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# Interleukin-6 enhances the induction of human lymphokine-activated killer Cells

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**Summary.** Human peripheral blood mononuclear cells develop a powerful lytic capacity when cultured in vitro with interleukin-2 (IL-2), becoming lymphokine-activated killer cells (LAK cells). As part of an investigation into means of influencing this process, the effect of other cytokines has been examined. In this study we describe the ability of interleukin-6 (IL-6) to regulate the induction and function of human LAK cells. The results show that substitution of IL-6 for IL-2 did not lead to the development of functional LAK cells, nor was IL-6 able to alter the lytic capacity of established LAK cells. However, when IL-6 was included with IL-2 during the induction phase of the LAK cells, the resulting cells displayed considerably greater lytic activity than those prepared with IL-2 alone. This effect was IL-6 dose-related. These results indicate that LAK cell development may be positively regulated in vitro; the implications of this observation for the clinical usage of LAK cells are discussed.

## Introduction

The ability of human peripheral blood mononuclear cells to be transformed by interleukin-2 (IL-2) into potent lytic effectors has attracted much interest as a therapeutic strategy for the treatment of advanced human tumours [13, 15]. It is of particular interest that in vitro they kill tumour cell targets in a non-MHC-restricted manner, while leaving normal tissue undamaged (as reviewed by Rosenberg and Lotze [14]).

It is difficult to isolate LAK cells ex vivo, but easy to generate them in vitro and this suggests that their development must be closely regulated within the body, if it is permitted at all. The generation of such potent effector cells may not be desirable physiologically, especially when they are not constrained by the usual requirement for antigen to be recognised in the context of self major histocompatibility complex (MHC) molecules. However, it would give a therapeutic advantage to the clinician if he were able to overcome this in vivo regulation in certain circumstances, such as the post-surgical treatment of advanced malignancies.

This question has been addressed by examining the ability of cytokines other than IL-2 to influence the induction of human LAK cells. Previous investigations have suggested that several cytokines are able to inhibit human LAK cell development severely; in particular, transforming growth factor β [9], IL-4 [3] and IL-3 or IL-4 [5]. Thus, it appears that several immuno-physiological signals can suppress LAK cell generation in the presence of IL-2. Natural materials that promote LAK cell induction have proved more difficult to identify but it has been shown that interferon γ, interferon β1 and IL-1β are able to prolong the induction time for LAK cells with resultant higher yields of cytolytic activity [11]. In this report we describe how recombinant human IL-6 can greatly increase the lytic activity of cells recovered after a 4-day induction period with IL-2.

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## Materials and methods

*Isolation and preparation of human LAK cells.* Samples of peripheral venous blood (20 ml) were obtained from healthy adult volunteers; both male and female donors were used. Peripheral blood mononuclear cells were isolated by density centrifugation over Ficoll-Hypaque (Pharmacia) then washed three times in culture medium. Cells were resuspended to a density of $2 \times 10^6$/ml and cultured in 1-ml volumes in 24-well plates (Sterilin); experiments were established in duplicate. The culture medium employed was Ham's F10, supplemented with 10% (v/v) foetal calf serum and containing 4 mM glutamine; cells were incubated at 37° C in a humidified atmosphere of 5% $CO_2$ in air. All media components were from Flow Laboratories (Rickmansworth, UK). IL-2 (human recombinant, from Genzyme, Boston, USA) was obtained from Koch-Light Ltd (Haverhill, UK) and added to a final concentration of 200 units/ml (40 ng/ml). Cells were cultured for 4 days, after which time they were harvested, counted and designated LAK cells. Where appropriate, human recombinant IL-6 (Genzyme) was added to final concentrations of 10, 50 or 100 ng/ml and anti-IL-6 (Genzyme) at 25 μg/ml.

*Cytotoxicity assay.* An 18 h assay was deliberately chosen to mimic in vivo conditions, where LAK cells are expected to function for long periods [5]. The target cell was the natural-killer – resistant ovarian tumour cell line OWmM1, which we have previously shown to have antigenic characteristics of primary human ovarian-tumour-derived cells [1]. These were cultured at a level of $2 \times 10^4$ cells/well in

96-well flat-bottom plates (Sterilin) and allowed to adhere overnight. LAK cells were then added to an effector-to-target cell ratio of 5:1. In some cases (see Results) IL-2 and/or IL-6 were present during the effector phase. At the end of this time, LAK cells and dead target cells were removed by gentle but thorough washing with complete culture medium and the remaining viable target cells quantified by the MTT assay [4, 5, 8]. In this method, the enzymic reduction over 4 h of the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Koch-Light Ltd) results in the production of blue crystals, which can be dissolved in dimethylsulphoxide. The resulting absorbance ($_{595}A$) is directly proportional to the number of viable cells present at the start of the 4 h incubation, and the percentage kill achieved in the various test wells was calculated by comparison with wells which contained only OWmM1 targets and no effectors.

## Results

### *Interleukin-6 enhances the induction of human LAK cells by IL-2*

We first examined the effect of stimulating peripheral blood lymphocytes with either 100 ng/ml IL-6 or 40 ng/ml IL-2 (previously seen to be optimal [5]) or a combination of the two. The resultant LAK activity was measured in the absence of cytokines, for the 18 h period described. The experimental results shown in Table 1 illustrate that IL-6 greatly enhanced the induction of LAK activity, as shown by a greater percentage killing of OwMm1 cells. In the case of donors 1–3, this effect of IL-6 on IL-2-driven LAK cell induction was shown to be dose-related (Fig. 1). In an additional experiment (Fig. 2) it was clearly demonstrated that 25 μg/ml rabbit anti-(human IL-6), when present with 100 ng/ml IL-6 and 40 ng/ml IL-2 during the induction phase, resulted in LAK activity no different from that expected with 40 ng/ml IL-2 alone.

These data suggest strongly that IL-6 is able to act in the presence of IL-2 to enhance the cytotoxic capability of the activated peripheral blood lymphocytes.

**Table 1.** Interleukin-6 augments the IL-2-driven induction of human lymphokine-activated killer cells[a]

| Donor | Killing (%) following cytokine stimulation | | | |
|---|---|---|---|---|
| | None | IL-6 | IL-2 | IL-6+IL-2 |
| 1 | 24±2 | 29±3 | 54± 6 | 86±14 |
| 2 | 18±7 | 18±2 | 32± 7 | 68± 9 |
| 3 | 17±3 | 18±4 | 26± 4 | 52±11 |
| 4 | ND | 28±9 | 41± 4 | 73±12 |
| 5 | ND | 11±5 | 29± 8 | 56± 4 |
| 6 | ND | 17±2 | 53± 3 | 67±13 |
| 7 | ND | 24±7 | 68± 9 | 82±10 |
| 8 | ND | 32±4 | 47±12 | 78± 7 |

[a] Human peripheral blood lymphocytes were exposed to IL-6 alone (100 ng/ml), IL-2 alone (40 ng/ml) or a combination of 100 ng/ml IL-6 plus 40 ng/ml IL-2. After 4 days the cells were treated for LAK cell activity as described in the text. The mean percentage killing±standard deviation of six replicate wells is shown

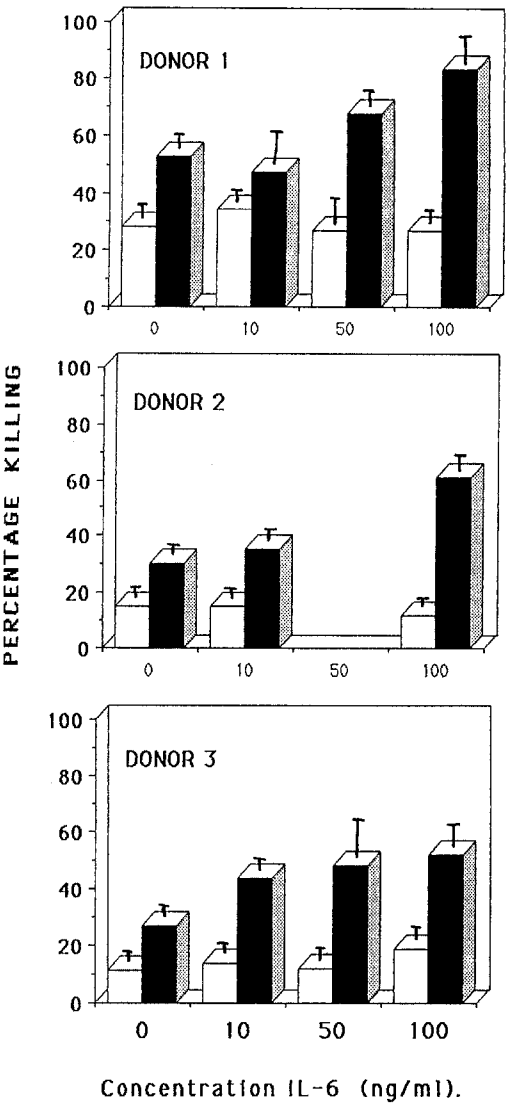


**Fig. 1.** Interleukin-6 enhances the production of LAK cell activity by interleukin-2 but does not support LAK cell production by itself. LAK cells were generated as described in the text and applied to target cells at an E:T = 5:1. Killing was allowed to proceed for 18 h in the absence of exogenous cytokines and the percentage killing achieved was calculated. The results show the mean±standard deviation of six replicate wells per test point. The *open bars* show results when increasing amounts of IL-6 alone were used during the induction phase, the *solid bars* illustrate the effect of titrating IL-6 in the presence of 40 ng/ml IL-2

### *Interleukin-6 does not increase the number of LAK cells recovered*

It was of interest to investigate whether the presence of IL-6 led to an increase in cell growth in the presence or absence of IL-2.

Table 2A shows the total number of cells recovered from test wells receiving IL-6 alone. These wells yielded the same number of cells as untreated wells ($2\times10^6$–$3\times10^6$ cells/ml recovered), this representing a minor increase over the $2\times10^6$ cells plated. In addition, cells recovered from wells treated with IL-6 alone were morphologically similar to the non-stimulated cells obtained from control wells (not shown). Table 2B compares the

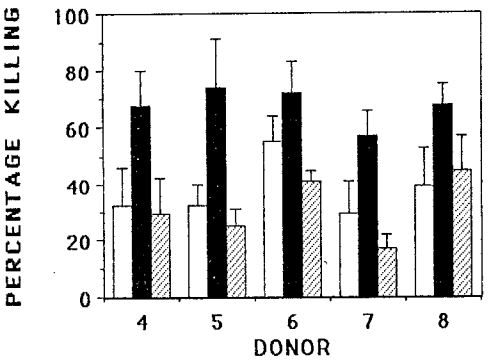


**Fig. 2.** Anti-IL-6 neutralises the effect of IL-6 on LAK cell induction. LAK cells were generated as described above and additionally in the presence of 25 μg/ml rabbit anti-(human IL-6). The *open bars* represent LAK activity generated by 40 ng/ml IL-2; the *closed bars* show the results with 40 μg/ml IL-2 + 100 ng/ml IL-6; the *hatched bars* give the killing generated by 40 ng/ml IL-2 + 100 ng/ml IL-6 + 25 μg/ml anti-IL-6 antibody. The mean ± SD of six replicate wells is shown for donors 4–7 and of four replicates for donor 8

number of cells obtained following IL-2 treatment in the presence and absence of IL-6. Again, no difference in cell numbers due to the presence of IL-6 was noted although there was an IL-2-induced cell proliferation ($2 \times 10^6$ cells/ml plated, $3.56 \times 10^6 - 5.88 \times 10^6$ cells/ml recovered). In addition, the cells all showed the activated morphology typical of IL-2 treatment.

*Interleukin-6 does not affect LAK cell function*

We next addressed the question of whether IL-6 was able to influence the effector phase of LAK cell function. This was achieved by inducing LAK cells in the presence of IL-2 alone, then performing the cytotoxicity assay in the presence of the test cytokines. The experimental results are shown in Table 3 and demonstrate that IL-6 has no effect

**Table 2.** Interleukin-6 does not affect the number of LAK cells harvested[a]

| Stimulation | $10^{-6} \times$ Cells recovered ($ml^{-1}$) | | |
|---|---|---|---|
| | Donor 1 | Donor 2 | Donor 3 |
| A | | | |
| None | 2.64 | 2.85 | 3.72 |
| IL-6 (10 ng/ml) | 2.66 | 2.56 | 2.96 |
| IL-6 (50 ng/ml) | 2.66 | ND | 2.54 |
| IL-6 (100 ng/ml) | 2.20 | 2.34 | 3.48 |
| B | | | |
| IL-2 (40 ng/ml) | 5.24 | 3.73 | 5.88 |
| IL-2 + IL-6 (10 ng/ml) | 4.95 | 3.56 | 5.06 |
| IL-2 + IL-6 (50 ng/ml) | 4.95 | ND | 5.23 |
| IL-2 + IL-6 (100 ng/ml) | 4.50 | 3.87 | 5.70 |

[a] LAK cells were generated in the presence of the above cytokines; $2 \times 10^6$ cells were plated into two wells of a 24-well plate per test point. After 4 days, cells from each point were harvested, pooled and counted. The average cell number thus obtained is shown

**Table 3.** Interleukin-6 does not affect the ability of LAK cells to kill the tumour cell target[a]

| Cytokines present | Killing (%) | | |
|---|---|---|---|
| | Donor 4 | Donor 5 | Donor 6 |
| None | 53 ± 8 | 32 ± 10 | 68 ± 12 |
| IL-6 (10 ng/ml) | 60 ± 5 | 30 ± 4 | 58 ± 10 |
| IL-6 (100 ng/ml) | 47 ± 5 | 32 ± 9 | 42 ± 9 |
| IL-2 (40 ng/ml) | 76 ± 13 | 42 ± 4 | >99 |
| IL-2 + IL-6 (10 ng/ml) | 81 ± 12 | 44 ± 13 | >99 |
| IL-2 + IL-6 (100 ng/ml) | 76 ± 5 | 36 ± 11 | 89 ± 14 |

[a] LAK cells were generated by exposure to IL-2 alone (40 ng/ml) and target cells were exposed as described for Fig. 1, but in the presence or absence of IL-2 and/or IL-6 as shown. IL-6 was unable to affect the LAK cell function, either positively or negatively. The mean ± standard deviation of six replicate wells per test point is shown

on the killing achieved by the cells of any of three donors, irrespective of whether IL-2 is also present.

## Discussion

We set out to investigate physiological signals that could influence the development of human LAK cells. In this report we describe how the cytokine interleukin-6 enhances the effect of IL-2, with an increase in the resulting cytolytic activity (Table 1).

LAK cells harvested after exposure to IL-2 and IL-6 showed a greatly increased killing ability, in a manner which was dependent upon the concentration of IL-6 present during the induction phase (Fig. 1).

Several possible explanations exist for this observation. First, that IL-6 was inducing a cytotoxic population additional to that stimulated by IL-2; however, no killing was achieved by cells stimulated by IL-6 alone and so this possibility can be discounted. Secondly, that IL-6 rendered the target cells more susceptible to LAK cell lysis, or that IL-6 enhanced the cytotoxic function of LAK cells; the results shown in Table 3 suggest that this is not the case. Thirdly, that increased proliferation of activated cells in response to the presence of IL-6 during the induction phase would lead to the development of a greater number of lytic effectors, which would be reflected in the functional assay of the fixed E:T ratio as an increase in measured killing. However, IL-6 did not influence the number of cells harvested after the induction period (Table 2).

Two probable explanations remain. First, that IL-6 and IL-2 act together to develop cytotoxic function in a subpopulation of peripheral blood mononuclear cells, which do not become active under the influence of either material alone. Secondly, that IL-6 acts on the same subpopulations as does IL-2 (but only in the presence of IL-2) in such a way as to amplify the lytic capability of these cells. At present we are unable to discriminate between these two possibilities and this is an object of active investigation, although it is of interest that in the mouse, IL-6 combines with IL-2 and interferon γ to promote the development of cytotoxic T cells from immature thymocytes [12].

The complex nature of the action of IL-6 within the "cytokine network" [17] has led to many activities of this

material being described; for example, IL-6 enhances the production of murine multipotent stem cells from bone-marrow culture [7]. Of particular interest is the pyhsiological role of IL-6 as the causative agent in the induction of acute-phase proteins in vitro [2] and in vivo [6]. Elevated serum and urine IL-6 levels are associated with acute-phase protein titres in burns patients [10] and following surgery [16]. Thus, IL-6 appears to be present systemically within the body during trauma (such as surgery) and as such represents one of the few natural instances where a cytokine reaches measurable levels in the peripheral circulation. If this were shown to be the case in cancer patients then it may allow the in vivo generation of specific cytotoxic T cells or LAK cells by post-surgical administration of IL-2.

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