-2 injection (Fig. 1). An analysis performed at early times after IL-2 infusion in 1 of these patients showed that: (a) CD3$^+$CD56$^-$ and CD3$^+$TCR-delta-1$^+$ lymphocytes virtually disappear from peripheral blood after 5 min; (b) CD56$^+$CD3$^+$ lymphocytes almost completely disappear from peripheral blood 15 min after the start of IL-2 injection (Fig. 1). These results indicate that lymphocyte

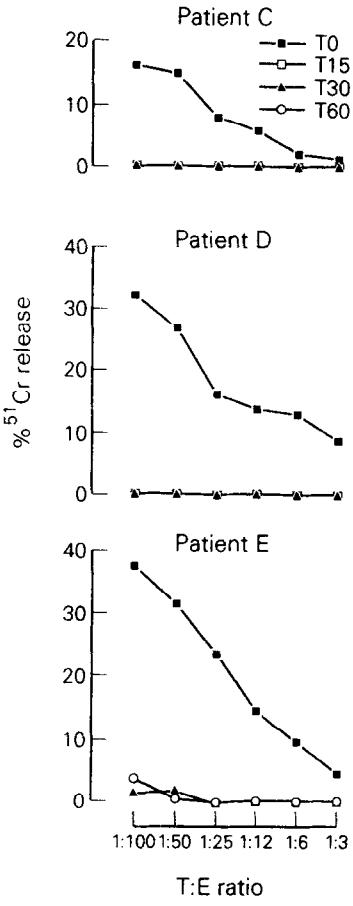

Fig. 2. NK activity of PBL derived from the blood of patients C, D and E at different times (0–90 min) after IL-2 bolus injection. NK cytotoxic activity was assayed using a standard 4-h ^{51}Cr release assay; K562 were used as NK-sensitive tumour targets.

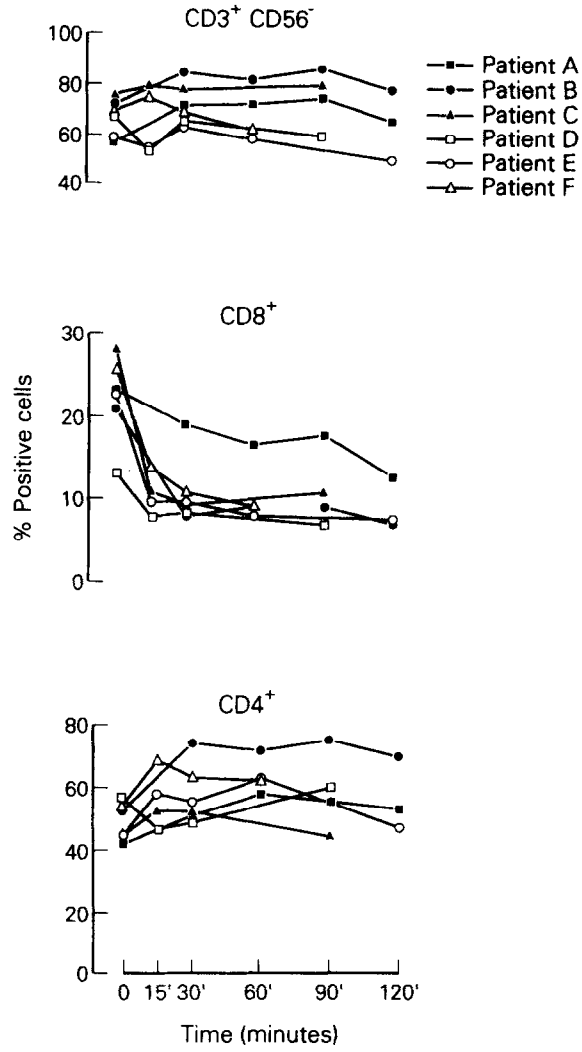

Fig. 3. Immunofluorescence labeling of CD4$^+$ and CD8$^+$ lymphocyte subpopulations at different times after IL-2 bolus administration. The CD4$^+$ and CD8$^+$ populations were identified by direct immunofluorescence using Leu 2 (anti-CD8) and Leu 3 (anti-CD4) Mab. Fluorescent cells were processed as in the legend to Fig. 1.

subpopulations *in vitro* exhibiting NK activity are rapidly induced to disappear from peripheral blood by IL-2.

The NK activity of PBMC from 3 patients, obtained before and after IL-2 injection, was evaluated by a standard ^{51}Cr release assay using K562 as target cells. A marked decrease in NK activity was observed as early as 15 min after the start of IL-2 infusion (Fig. 2).

Effect of IL-2 bolus infusion on CD4$^+$ and CD8$^+$ lymphocytes

The effect of IL-2 bolus infusion on the number of circulating CD4$^+$ and CD8$^+$ lymphocytes was also investigated using the procedure described for NK lymphocyte subpopulations. Results presented in Fig. 3 show that: (a) the percentage of total lymphocytes (CD3$^+$CD56$^-$) was essentially unmodified after IL-2 infusion; (b) the percentage of CD4$^+$ cells showed a slight increase in the early minutes following IL-2 infusion; (c) the percentage of CD8$^+$ lymphocytes rapidly diminishes after IL-2 injection, reaching nadir values corresponding to approximately 50% of starting levels. Analysis of the fluorescence intensity of CD8$^+$ cells showed two distinct subpopulations defined as 'bright' or 'dim' (see Fig. 3). Evaluation of PBL at different times after IL-2 infusion showed that the dim CD8$^+$ population virtually disappeared from peripheral blood after IL-2 infusion, whereas the bright CD8$^+$ lymphocytes remained unmodified (data not shown).

Further experiments were performed to characterise the CD8$^+$ lymphocyte subpopulation induced to disappear from peripheral blood by IL-2. Since T NK lymphocytes are CD57$^+$CD8$^+$ double-labeling experiments were carried out using anti-CD8 and anti-CD57 Mab: after IL-2 infusion all CD57$^+$CD8$^+$ lymphocytes rapidly disappear from peripheral blood, in contrast to only 20–40% of CD8$^+$CD57$^-$ lymphocytes (data not shown).

Effect of IL-2 bolus infusion on the release of secondary cytokines

In vitro incubation of endothelial cells with TNF-α, IFN-γ or IL-1-β leads to an increased binding of resting or IL-2-stimulated NK cells [9–13]. On the other hand, it has been shown that IL-2 infusion elicits secretion of secondary cytokines including TNF-α, IFN-γ and IL-1-β [14–17]. On the basis of these findings, we evaluated the plasma concentration of TNF-α, IFN-γ and IL-1-β in the early minutes following bolus IL-2 infusion, thus expanding and confirming our preliminary data on the first two cytokines [17].

We observed that within 2–5 min IL-2 induced a significant rise of TNF-α plasma concentration, whereas IFN-γ and IL-1

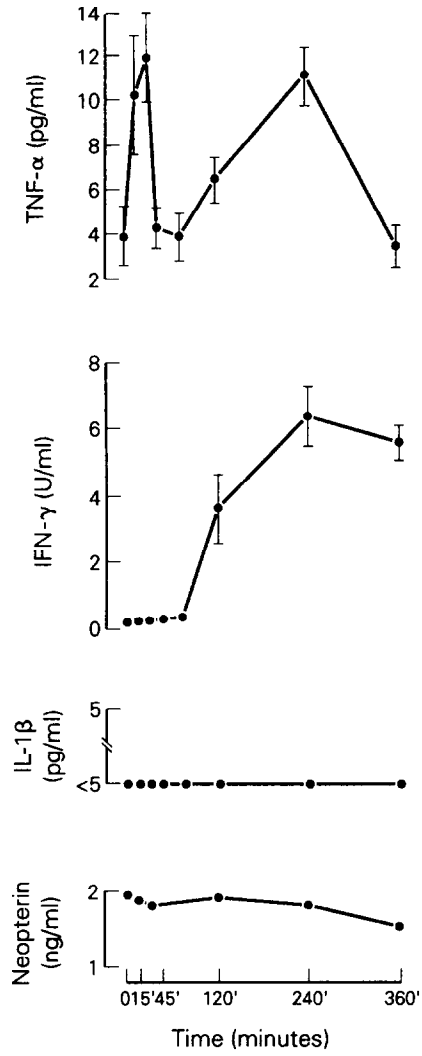

Fig. 4. Plasma TNF-α, IFN-γ and IL-1-β levels at early times after IL-2 bolus infusion. Plasma samples were collected just before the infusion and at sequential minutes following the start of treatment. Plasma TNF-alpha, IFN-gamma and IL-1 beta levels were evaluated by means of a sensitive immunoenzymatic method. Mean (S.E.) values from 6 patients are presented.

β levels remained unmodified (Fig. 4). An increase in plasma IFN-γ was observed after 120 min, thus confirming our previous results [20]. These findings suggest that IL-2-induced TNF-α release may play a key role in the disappearance of NK cells mediated by IL-2.

In order to identify the cell type(s) responsible for the IL-2-mediated TNF-α production, total PBMC, purified T lymphocytes or NK cells were incubated *in vitro* with IL-2 and the TNF-α level was evaluated in the culture supernatants at different times thereafter. The results suggest that PBMC and NK lymphocytes release significant amounts of TNF-α (data not shown). However, this phenomenon is monitored only at 3 h of *in vitro* culture, as compared to the early rise of TNF-α level after the start of IL-2 *in vivo* infusion.

Effect of IL-2 and TNF-α on the in vitro *adherence of PBMC to endothelium*

In order to evaluate whether the IL-2-induced disappearance of NK lymphocytes is due to massive adherence of these cells to endothelium, the *in vitro* adherence of PBMC or purified NK

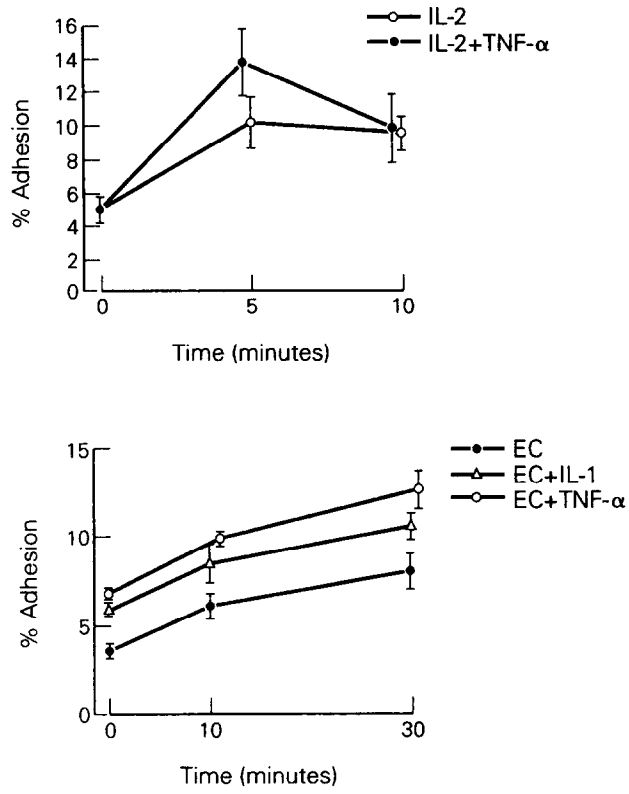

Fig. 5. Adhesion of IL-2-activated or IL-2 + TNF-α-activated NK cells to resting human endothelial cells (top panel). Percentage adhesion was determined after 30 min incubation at 37°C of ^{51}Cr-labeled cells. Values represent mean (S.D.) of two separate experiments (each point was evaluated in triplicate). The percentage of cell adhesion of NK cells stimulated for 5, 10 min with IL-2 or IL-2 plus TNF-α is significantly different from that of resting NK lymphocytes ($P < 0.01$). Cell adhesion of NK cells stimulated with IL-2 alone is significantly lower than that of cells stimulated with IL-2 + TNF-α ($P < 0.01$). The cells were incubated for different times in the presence of 10^3 U/ml IL-2 alone or together with 10^3 U/ml TNF-α. Adhesion of IL-2 activated NK cells to either resting or IL-1β or TNF-α activated human endothelial cells (lower panel). EC were incubated 30 min at 37°C in medium without additives (EC) or in medium containing 10^3 U/ml IL-1β (EC+IL-1) or 10^3 U/ml TNF-α (EC+TNF-α) and then utilised for the NK adhesion assay performed as above; NK cells were incubated for different periods of time in the presence of 10^3 U/ml IL-2 under the conditions reported above.

to endothelium in the presence of IL-2 and TNF-alpha was analysed.

Resting NK lymphocytes exhibit a relatively low level of 'spontaneous' adhesion to EC (Fig. 5), thus in line with a previous report [12]. NK lymphocytes activated for 5–10 min by IL-2 (10^3 U/ml) exhibit a 2-fold increase in their binding to EC (Fig. 5). Combined addition of IL-2 and TNF-α (10^2 U/ml) elicited at 5 min a further increase in the adherence of activated NK cells (Fig. 5a). Control experiments indicated that treatment with TNF-α alone does not exert any significant effect (data not shown). Furthermore, NK cells preincubated with IL-2, for longer times (i.e. 20 h) exhibited a marked enhancement of their adhesion to EC (data not shown), as observed previously [12]. Finally, dose–response studies showed that peak-stimulation of NK adhesion to endothelial cells was observed using IL-2 at 10^3 U/ml, a significant effect with IL-2 at 10^2 U/ml and no effect using lower IL-2 concentrations (i.e. 10 or 1 U/ml) (data not shown). In a second series of experiments it was shown that, a short preincubation (30 min) of EC with IL-1 β (10^3 U/ml) or

Table 2. Phenotype analysis of EC non-adherent PBL after incubation with EC monolayer: effect of IL-2 + TNF-α preincubation

	% Positive cells							
	CD14		CD3		CD16		CD56	
Cell population	A	B	A	B	A	B	A	B
Total PBL	1.1	1.5	65.9	75.7	14.9	15	17	23.5
EC non-adherent PBL	2.3	0.9	64.7	78.7	14.1	17.9	16	21.6
EC non-adherent PBL + IL-2 + TNF-α	0.9	0.8	71	81.2	9.9	12.4	11.2	14

PBL were isolated from PBMC of 2 different subjects (A and B) after two cycles of plastic adherence (Total PBL). PBL were then incubated 5 min at 37°C in medium alone or in the presence of IL-2 (10^3 U/ml) and TNF-α (10^3 U/ml) and then plated without washing 30 min at 37°C on EC; at the end of the incubation, non-adherent PBL pre-incubated without additives (EC non-adherent PBL) or with IL-2 plus TNF-α (EC non-adherent PBL + IL-2 plus TNF-α), were recovered and analysed by indirect immunofluorescence using anti-CD14, -CD3, -CD16 and -CD56 Mabs.

TNF-α leads to a slight, but significant, rise of the adhesion of NK cells to endothelium (Fig. 5b).

In order to investigate whether the stimulatory effect of IL-2 on the adhesion to endothelium selectively affects NK cells, PBMC depleted of monocytes (peripheral blood lymphocytes, PBL) were first activated (10 min in the presence of IL-2 plus TNF-α), and then incubated with EC. The percentage of the different lymphocyte subpopulations among non-adherent cells was finally assayed. After binding of PBMC to EC the percentage of unbound $CD3^+$ T lymphocytes and $CD16^+/CD56^+$ NK lymphocytes remained unmodified, as compared to the values observed before incubation with EC (Table 2). In contrast, treatment with IL-2 combined with TNF-α induced a slight increase of the percentage of T lymphocytes and a marked decrease of NK cells in the non-adherent population (Table 2).

Expression of adhesion molecules on the membrane of NK cells after IL-2 activation

To characterise the dynamic changes in cell adhesion molecules (CAM) expression induced by *in vitro* IL-2 activation, purified NK lymphocytes were incubated in medium containing IL-2 (10^3 U/ml) and then analysed at 10 min, 30 min and 18 h. NK cells demonstrated consistent changes in their CAM phenotype after activation with IL-2. Thus, CD11a antigen expression showed a slight increase early after IL-2 activation and a marked elevation at later times (18 h) (data not shown). Furthermore, CD54 (ICAM-1) antigen expression is substantially increased at both 10–30 min and 18 h after IL-2 activation (data not shown). The expression of other CAM, i.e. CD56 or VLA-4 antigens, remained unmodified at all times of observation (data not shown here).

DISCUSSION

In the present study we have investigated the kinetics of lymphocyte subpopulations immediately after high-dose IL-2 infusion. The results indicate that as early as 5–15 min after the start of IL-2 bolus infusion the three subpopulations exhibiting *in vitro* NK activity virtually disappear from peripheral blood. It is of interest that these populations are highly heterogeneous: some are T cells (e.g. gamma–delta lymphocytes), others represent the 'NK lineage' ($CD3^-$ NK cells) and finally other ones pertain to both T and NK lineages ($CD4^+$TCR α/β^+ lymphocytes). In spite of this diversity, all of these lymphocyte types exhibiting NK activity were similarly induced by IL-2 to rapidly migrate from peripheral blood. In parallel, we observed that NK activity, as assayed by a standard ^{51}Cr release assay, is virtually undetectable in PBMC obtained a few minutes after the start of IL-2 infusion, as expected on the basis of the rapid and complete disappearance of the different NK subpopulations. Conversely, T lymphocytes not displaying NK activity (i.e. $CD56^-CD3^+$) are not induced by IL-2 to rapidly disappear from peripheral blood.

Our findings extend previous data indicating that NK lymphocytes disappear from peripheral blood as a consequence of *in vivo* IL-2 administration [6, 7]. In these studies, NK lymphocytes were evaluated (i) at later times than in the present study (i.e. at 30 min–1 h, 5–15 min), (ii) on the basis of PBMC reactivity with anti-CD16 and -CD57 Mab [6, 7], thus rendering it impossible to monitor the kinetics of the three NK subpopulations mentioned above.

IL-2 elicited the rapid disappearance of only a fraction of $CD8^+$ lymphocytes, which were identified on the basis of fluorescence intensity labeling and expression of NK markers (e.g. CD57). Previous studies showed that at later times (days and weeks) IL-2 therapy causes a marked decrease in the relative number of bright $CD8^+$ cells with an accompanying rise in dim $CD8^+$ cells [18]. Altogether, these results suggest that IL-2 administration exerts a biphasic effect on two subpopulations of $CD8^+$ cells: (a) at early times IL-2 induces a complete disappearance of dim $CD8^+$ cells associated with only a moderate decrease of bright $CD8^+$ cells; (b) at later times, IL-2 causes an increase of circulating dim $CD8^+$ cells, associated with a pronounced decline of bright $CD8^+$ cells.

It has been suggested that the IL-2 induced disappearance of NK lymphocytes may be related to massive adhesion of these cells to activated endothelium [9]. Indeed, NK cells exhibit a pronounced capacity to adhere to activated endothelium [8–19]. TNF-α, IFN-γ and IL-1-β may activate endothelial cells, *in vitro* [13] and *in vivo* [20], as shown by induction of endothelial adhesion structures. Interestingly, these cytokines are secondarily released after high-dose IL-2 *in vivo* administration [14–17]. Thus, the hypothesis may be considered that the complete, rapid and selective disappearance of NK cells occurring after IL-2 administration may be mediated by two alternative or complementary mechanisms: (i) the activation of NK cells by IL-2 and/or TNF-α, (ii) the activation of EC by IL-2, TNF-α and/or other secondary cytokines rapidly released after IL-2 infusion.

The first hypothesis is directly supported by *in vitro* experiments of cell adhesion to normal human endothelium showing that: (i) a short incubation (5–10 min) of NK cells with IL-2 alone or better combined with TNF-α stimulates the adhesion of these cells to EC; (ii) a 10-min incubation of PBMC with IL-2 alone or combined with TNF-α selectively induces the EC adhesion of NK lymphocytes, but not of other lymphocyte subpopulations.

The first hypothesis is also supported by experiments showing that IL-2 rapidly elicited a slight, but significant, increase in the expression of CAM, i.e. CD11a and CD54 [intercellular adhesion molecule-I (ICAM-1)] on NK cells. These two molecules are involved in mediating the interaction of both resting and IL-2-activated NK cells to endothelium [12]: it is hence tempting to

suggest that their increased expression may at least in part mediate the IL-induced adhesion of NK cells to endothelium. It is noteworthy that the early effects of IL-2 on CAM expression on NK cells described here are in line with a previous analysis at later times (>20 h) of incubation with IL-2 [21].

The second hypothesis is also supported by NK cells/EC adhesion experiments involving preincubation of EC wtih IL-1 and TNF-α. However, in quantitative terms this second mechanism seems to be less relevant as compared to the first one. The possibility cannot be excluded that other secondary cytokines rapidly released by activated NK and/or other cells may activate EC, thus contributing to the IL-2-induced adhesion of NK cells to endothelium. In this regard, agonists such as thrombin or histamine induce a rapid expression on EC surface of adhesion structures such as PAF and gp140.

It has to be emphasised that a discrepancy does exist between the *in vivo* and *in vitro* data; in fact, *in vivo* IL-2 induced a rapid and complete disappearance of all NK lymphocyte subpopulations, whereas *in vitro* IL-2 elicited after 5–30 min of incubation the adherence of not more than 20% of NK cells. This discrepancy may be related to the fact that the *in vitro* assay does not entirely reproduce the conditions occurring *in vivo* (i.e. presence of 'specialised' endothelial cells promoting the adherence of activated NK cells and/or release of specific humoral mediators).

Lymphocyte migration from peripheral blood into lymphoid tissues is dependent upon receptor–ligand interactions between lymphocytes and specialised EC in the high venules of lymph nodes [22, 23]. Although normal EC exhibit a low affinity for circulating lymphocytes, cytokines released at sites of inflammation and in the course of immune reactions, may cause endothelial activation, associated with an increased expression of surface antigens [13]: these include the adhesion protein ELAM-1 (endothelial leucocyte adhesion molecule), which binds neutrophils and monocytes [24], ICAM-1 and ICAM-2 which interact with all leucocytes [25] and VCAM-1 (vascular CAM), which binds lymphocytes and monocytes [26].

The lymphocyte membrane molecules mediating the interaction with EC pertain to the integrin family. Integrins have been subdivided into three major subgroups: the beta-2 subfamily (CD11a, CD11b and CD11c) is mostly involved in cell–cell interactions with the immune system [27–30], whereas beta 1 and beta 3 integrins subfamilies predominantly mediate cell attachment to the extracellular matrix [27–30]. In this context, it is of interest that integrin molecules are differentially expressed on NK vs. T cells: thus, CD11a/CD18 is the only heterodimer normally present on T lymphocytes, whereas all three CD11/CD18 heterodimers (a, b and c) are expressed on NK cells [27–30].

Further studies are in progress in an attempt to elucidate the membrane molecules involved in the *in vivo* IL-2-induced adherence of activated NK cells to endothelium. In this regard, a recent study allowed to identify the membrane molecules involved in the *in vitro* interaction of both resting and IL-2-activated NK lymphocytes to resting or activated EC [16]. The β-2 leukocyte integrin CD18/CD11a was identified as the major adhesion pathway of both resting and IL-2-activated NK cells to unstimulated EC. In addition, the interaction of resting or IL-2-activated NK cells to activated EC involves the VLA-4 (α-4 β-1) with vascular CAM (1 receptor/counter receptor pair).

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Does Extragonadal Presentation Impart a Worse Prognosis to Abdominal Germ-cell Tumours?

James J.A. McAleer, Judy Nicholls and Alan Horwich

The prognostic significance of extragonadal rather than gonadal presentation of germ-cell tumour in 51 patients presenting between 1979 and 1988 with abdominal tumours was compared with that of 51 control patients with testicular primary tumours matched for bulk of disease, serum tumour marker concentration, age and year of treatment. Very large volume tumour was found at initial staging in 24 extra-gonadal cases (47%) and high tumour markers in 29 (57%). Actuarial survival at 2 and 5 years was 82% and 70% for cases and 78% and 63%, respectively, for controls. These outcomes were not significantly different and the relative hazard of death for cases compared with controls was 0.7 (95% confidence intervals 0.3–1.5). Thus the presentation of germ-cell tumours with a retroperitoneal mass does not itself adversely influence prognosis compared with testicular presentation with equivalent disease extent. However it is rare for extragonadal presentation to be associated with small volume disease.
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INTRODUCTION

APPROXIMATELY 5–10% of male patients with germ-cell tumours present without an overt testicular primary [1]. These presentations are termed "extragonadal" although in some cases they may arise from a small occult testicular primary tumour. It has been suggested that extragonadal presentation is associated with a poor prognosis [2, 3] and in the classifications used by the Memorial Sloan Kettering Cancer Center and by the National Cancer Institute all such patients are classified as having an adverse prognosis regardless of other features of their illness [4]. However, the lack of early symptoms with extragonadal presentation gives rise to a pattern of bulkier and more advanced disease at presentation than with testicular primary tumour and may account for the perception that the prognosis is worse. We have therefore compared the prognosis of patients with extragonadal germ-cell tumour (EGCT) to that of matched controls presenting with a testicular primary tumour to determine if extragonadal presentation is an independent indicator of poor prognosis, or merely linked to extent of metastatic disease.

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PATIENTS AND METHODS

Patient selection

Patients selected for this study were diagnosed in the years 1979 to 1988 inclusive and met the following criteria: diagnosis of germ-cell malignancy (histology or raised serum levels of alphafetoprotein (AFP) or beta sub-unit of human chorionic gonadotropin (HCG); and both testes present in scrotum without palpable testicular tumour. Patients with disease limited to the mediastinum or pineal area were excluded as there were inadequate numbers of matched controls. 51 patients fulfilled