

# ANTI-TUMOR EFFECT OF HUMAN LYMPHOCYTES AND INTERLEUKIN-2

Pages with reference to book, From 45 To 49

Farzal Anwer ( Al Hada Armed Forces Hospital, Tail, Saudi Arabia. )

Mehdi Mesri, Robert C. Rees ( Department of Experimental and Clinical Microbiology, Sheffield Medical School, Sheffield, England. )

Shahana U. Kazmi ( Department of Microbiology, University of Karachi, Karachi. )

## ABSTRACT

We studied the anti-tumor effect of control human lymphocytes and interleukin-2 (IL-2) activated lymphocytes (lymphokine activated killer cells, LAK-cells), on two different cell lines: SW742 human colon adenocarcinoma and K562 human myeloid leukaemia cell line. Our results indicate that IL-2 augments the anti-tumor activity of human lymphocytes and these LAK-cells lyse the tumor cells very efficiently. Furthermore, we treated the target cells (SW742 and K562) with different cytokines in order to establish whether these cytokines have any effect on susceptibility to lysis by LAK-cells. Anti-tumor activity of human lymphocytes and IL-2 is discussed in this study (JPMA 43: 45, 1993).

## INTRODUCTION

Interleukin-2 (IL-2) was originally described as T cell growth factor and is significant to the growth and function of a variety of cell types of the immune system. IL-2 stimulates proliferation of activated cells of the lymphoid lineage and cells responsive to its action include activated T lymphocytes, natural killer cells (NK), lymphokine activated killer (LAK) cells, B lymphocytes and macrophages<sup>1-5</sup>. Grimm et al reported that human peripheral lymphocytes cultured for four days with 1,000 units of interleukin-2 become able to lyse fresh tumor cells with very high efficiency<sup>6</sup>. In experimental murine system, the adoptive transfer of the lymphokine activated killer cells along with repeated high doses of IL-2 induces a dramatic reduction in established tumor metastasis<sup>7</sup>. Clinical trials have shown that impressive tumor regression can be obtained with this protocol in about 20% of patients with advanced metastatic renal cell carcinoma and melanoma<sup>8,9</sup>. There are several types of lymphoid cells capable of mediating target cell lysis. These include classic cytotoxic T cells (CTL), natural killer (NK) cells, lymphokine-activated killer (LAK) cells and the antibody-dependent cell-mediated cytotoxicity (ADCC) effector population. Different types of effect or cells can be differentiated from one another because they carry cell surface proteins that act as identifying markers. Extensive studies have now been done on the phenotype of both the lymphocytes which develop LAK activity after culture with IL-2 (i.e., progenitor of cells with LAK activity) and the effector cells themselves. Although the initial studies on LAK activity suggested a shift in phenotype, from progenitor lacking T-cell as well as NK-cell markers to effector cells with T-cell marker, most of the subsequent studies have indicated that the progenitors and effector cells express a very similar pattern of markers<sup>10-12</sup>. It is now clear that most of the LAK activity from blood lymphocytes is generated from cells with the same characteristics as NK cells. NK cells have been closely associated with large granular lymphocytes (LGL) and the CD16 antigen has been found on almost all NK active cells<sup>13</sup>. The progenitor of LAK activity in human peripheral blood are mainly LGL with the CD3, CD16+, NKHY phenotype. In the present study we have reported the anti-tumor effects of control lymphocytes (without IL-2) and IL-2 activated lymphocytes on two different cell lines: SW742 colon adenocarcinoma cell line and K562 myeloid leukaemia. We also investigated the susceptibility to lysis of LAK cells of cytokine treated (TNF alpha, IL-1alpha and INF

alpha), K562 and SW742 cell lines. This was based on the hypothesis that the differences between the cell lines (in their susceptibility to lysis) could result from the expression or accessibility of some membrane structures (by cytokines treatment of a cell line), which play an important role in the formation of conjugates between targets and CD3 effect or lymphocytes followed by lysis.

## **MATERIAL AND METHODS**

### **K562 cell line**

K562 myeloid leukaemia cell line was a generous gift from Imperial Cancer Research Fund Laboratories, London and maintained as anon-adherent cell line in RPM I-I640 plus 10% new born calf serum.

### **SW742 cell line**

The SW742 colon adenocarcinoma cell line was a generous gift from Dr. Russel Greig, Smith Mine and Beecham\`s Laboratories and maintained as adherent cell line in RPMI-1640 medium plus 10% foetal calf serum.

### **Preparation of peripheral blood lymphocytes**

Peripheral blood was collected from normal healthy volunteer donors by venepuncture into sterile glass bottles containing the anticoagulant heparin (iO u/ml blood). The blood was diluted 1:1 with sterile PBS and carefully layered over LSM (lymphocytes separation medium) in a plastic universal at a ratio of 1:2, LSM:diluted blood with a sterile plastic 10 ml pipette. The density gradients were centrifuged at 400g for 35 mins. at room temperature. The rich buffy coat interface, between the LSM and plasma, was harvested carefully with a sterile glass pasteur pipette. The cells were washed once with PBS-FCS at 4°C 550g for 15 mins. and twice at 4°C, 400g for 6 mins. The lymphocytes were resuspended in RPMI and counted using the trypan blue exclusion method.

### **Generation of cytotoxic effector cells**

Lymphokine-activated killer (LAX) cells. PBMCs (peripheral blood mononuclear cells) obtained from peripheral blood of healthy donors were resuspended at the concentration of 4X10<sup>6</sup>/ml in RPMI containing 10% AB serum and IL-2 (500 u/ml). Following a 4 day incubation in a 24 well tissue culture plate, at 37°C in a humidified CO<sub>2</sub> atmosphere, cells were harvested, washed twice in PBS, resuspended at the concentration of 3X10<sup>6</sup>/ml and used as LAK effectors in the cytotoxicity assay.

### **4hours<sup>51</sup> Cr-release test (cytotoxicity assay)**

Target cells (K562, SW742) were grown and treated with cytokines (tumor necrosing factor 1000U/ml, interleukin-1 1000Ij/ml, interferon alpha 500U/ml) for 24 hrs. Target cells (in a 0.2 ml volume) were labelled for 1 h at 37°C with 60 mCi of <sup>51</sup>Cr as sodium chromate (Na<sup>251</sup>CrO<sub>4</sub>), washed twice with RPMI containing 10% NBS, resuspended in 10 ml of medium and incubated for a further 1 h at 37°C. Target cells were washed twice again and resuspended at the concentration of 1X10<sup>5</sup>/ml.

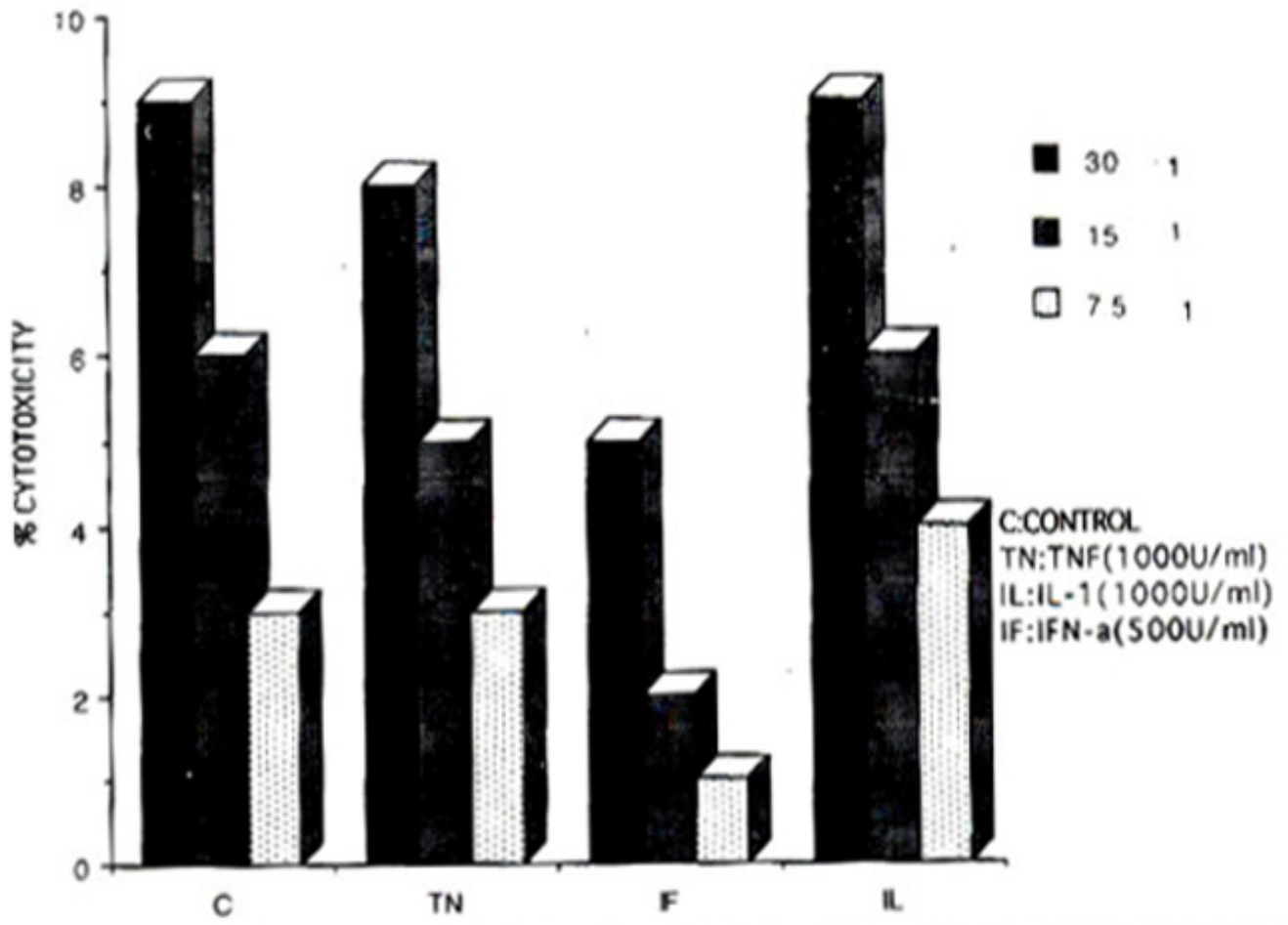
Cytotoxicity tests were performed in triplicate in round bottomed 96 well flexible assay plates. Effector cells (0.1 ml/well) were incubated with target cells (0.1 ml/well) at ratios of 30 to 1, 15 to 1 and 7.5 to 1 and the plates incubated at 37°C for 4 h in humidified 5% CO<sub>2</sub> atmosphere. Supernatant (0.1 ml) was harvested from each well and counted in a gamma spectrophotometer, Canberra Packard Ltd. The percent <sup>51</sup>Cr release was determined for each group following subtraction of the spontaneous release and the percentage cytotoxicity calculated by the formula:

(test release) — (spontaneous release) % cytotoxicity =  $\frac{\text{test release} - \text{spontaneous release}}{100 - \text{spontaneous release}} \times 100$

## **RESULTS**

Effect of cytokines on K562 susceptibility to lysis by human lymphocytes

Human lymphocytes and K562 cell line (with IL-1a, TNFa and IFNa and without cytokines) were prepared and used as effectors and targets in a 4 h <sup>51</sup>Cr-release assay at a maximum effector to target ratio of 30:1.



**Figure 1. % cytotoxicity of 24 h cytokine treated K562 cell line with lymphocytes.**

As shown in Figure 1 there was no significant difference in lysis of cytokine treated and untreated K562 cells by lymphocytes, lysis was less than 10% in all cases.

**Effect of cytokines on K562 susceptibility to lysis by IL-2 activated lymphocytes (LAK cells)**

The LAIC cells were produced by IL-2 treatment of PBMC and were used as effectors in a 4 h <sup>51</sup>Cr-release assay at a maximum effector to target ratio of 30:1 as before. There was about seven fold increase in lysis of K562 cells by IL-2 activated lymphocytes as compared to lysis by control lymphocytes (Figure 2, Table 1).

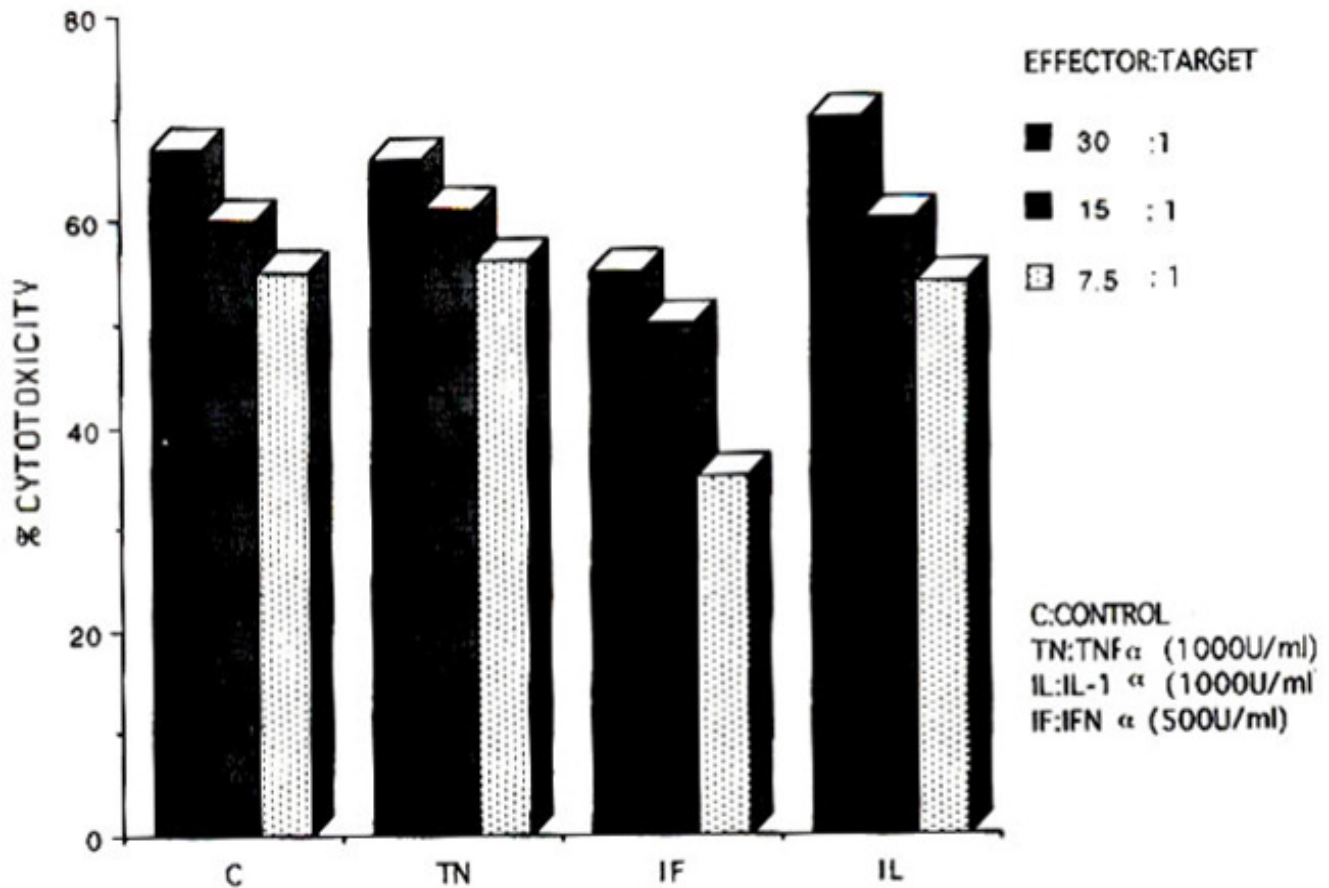


Figure 2. % cytotoxicity of 24 h cytokine treated K562 cell line with LAK-cells.

TABLE I. % cytotoxicity of cytokine treated K562 cell line with control and IL-2 activated lymphocytes, LAK-cells\*.

K562	Control lymphocytes	LAK-cells
Control	9%	67%
TNF alpha	8%	67%
IFN alpha	5%	55%
IL-1 alpha	9%	70%

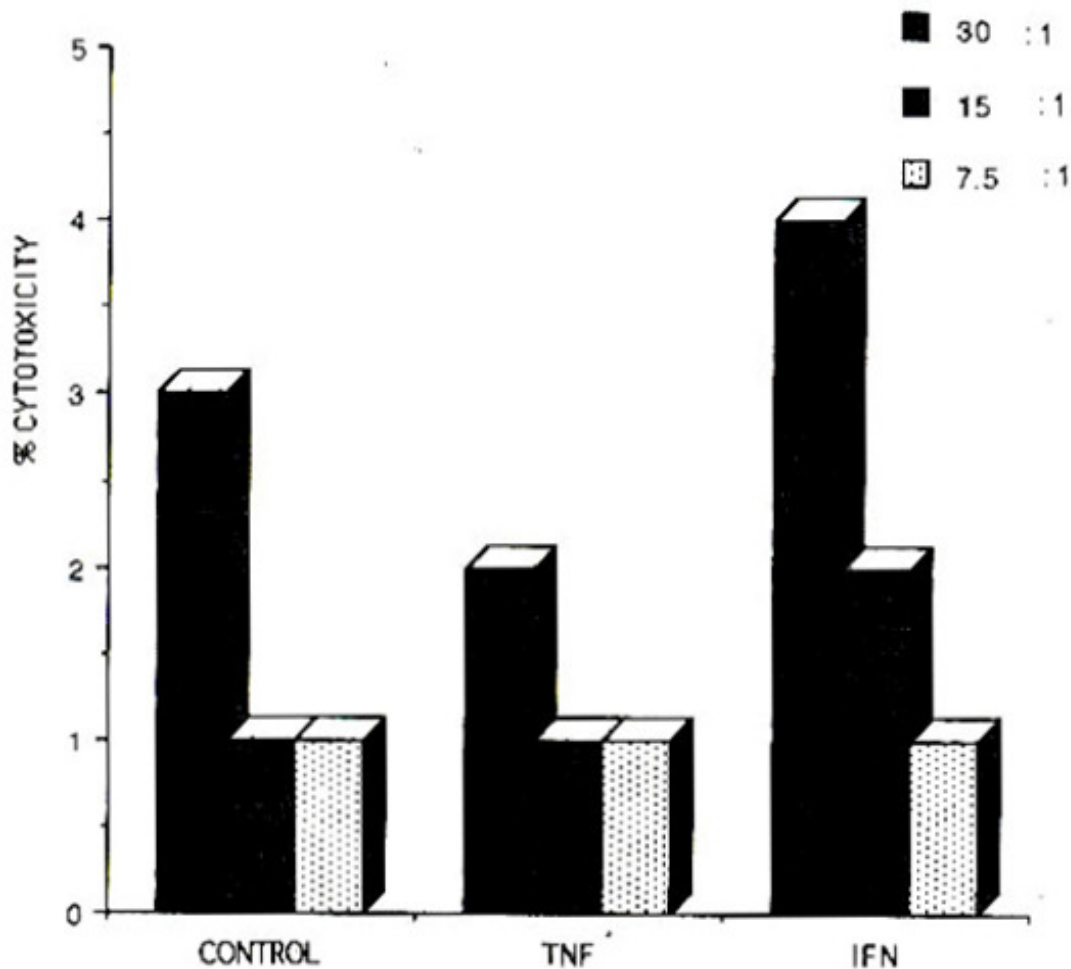
\*at 30:1, effector-target ratio.

Results are representative of several serially carried out experimental runs.

As shown in Figure 2, TNF  $\alpha$  and IL-1 $\alpha$  treated K562 cells did not show any considerable change in lysis by LAK cells, although some decrease was noticed in IFN $\alpha$ . Enhanced expression of MHC class I antigens with TNF $\alpha$  and IFN $\alpha$  did not correlate with a decrease in K562 susceptibility to lysis by LAK cells.

Effect of cytokines on SW742 cell line susceptibility to lysis by human lymphocytes

Cytokine treated SW742 cell line (TNF  $\alpha$  1000 u/ml and IFN  $\alpha$  500 u/ml for 24 hours) and without cytokines was used as target for lymphocytes in this experiment. A 4 h <sup>51</sup>Cr-release assay was set up as described before.



**Figure 3. % cytotoxicity of 24 h cytokine treated SW742 cell line with lymphocytes.**

As it is shown in Figure 3 that there was no significant killing of SW742 cell line by lymphocytes nor there was any effect on killing by cytokine treated SW742 cells. Lysis was less than 5% in all cases.

**Effect of cytokines on SW742 susceptibility to lysis by IL-2 activated lymphocytes (LAK cells)**

The LAK cells were used as effectors in a 4 h <sup>51</sup>Cr release assay at a maximum effector to target ratio of 30:1 as before. There was extensive killing of SW742 cells by IL-2 activated lymphocytes (compare Figure 4 with Figure 3, also see Table II).

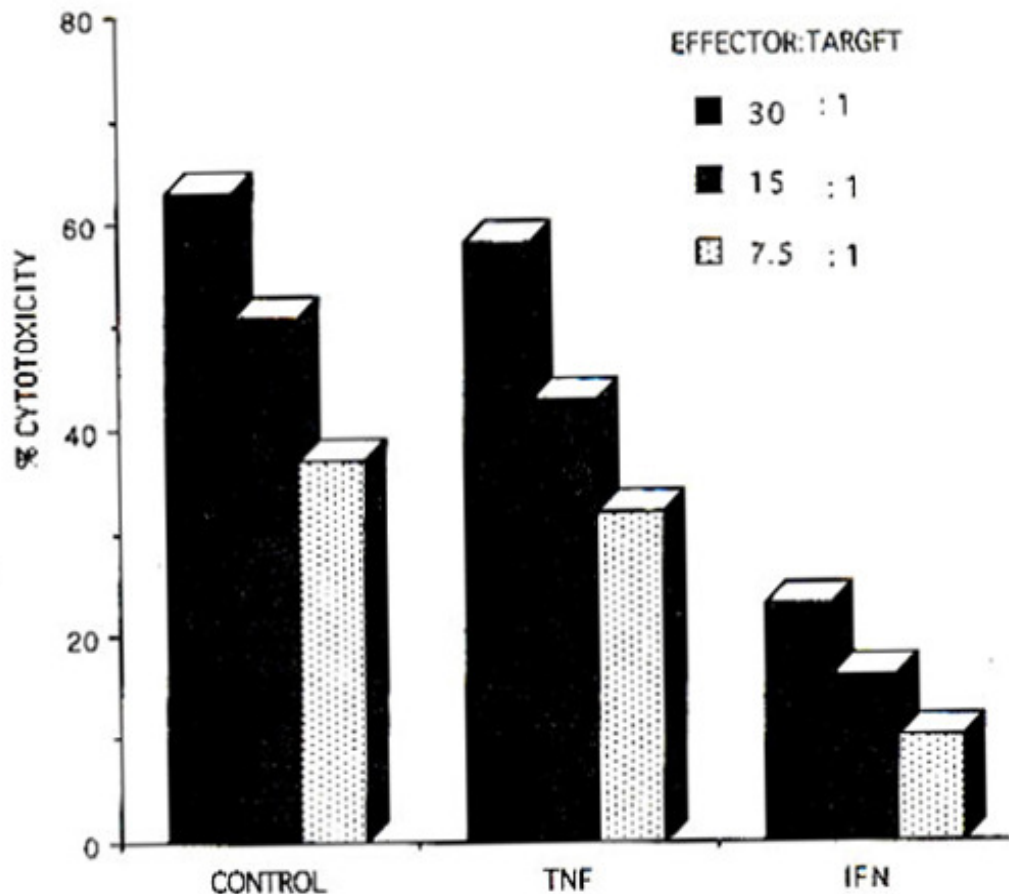
**TABLE II. % cytotoxicity of cytokine treated SW742 cell line with control and IL-2 activated lymphocytes, LAK-cells\***

SW742	Control lymphocytes	LAK-cells
Control	3%	63%
TNF alpha	2%	58%
IFN alpha	4%	23%

\*at 30:1, effector-target ratio.

Results are representative of several serially carried out experimental runs.

As it is shown in Figure 4.



**Figure 4. % cytotoxicity of 24 h cytokine treated (TNF 1000u/ml, IFN 500u/ml) SW742 cell line with LAK-cells.**

TNF alpha did not appear to alter susceptibility to lysis by LAK cells. IFN alpha, however, considerably decreased SW742 susceptibility. Although both TNF alpha and IFN alpha increased MCH class I expression, decrease in cytolytic susceptibility was only noticed with IFN alpha treatment of the target cells. This may further indicate that LAK cells have different specificities compared to NK or IFN alpha activated NK cells.

## DISCUSSION

For the past two decades attempts have been made to develop immune therapies for cancer treatments on the basis of stimulating the host immune response to the tumor. These approaches have been based on attempting to immunize against specific tumor cells or to immunize with non-specific stimulants (like BCG) in the hope that general immune stimulation would concomitantly increase the host anti-tumor response. The inability to stimulate sufficiently strong response to putative tumor antigens and the general immune incompetence of the tumor bearing host were the factors that argued against the success of this approach. In fact these clinical attempts were unsuccessful and have largely been abandoned<sup>15</sup>. An alternative therapeutic approach to the immunologic treatment of cancer is that of adoptive transfer of immune cells<sup>7</sup>. Adoptive immunotherapy is defined as the transfer of active immunologic agents, such as cells with anti-tumor reactivity that can mediate, either directly or indirectly anti-tumor effects in the tumor-bearing host. The major obstacle to the development of successful adoptive immune therapies has been the availability of appropriate cells for use in adoptive transfer. The cells of immune system are best to be used as an important tool for their possible



advantages in the adoptive immunotherapy. It is not technically precise to use the term 'LAK cell', since a variety of cells can mediate to LAK activity although CD3 lymphocytes constitute the major population of IL-2- induced cytotoxic effectors<sup>16</sup>. Flow cytometric analysis has shown that the expression of both ICAM-1 and CD2 increases on IL-2 activated LAX cells, resulting in increased binding of these cells to tumor cells such as lymphomas<sup>17</sup>. These results are in agreement with the report by Wiekbe et al<sup>16</sup>. who showed that LAK susceptibility is usually unaltered after K562 TNF treatment. These results may emphasise the hypothesis that LAX cells are "ultra activated" entities with different specificities. The nature of the molecules involved in recognition of targets by LAX cells is not known, although the existence of two new cell surface molecules have been reported<sup>18</sup>. The possibility exists that the CD3 cytotoxic effectors recognise their targets through non-specific adhesion mechanisms possibly LFA-1/ICAM-1 and/or CD2/LFA-3. Since the natural or IL-2 activated CD3 cytotoxic cells are armed lymphocytes which contain granules or cytotoxic proteins, the stable adhesion of effector cells to the targets might be the triggering signal inducing the destruction of the targets<sup>19</sup>. Lymphotoxicity is mediated by various subsets of effector lymphocytes. One type of cytotoxicity, for instance, is mediated by well characterised immune killer cells which bind to their target via an antigen-specific heterodimeric T-cell receptor and recognise an antigen associated with histocompatibility molecules. Another type of cellular cytotoxicity is mediated by the CD2+ CD3 lymphocyte subset which does not express any known specific recognition structure and represents the majority of natural killer (NK) lymphocytes and lymphokine activated killer cells (LAK)". Most of the cells of this subset express surface markers such as CD16 and CD56. However, NK/LAK cytotoxicity is often designated as non-immune and non-MHC-restricted cytotoxicity. A population of CTL (CD3 ~) is also involved in non- MHC restricted killing, termed non-MIIC restricted cytotoxic T cells. The CDY lymphocytes appear to be extremely important for neoplastic surveillance, since LAK effectors can efficiently destroy certain tumour cells in vitro and in vivo. Cytotoxic effector cells mediate target cell lysis through a series of stages: (1) target cell recognition, (2) cell adhesion, (3) effector cell triggering, (4) effector cell activation, (5) secretion of cytotoxic factors, (6) factor binding to the target cell, (7) target cell changes, (8) cell death and (9) effector cell recycling. T-lymphocytes recognise peptide sequences associated with MHC class I antigen via their CD3/TCR complex, whereas other surface molecules (yet unknown) are associated with target recognition by NK cells<sup>4</sup>. Adhesion of lymphocytes to a target cell occurs through a series of adhesion proteins present on the effector cell surface. Two major pathways of adhesion have been identified in T-lymphocyte adhesion to target cells. Firstly, LFA- 1 on CTh interacts with ICAM-1 on the target cell<sup>20</sup>. Secondly, the effector cell CD2 molecule interacts with LFA-3 on the target cell<sup>21</sup>. CD56 molecule (NKH-I/Leu 19 antigen<sup>2</sup> appears to be involved in target cell binding on NK cells. Following the binding of effector cells to targets, trans-membrane signalling activates certain intracellular processes, leading to the secretion of granules from cytotoxic T-cells or NK-cells which are responsible for delivering the 'lethal hit' to the target. Although the process of killing by NK-cells and cytotoxic T- lymphocytes is in many respects similar, granules only appear in CTL upon activation, whereas lytic LGL (NK cells) already contain these granules<sup>4</sup>. It has been shown that treatment of target cells with IFN results in target cell resistance to cytotoxic attack (Figure 4), due to the failure of the effector granules to become reoriented after target cell binding and hence preventing the triggering of already bound LAX cells. The data presented in this study shows that the inhibitory effect of TNF $\alpha$  on K562 and SW742 susceptibility to lysis by LAK cells is negligible as compared to the effect of IFN $\alpha$ . Therefore, it may be possible to speculate that although LAX activities are similar in their lytic properties (e.g., target selection), their mechanism of recognition and lytic attack may be divergent. Furthermore, cytokine treatment of targets may greatly alter the recognition structures on targets. Preliminary investigation of recognition events by Ortaldo and Longo indicates that IL-2 activated effector cells do not recognise the same structure that is

recognised by freshly isolated NK cells<sup>22</sup>. Extensive amount of research is being done to understand the exact mechanisms of immunotherapy of cancer by using immune cells, because the normally functioning immune system protects against disease, it is intriguing to think that therapeutic manipulation of the immune response can combat malignancy. Our understanding of the immune system has expanded dramatically during the past decade, revealing an immensely complicated network of effector cell-target cell interactions. We are still far from precise understanding of the relationships between tumor cells and immune effector cells, perhaps exogenously administered agents, such as lymphokines and tumor-specific antibodies, can lead to effective anti-tumor response from a patient's own immune effector cells. On the other hand, successful immunotherapy may involve this present approach, with adoptive transfer of in vitro-activated immune competent cells. In this model the task for the researchers is to define relevant T-cell subpopulations that will participate in tumor regression in vivo. Optimal immunotherapy will require significant understanding of tumor cell biology, as well as the long list of lymphokine signals capable of modulating NK cells, LAK cells and CTL function. The problem of homing of effector populations to tumor sites may also be addressed through knowledge of the role of adhesion molecules. It is important to note that none of the immunotherapeutic trials to date addresses the issue of immune system maintenance after therapy. Immune system management of survivors of treatment will be an increasingly important clinical research problem. Until the immunologic deficits characteristic of patients with cancer are reversed, it is expected that complete responders will represent the minority of clinical trials. As our knowledge of the immune system increases, so must clinical research expand to explore new possibilities for therapy. It is anticipated that through the use of new approaches to immunotherapy, including autologous transfer of anti-tumor effector cells, IL-2 therapy, monoclonal antibodies, retrovirus-mediated gene transfer and chemotherapeutic agents, future patients with cancer will realise significant increases in survival.

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