Clin. exp. Immunol. (1988) **74**, 166–170

Interleukin-3 and interleukin-4 each strongly inhibit the induction and function of human LAK cells

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(Accepted for publication 11 June 1988)

SUMMARY

We have examined some of the factors which may regulate the generation and function of human lymphokine-activated killer (LAK) cells. In particular we have studied the effect of cytokines other than interleukin-2 on the ability of human LAK cells to kill human tumour cells. By exposing human tumour cells to human LAK-cells in the presence and absence of interleukin-3 or interleukin-4, we were able to demonstrate that each of these cytokines was able to severely reduce the amount of tumour cell killing. Additionally, we examined the effect of interleukin-3 or interleukin-4 on the production *in vitro* of human LAK cells by including these materials with interleukin-2 while LAK-cells were being induced. These results showed that not only were these cytokines able to inhibit human LAK-cell function, but they were also able to strongly reduce the ability of interleukin-2 to generate human LAK-cells.

Keywords interleukin-3 interleukin-4 LAC-cells

INTRODUCTION

The recent observation that normal splenic or peripheral leucocytes can be activated to become potent killing cells by prolonged exposure to interleukin-2 (IL-2) has formed the basis for a large body of work on so-called 'lymphokine-activated killer cells'—LAK-cells. LAK-cells have been described in both mouse and human systems and are defined as being able to kill a wide range of tumour cell targets, while leaving normal cells undamaged. Therefore, they have naturally aroused much interest as potential tools in cancer therapies. As recently reviewed (Rosenberg & Lotze, 1986), LAK-cells have been used to treat advanced tumours in humans with some success, notably in the case of malignant melanoma. The technique has not been successful with a wide range of tumour types however and a number of novel approaches are being developed which build on the LAK-cell concept (Kradin & Kurnick, 1986; Rosenberg, 1988).

One reason for the failure of such treatments to live up to expectations may be the influence of cytokines on the LAK-cells once they are re-introduced into the body. For this reason we have begun to study the ability of cytokines (other than IL-2) to regulate the induction of human LAK-cells and their function. We were particularly interested in the effects of interleukin-3 and interleukin-4 (IL-3, IL-4) because both of these molecules have been shown to have powerful growth and activation effects on many cell-types of the immune system (Ihle & Weinstein, 1986; Lee *et al.*, 1986). In particular, IL-4 is able to promote the growth of human T cells (Spits, *et al.*, 1987), which are known to form a proportion of LAK-cell precursors (Tilden, Itoh & Balch, 1987). Additionally, IL-3 can regulate mouse NK-cell function (Kalland, 1986) and NK-like cells also form a component of the LAK effector population (Kalland, *et al.*, 1987).

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MATERIALS AND METHODS

Induction of human LAK-cells

Leucocytes were isolated from the peripheral blood of healthy adult volunteers by density gradient centrifugation over Ficoll-Hypaque (Pharmacia). After washing, the cells were suspended at a density of 10^6/ml in Ham's F10 culture medium containing 10% (v/v) fetal calf serum, 4 mM glutamine and 1% (v/v) penicillin/streptomycin mixture (all components from Flow Laboratories, Rickmansworth, UK). To this was added a variety of human cytokines as described below. LAK-cells were induced over a 3 day period at 37°C in a humidified atmosphere containing 5% CO_2.

Cytokines

The human cytokines IL-2, IL-3 and IL-4, produced by recombinant DNA technology, were obtained from Koch-Light Ltd. (Haverhill, UK). The specific activities of these materials were: IL-2, 5·0 units/ng; IL-3, 100·0 units/ng and IL-4, 100·0 units/ng. For LAK-cell induction, IL-2 was routinely used at 40

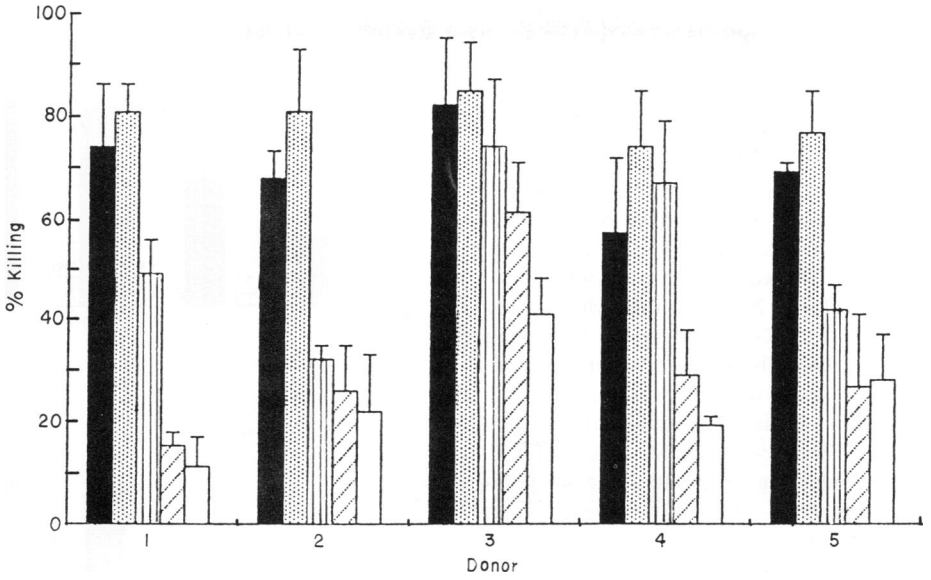

Fig. 1. Interleukin-4 inhibits human LAK-cell function. OVM-1 target cells were grown to confluence (2×10^4 cells/well) and exposed to 10×10^4 human LAK-cells (E:T = 5:1) for 18 h in the presence or absence of a range of concentrations of human IL-4. Five LAK-cell donors were tested and in all cases IL-4 was shown to mediate a dose-dependent inhibition of LAK-cell killing. The mean ± s.d. of four replicates is shown. Concentrations of IL-4 (ng/ml): ■ 0; ▨ 2·5; ■ 5·0; ■ 10·0; □ 20·0.

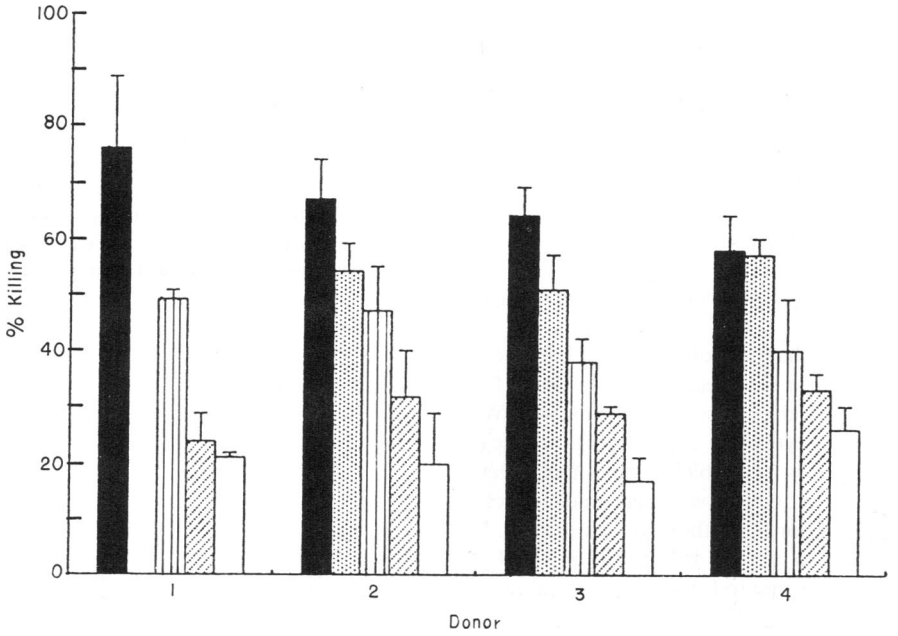

Fig. 2. Interleukin-3 inhibits human LAK-cell function. OVM-1 target cells were exposed to LAK-cells from four donors in the presence of a range of concentrations of IL-3, as described for Fig. 1. In each case IL-3 was able to inhibit LAK-cell killing in a dose-related manner.

ng/ml (200 U/ml); IL-3 and IL-4 were both added at 2·5–20 ng/ml (250–2,000 U/ml). These concentrations were chosen to straddle the optimal concentrations for IL-3 and IL-4 in their usual test systems (1,000 U/ml for IL-3 and IL-4) as quoted by the suppliers.

Target cells

Two NK-resistant target cell-lines were used, both of which were derived from human ovarian tumours; OVM-1 and SCC-1. We have previously shown that these lines are immunologically similar to primary human tumours of this type (Al-Azzawi, Stimson & Govan, 1987).

Cytotoxicity assay

The cytotoxicity assay was carried out over a period of 18 h by an adaptation of the method of Johnson & Adams (1986). The prolonged period was specifically chosen because we wanted to

evaluate the effects of cytokines on human LAK-cell function under conditions which would allow cytokines to exert an influence. Also, it was perhaps more representative of the current clinical usage of LAK-cells, where they are expected to function *in vivo* over several days.

OVM-1 or SCC-1 cells were grown to confluence in 96-well plates (Sterilin) then exposed to human LAK-cells in the presence and absence of IL-4 or IL-3 as described below. When examining the effects of IL-3 and IL-4 on LAK-cell function, an effector-to-target cell ratio of 5:1 was used throughout (E:T = 5:1). A range of E:T ratios (1:1 to 20:1) was used when testing the effects of cytokines of LAK-cell induction. Following overnight incubation, the LAK-cells and dead target cells were removed by a gentle but thorough washing procedure.

The number of live cells remaining in each well was then assessed directly by the MTT method of Mosmann (1983). Culture medium (200 μl) containing 1·0 mg/ml of the histological stain (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Koch-Light) was added to each well and incubated for 4 h at 37°C. Coloured crystals develop round functioning mitochondria as a result of enzymatic cleavage of the MTT tetrazolium ring to form a formazan. Following the 4 h incubation period all the medium was carefully removed and the formazan crystals dissolved by the addition of 200 μl of dimethylsulphoxide (DMSO; Sigma). The resulting blue/purple colour was measured in a multiwell spectrophotometer (Flow Laboratories) by reading the optical density at 595 nm.

This colour intensity was converted into a cell number by comparison with a standard curve of the appropriate target cell type, set up for each assay as described previously (Gallagher, Taylor & Willdridge, 1987). Four replicate wells were established for each test point.

RESULTS

IL-4 and IL-3 do not affect target cell growth or susceptibility to lysis

As a prelude to investigating the function of human LAK-cells in the presence of cytokines, it was necessary to evaluate the effects of these cytokines on the target cells themselves. We examined the ability of the cytokines to kill the target cells directly and also whether they were able to make the targets more resistant to LAK-cell activity. It was clear that for both target cell types, neither IL-4 nor IL-3 inhibited their growth. In addition, pre-incubation with a high level of IL-4 or IL-3 (followed by washing and exposure to LAK-cells in the absence of any cytokine) failed to interfere with successful killing of these targets by LAK-cells (data not shown).

IL-4 and IL-3 inhibit human LAK-cell effector function

We next examined the effect of IL-4 or IL-3 on the killing ability of human LAK-cells by including one or other of these cytokines in the cytotoxicity assay. The experimental results are shown in Fig. 1 and Fig. 2 respectively.

Figure 1 describes the inhibition of killing observed when the cytotoxicity assay was carried out in the presence of a range (2·5 ng/ml to 20 ng/ml) of concentrations of IL-4. Results from five donors are shown and clearly illustrate a severe, dose-dependent inhibition of killing by IL-4. The degree of inhibition varied from donor to donor. The most powerful inhibition was

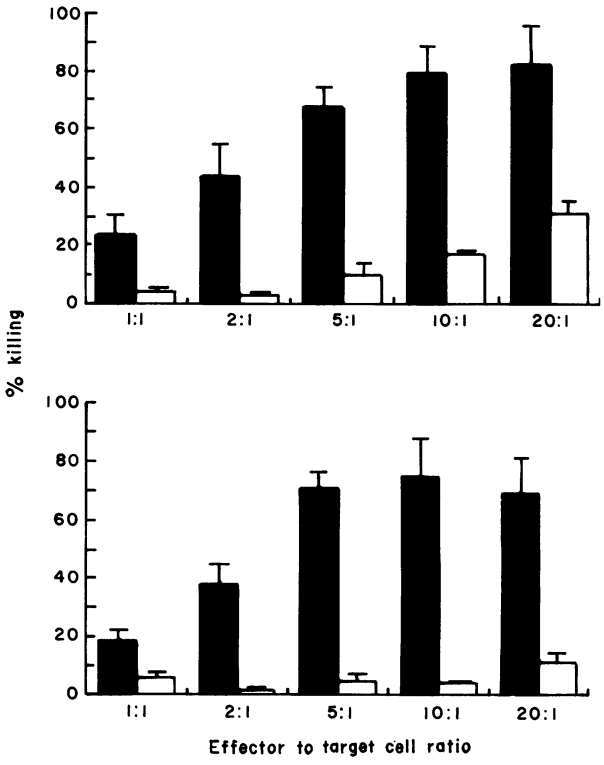

Fig. 3. Interleukin-4 inhibits the development of functional human LAK-cells. Peripheral blood cells from five donors were exposed to 40 ng/ml IL-2, in the presence or absence of 2·5 ng/ml IL-4, for 72 h then tested for the ability to lyse OVM-1 cells by examining the percentage kill achieved at different effector-to-target cell ratios. Four of the five donors showed a severe inhibition of LAK development. In three of these (upper) the percentage killing is reduced by almost two-thirds (E:T = 20:1) by 2·5 ng/ml IL-4. However, in one case (lower) the killing was reduced to the point where it was barely detectable. The mean ± s.d. of four replicates is shown. ■ IL-2 only; □ IL-2 and IL-4.

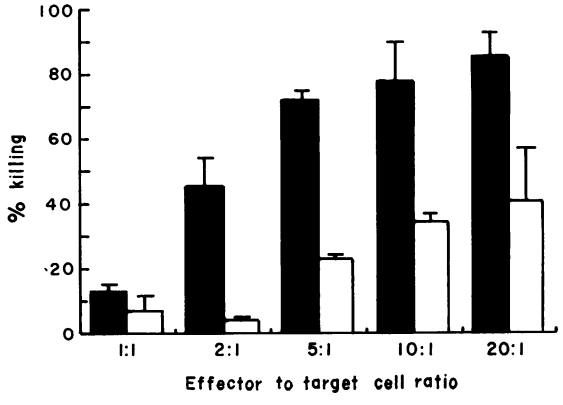

Fig. 4. Interleukin-3 inhibits the development of functional human LAK-cells. These experiments were carried out exactly as described for Fig. 3. The results show that at 5 ng/ml IL-3 is able to inhibit the development of LAK activity by over one-half (all donors). ■ IL-2 only; □ IL-2 and IL-3.

observed in the case of donor 1 (74% killing reduced to 11% in the presence of 20 ng/ml IL-4).

Figure 2 describes the inhibition of LAK-cell function observed in the presence of IL-3 for four donors. This effect was also dose-dependent over the IL-3 concentrations 2·5 ng/ml to 20 ng/ml. These results clearly show that IL-3 was able to severely reduce LAK-cell function. In particular, while human LAK-cells were able to cause 60–75% killing of OVM-1 cells in the absence of any cytokine (depending on the donor), this was reduced from 63% to 17% killing in the case of donor 3 (representing an 88% inhibition of LAK cell function for this donor) at the highest concentration of IL-3.

From these results it is clear that both IL-4 and IL-3 were able to strongly inhibit human LAK-cell function.

IL-4 and IL-3 inhibit human LAK-cell induction

We were unable to generate human LAK-cells under the influence of IL-4 or IL-3 alone (data not shown). This result contrasted with the observation of Mule, Smith & Rosenberg (1987), for murine LAK cells and mouse IL-4.

We next examined the generation of functional LAK cells when IL-4 or IL-3 was present, in addition to IL-2, during the three-day induction period. The resulting number of functional LAK-cells was then assessed by determining the percentage killing achieved at a range of effector-to-target cell ratios (E:T = 1:1 to 20:1). A number of concentrations of IL-4 and IL-3 were examined and IL-4 was found to be slightly more potent at inhibiting LAK-cell induction than was IL-3 (data not shown). The results presented here represent the effect of IL-4 at 2·5 ng/ml and IL-3 at 5 ng/ml each, in addition to IL-2 at 40 ng/ml. For each case, the percentage killing achieved by cells induced with IL-2 alone or IL-2 plus another material is shown, at a number of effector-to-target cell ratios. The experimental results for IL-4 and IL-3 are shown in Fig. 3 and Fig. 4 respectively.

Figure 3 shows that exposure to IL-4 during the induction-phase dramatically reduced the ability of the resultant cells to lyse OVM-1 targets, even at high (20:1) effector-to-target cell ratios. This effect has been observed in four out of five donors tested. In three cases, the reduction in killing was of the degree shown in Fig. 3a (82% killing reduced to 31% at E:T = 20:1). However, in one case (Fig. 3b) the generation of killing ability was almost completely inhibited, with no detectable killing observed even at E:T = 10:1, and only 11% at E:T = 20:1. In one case out of five LAK-cell induction was not affected by the presence of IL-4 (data not shown).

Figure 4 shows what happens when leucocytes are subjected to the influence of both IL-2 and IL-3 during the induction period. These results indicate that like IL-4, IL-3 is able to significantly inhibit the generation of human LAK-cells. This inhibition is not as great as that seen in the presence of IL-4, but is none the less severe. The results shown are for one donor whose results are representative of the three donors examined in this way. IL-3 appears to reduce the number of functional LAK-cells by one-half to two-thirds of that generated in the presence of IL-2 alone.

DISCUSSION

Lymphokine-activated killer cells appear to hold great potential as therapeutic tools for the management of advanced human tumours. However, despite impressive animal data, their success has not been transferred to the clinic, except perhaps in the case of malignant melanoma. While LAK-cells develop and are very active in the presence of IL-2, we felt that a knowledge of the effects of other cytokines on LAK-cell function might hold some key to improving their performance in therapy. It might also lead to an understanding of why LAK-cells appear to be a wholly *in vitro* phenomena; the cells being stimulated with one cytokine (IL-2) in the absence of the others which would normally be present in the body.

For these reasons we studied the effects on human LAK-cells of cytokines which are known to deliver activation and maturation signals to a wide range of cell types. We brought the IL-4 and IL-3 results together in this report because of their similarity; it is interesting that two materials which have strong growth promoting properties in some systems should be so inhibitory to human LAK-cells.

It is clear from the results described here that IL-4 and IL-3 are each able to inhibit both LAK-cell induction and LAK-cell function *in vitro* in the human system. The precursor cell type for the LAK-cell has not been completely defined but cells of the natural killer (NK-cell) lineage are known to represent a substantial proportion of 'pre-LAK' cells (Kalland *et al.*, 1987). Recent studies have shown that mouse NK-cell development is suppressed by IL-3, both *in vitro* and *in vivo* (Kalland, 1986; 1987). Interleukin-3 was shown to prevent the generation of NK-cells by acting at an early stage of their differentiation, although no regulatory effect on NK-cell function *in vitro* was recorded. Our data on human LAK-cells show that both induction and function are regulated by IL-3 and, while this appears to support the association between LAK-cells and NK-cells, there are a number of differences between the two sets of work. In particular, it is the difference in the cell source (mouse spleen/bone marrow versus human peripheral blood) which makes us wary of suggesting too great an association.

The difference in cell-source may account for the striking difference between our data with human LAK-cells and IL-4, and the mouse LAK-cell/IL-4 results of Mule *et al.* (1987). These authors show that the development of LAK-cells from mouse spleen is significantly enhanced by the addition of IL-4 to IL-2, and that IL-4 alone can result in LAK-cell generation. Our results for human cells are almost exactly the opposite: IL-4 alone cannot generate LAK-cells (data not shown) and can completely inhibit the IL-2-mediated induction of human LAK-cells when the two cytokines are present together. Furthermore, we have shown that IL-4 is able to inhibit human LAK-cell effector function by severely reducing the killing of two NK-resistant human ovarian cancer cell lines, when the assay was carried out in the presence of this cytokine. It was interesting to note however, that for all donors tested 2·5 ng/ml IL-4 was able to moderately augment LAK-cell *function* (Fig. 1), even though it severely inhibited LAK-cell *induction* at that concentration (Fig. 3). Higher concentrations of IL-4 were inhibitory in the cytotoxicity assay, as discussed. The reasons for this are as yet undefined but may relate to different effects of IL-4 on the two types of LAK-precursors (T cell-like and NK cell-like).

Thus, IL-3 and IL-4 strongly inhibit human LAK-cells *in vitro* and it is interesting to speculate on the implications of this inhibition for LAK-cell therapy. We feel it is important to know if materials likely to be present in the body will adversely affect LAK-cell function. Tumours are known to elicit both T cell and

B cell responses from the host *in vivo* (Al-Azzawi *et al.*, 1987; Rosenberg, 1988) and almost without exception any immune response *in vivo* results in the generation of a diverse range of helper T cell clones, necessary to drive B cell and cytotoxic T cell maturation. While the production of IL-2 and IL-4 in these clones appears to be mutually exclusive (Mosmann & Coffman, 1987), most helper clones make IL-3 as well as a variety of other cytokines; one might therefore expect to find IL-3 and IL-4 produced in the vicinity of tumours. When LAK-cells are introduced our results suggest that their function will be influenced by local cytokine production. Indeed, the IL-2 administered with LAK-cells may contribute to this by promoting the growth of such cytokine-secreting clones.

The results presented in this report suggest that *in vitro* studies on human LAK-cells and how their responses are influenced by cytokines other than IL-2 may improve our knowledge about how LAK-cells behave *in vivo* and therefore lead to their more efficient therapeutic use.

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