Lymphokine activated killer (LAK) cells in patients with gastrointestinal cancer

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SUMMARY Lymphokine activated killer (LAK) cells are a recently described cellular immune phenomenon with exciting potential for the treatment of tumours arising from solid organs. A comparison of some aspects of LAK cell precursors and LAK cell function was undertaken in 44 control subjects and 44 preoperative patients suffering from gastrointestinal cancer (20 localised and 24 advanced). Lymphokine activated killer cell precursor (natural killer (NK) cell) activity was significantly diminished in patients with advanced tumours ($p < 0.02$) as was fully mature LAK cell activity against an NK resistant target cell ($p < 0.012$). T-lymphocyte responses were not significantly different between the three groups. The reduced LAK cell generation was associated with a significantly diminished proliferative response of LAK precursors to stimulation with high dose IL-2 *in vitro* ($p < 0.012$). Impaired LAK cell generation may explain the failure of adoptive cellular immunotherapy with LAK cells in some patients with advanced gastrointestinal cancer and prompts the search for means of augmenting this activity in such patients.

Recurrent tumours of gastrointestinal origin are frequently resistant to conventional treatment with radiotherapy or chemotherapy. The recent reports on the successful use of adoptive cellular immunotherapy in patients with advanced solid organ malignancy have, however, rekindled interest in this approach.^{1,2} The principle of this treatment is to incubate large numbers of the patient's lymphocytes with high concentrations of the T-cell derived growth factor Interleukin-2 (IL-2) *in vitro* for several days. This procedure yields a population of lymphoblasts which are highly cytotoxic against a wide variety of tumour cells, including autologous tumour, but not normal cells. These lymphokine activated killer (LAK) cells are then reinfused into the patient in high numbers together with IL-2 and have been shown to produce regression and in some cases cure, in most experimental animals and in a proportion of patients suffering from disseminated solid organ malignancy of a variety of origins.³⁻⁵

The almost universal success of this treatment in animals receiving syngeneic LAK cells (generated from healthy animals) contrasts with the failure of this treatment in about 50% of patients receiving autologous LAK cells.⁶ The study of these failures may provide insight into means of augmenting the efficacy of this procedure since we, and others, have previously shown that patients with advanced gastrointestinal cancer exhibit impaired specific and non-specific immunological responses.^{7,8} More recently we have suggested that this may be a consequence of a failure of these patients' lymphocytes to generate adequate levels of the lymphokine IL-2.⁹ Interleukin-2 is known to positively regulate the expression of its own high affinity receptors¹⁰ and it may therefore be possible that LAK cell therapy fails in these patients because of their incapacity to generate a lymphocytotoxic response on exposure to exogenous lymphokine. The present investigations were designed to examine this possibility.

The term LAK cell is a functional denomination for cytotoxic lymphocytes whose phenotypic characteristics remain a matter of some controversy. Some workers suggest that LAK cell activity is subserved by lymphocytes whose precursors are natural killer

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(NK) cells.^{11,12} Others claim that the precursors are of T-lymphocyte lineage although they do not bind the classical markers of cytotoxic T-lymphocytes.^{13,14} In this study we have examined some functional attributes of both T-lymphocytes and NK cells as well as LAK cell activity in patients with gastrointestinal cancer. In addition lymphocyte proliferation in response to IL-2 has also been measured.

Methods

PATIENTS

Lymphocyte function was measured in three groups of individuals. Group 1 (controls) consisted of 44 patients suffering from non-malignant conditions (gall stones, peptic ulceration or peripheral vascular disease) admitted to the general surgical wards for operation. Forty four preoperative patients suffering from gastrointestinal cancer arising in the stomach, colon or rectum were also studied. Upon receipt of the surgical and pathological findings these patients were assigned either to the localised gastrointestinal cancer group (group 2) or were classified as suffering from advanced disease (group 3) without previous knowledge of the results of the lymphocyte function tests. Group 3 patients contained all those whose tumours were found to have breached the serosa or involved the regional lymph nodes, invaded local structures or had metastasised to the liver. Thus the only patients who were eligible for inclusion in group 2 were those with Dukes' stages A or B colorectal tumours or those with a mucosal gastric cancer. The final distribution of these patients is shown in the Table.

LYMPHOCYTE PREPARATION

Heparinised venous blood was obtained from all patients under study at the same time on the morning of operation before administration of any pre-medication. Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-hypaque centrifugation and corrected to a cell density of 1.5×10^7 cells/ml in RPMI 1640 medium containing gentamicin (160 µg/ml), Amphotericin (2.5 µg/ml),

glutamine (2 mM) and 10% fetal calf serum (complete medium). These cells were then further diluted in complete medium for use in the following tests as described below.

BASAL NATURAL KILLER (NK) CELL ASSAY

Natural killer activity was measured in these subjects using a four hour chromium release assay as previously described.¹⁵ The target cell used was the erythroleukemoid cell line K562. Briefly 5×10^5 PBMC were incubated with 10^4 ⁵¹chromium labelled K562 target cells in triplicate round bottomed microtitre plates in a total reaction volume of 200 µl in complete medium. Serial doubling dilutions of the effector cells were done so that the effector:target ratios ranged from 50:1 down to 6:25:1. After four hours' incubation at 37°C in a humidified atmosphere of 5% CO₂ in air, 100 µl of the supernatant were removed from each well and counted for radioactivity in a gamma counter. Spontaneous ⁵¹Cr-release and maximal ⁵¹Cr incorporation were obtained by incubating 10^4 target cells alone in 200 µl medium without any effector cells or in the presence of 100 µl detergent respectively. The percentage specific ⁵¹Cr-release at each effector:target cell ratio was then calculated according to the usual formula:

$$\frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}} \times 100$$

T-lymphocyte stimulation with Concanavalin-A

Peripheral blood mononuclear cells at 10^6 cells/ml were distributed in 200 µl aliquots into 10 replicate wells of flat bottomed microtitre plates. Concanavalin A (20 µg/ml) was added to five of the wells and the plate was incubated at 37°C in a humidified atmosphere of 5% CO₂ in air for 48 hours, with ³H-thymidine (1 µCi/well) being added for the last 12 hours of culture. At the end of this period the cells were harvested and proliferation measured by counting the ³H-thymidine uptake in a beta counter. Thus for each patient studied the proliferation of unstimulated cells and Con-A stimulated cells was obtained in counts per minute and from these a stimulation index was calculated – that is,

$$SI = \frac{{}^3\text{H-Thymidine uptake in stimulated cells (cpm)}}{{}^3\text{H-Thymidine uptake in unstimulated cells (cpm)}}$$

IL-2 activation of PBMC

For each individual 20 millilitres complete medium containing 1.5×10^6 PBMC per ml were prepared in 80 cm² upstanding tissue culture flasks. To this cell suspension was added 200 units/ml of highly purified lectin free human lymphocyte derived Interleukin-2 (Lymphocult-HP, Biotest Pharma GmbH, Frankfurt). This reagent is 95% pure and devoid of all biological activities attributable to interleukin 1,

Table Details of patients

	n	Mean age (years) (±SE)	M:F	Tumour site
Controls	44	59.5±2.9	29:15	—
Localised GI cancer	20	67 ±3.1	11: 9	19 colorectal 1 gastric
Advanced GI cancer	24	69 ±3.2	13:11	11 colorectal 13 gastric

Interferon, migratory inhibitory factor or colony stimulating factors. This concentration was chosen following preliminary dose-response studies of both LAK cell generation and lymphocyte proliferation. No consistent increase in LAK cell generation was observed with concentrations of IL-2 above 200 U/ml, whereas higher concentrations were associated with a lower cell yield and possible toxicity. The flasks were incubated for 72 hours, the cells being agitated for two minutes twice daily. At the end of the three day period the cells were harvested, corrected to a cell density of 5×10^6 /ml and used in the assays of IL-2 activated cytotoxicity as detailed below. In addition, five replicate 100 μ l aliquots of this cell suspension were also incubated for a further 12 hours with 0.1 μ Ci of 3 H-thymidine for the assessment of IL-2 induced lymphocyte proliferation. These results are recorded as the mean 3 H-thymidine uptake of 5 replicate wells in counts per minute.

Cytotoxicity assays for IL-2 activated PBMC

Interleukin-2 activated PBMC at a cell density of 5×10^6 /ml complete medium were used as effector cells in four hour 51 chromium release assays against two target cells in an identical manner to that described above for basal NK activity. The cytotoxicity of these lymphokine activated cells was measured against the NK sensitive cell line K562 and this is referred to as 'activated' NK activity. Lymphokine activated killer cell activity was also measured against the archetypal LAK cell target cell DAUDI. This is an NK-resistant lymphoblastoid cell line originating from a patient suffering from a Burkitt lymphoma and has not been found to be susceptible to any spontaneous allogeneic lymphocytotoxicity other than as a LAK cell target.^{13,14}

STATISTICAL ANALYSIS

All data are expressed as mean $\pm$ SE for each group under consideration. Statistical analysis was by Student's *t* test after logarithmic transformation to normalise the data.

Results

BASAL NK ACTIVITY (Fig. 1)

The mean basal NK activity exerted against the cell line K562 for all three study groups is summarised in Figure 1. The mean ($\pm$ SE) percentage cytotoxicity of control lymphocytes at an effector:target cell ratio of 50:1 was $32.1 \pm 2.26\%$. This was not significantly different from the corresponding mean figure of $37.7 \pm 3.98\%$ for lymphocytes from the patients suffering from localised gastrointestinal cancer ($t=1.3$, $p=0.099$, NS). The mean NK activity in control subjects, however, was significantly higher

Fig. 1 Basal and IL-2-activated cellular cytotoxicity against the natural killer cell target K562 in controls and patients suffering from gastrointestinal cancer.

than that of patients suffering from advanced gastrointestinal cancer ($23.25 \pm 3.2\%$, $t=2.3$, $p=0.012$) as was that of the patients suffering from localised disease ($t=2.85$, $p=0.0034$).

IL-2 ACTIVATED LYMPHOCYTOTOXICITY AGAINST K562. (ACTIVATED NK ACTIVITY)

At an effector:target cell ratio of 50:1 the mean activated NK activity against the K562 cell line was $69.1 \pm 4.36\%$ for control subjects, $66.2 \pm 4.36\%$ for patients with localised disease and $62.1 \pm 4.17\%$ for patients with advanced GI cancer. None of these figures was significantly different from one another (Fig. 1).

LYMPHOCYTE PROLIFERATION

Figure 2 reveals that the 3 H-thymidine uptake of unstimulated lymphocytes from all three patient groups were not significantly different from one another indicating no increase or decrease in spontaneous lymphocyte proliferation in any of the three groups. Although there was a trend towards lower Concanavalin-A stimulated lymphocyte proliferation in the two cancer groups the mean responses were again not significantly different from those seen in the control subjects. This also is the case when these data are expressed as a stimulation index.

These observations are in marked contrast to the proliferative responses observed for the lymphocytes exposed to pure IL-2 alone *in vitro* (Fig. 3). The mean 3 H-thymidine uptake (expressed as $\text{cpm} \times 10^{-3}$) for lymphocytes from control subjects was 25.176 ± 2.743

Fig. 2 Spontaneous (basal) proliferation, lectin (Con A) stimulated proliferation and stimulation indices of control subjects and patients suffering from gastrointestinal cancer.

and was not significantly different from the figure of 27.365 ± 5.82 observed for the patients with localised cancer ($t=0.22$, $p=0.414$). The corresponding mean ^3H -thymidine uptake of cells from patients with advanced GI cancer was 13.74 ± 2.52 , this being significantly lower than that obtained for control subjects ($t=2.59$, $p=0.007$) as well as that obtained for the patients with localised gastrointestinal cancer ($t=2.0795$, $p=0.025$).

LYMPHOKINE ACTIVATED KILLER (LAK) CELL ACTIVITY

The mean (± 2 SE) LAK cell activity at an effector:target cell ratio of 50:1 for each of the three subject groups is also displayed in Figure 3. The mean LAK cell activity exerted by IL-2 stimulated lymphocytes from control subjects was $25.8 \pm 2.42\%$ compared with a mean figure of $30.83 \pm 4.25\%$ for lymphocytes from patients with localised GI cancer ($t=0.715$, $p=0.24$). In contrast the mean LAK activity for the lymphocytes obtained from patients suffering from advanced GI cancer was $16.8 \pm 4.23\%$ which was significantly lower than both that of the control group ($t=3.87$, $p=0.0001$) and of those with localised disease ($t=2.33$, $p=0.012$).

Fig. 3 Cytotoxic activity and proliferation of lymphokine activated killer (LAK) cells in control subjects and patients suffering from gastrointestinal cancer.

Discussion

The upsurge of interest in the LAK cell phenomenon results from the fascinating results which have been claimed for adoptive cellular immunotherapy using autologous LAK cells in patients suffering from solid organ malignancies which have proved resistant to all other types of therapy.¹⁻¹⁶ This approach has evolved from the results of exhaustive experimental animal studies which have used LAK cell infusions derived from non-tumour bearing animals and in such systems complete regression of tumour metastases has been repeatedly shown.³⁻⁵ This contrasts with clinical experience where although partial, and occasionally, complete regression of solid tumour metastases are seen, a number of patients with advanced tumour burdens seem not to respond to autologous LAK cell therapy.¹⁶⁻¹⁸ The experimental models clearly do not wholly reflect the clinical scenario and thus before this treatment is widely attempted, further information on the capacity of patients with heavy tumour loads to generate adequate LAK cell cytotoxicity is required.

Several workers have previously described abnormalities in NK cell function in patients suffering from malignant disease¹⁷⁻¹⁹ This is important because of the widely held view that NK cells are the main precursors of LAK cell activity.^{11,12} Our results on basal NK activity are in keeping with previous studies in gastrointestinal cancer patients and may partially explain the diminished LAK cell activity seen in our patients with advanced gastrointestinal cancer. It is

noteworthy, however, that even though the NK activity is diminished in patients with advanced disease, lymphokine activated killing of the NK-susceptible target cell K562 is almost identical in all three groups and suggests that these patients at least have the potential to generate higher levels of lymphocytotoxicity even if this is relatively diminished when these stimulated cells are confronted with an NK-resistant target cell – that is, exert impaired LAK activity.

An alternative view of LAK cell precursors is that they represent a T-lymphocyte which is quite distinct from classical cytotoxic T-lymphocytes.^{13,14} If a generalised T-cell defect does exist in these patients, then this may also partially explain the failure of LAK cell generation in our group of patients with advanced disease. In the present study the proliferation of unstimulated T-cells is similar in all three groups but, although not statistically significant, the response to a T-cell mitogen is on average diminished in the two cancer groups. We have previously shown that a helper T-cell defect exists in these patients,⁹ and this may well influence other T-cell functions including T-cell-mediated LAK activity. As IL-2 stimulated LAK cell activity is derived from the proliferation of NK-like cells and T-cells after exposure of their IL-2 receptors to a high local concentration of IL-2, we have also examined the proliferation of LAK precursor cells during the LAK cell generation phase. It is evident that the ³H-thymidine uptake by these cells during the three day incubation period with IL-2 is markedly diminished in those patients suffering from advanced disease. Considering all these data as a whole, therefore, we would suggest that the reported failure of LAK cell therapy to influence the course of the disease in some patients with advanced gastrointestinal malignancy is because of an inherent incapacity of these patients to generate adequate LAK cell cytotoxicity in the *in vitro* induction phase. Furthermore, the results presented in this study would incline to the conclusion that this is attributable to the presence of a diminished proliferative capacity of LAK cell precursors.

It is possible that this reduced IL-2-activated lymphocyte proliferation is attributable to diminished numbers of either the T-cell or NK cell component of LAK precursors. We have previously reported,⁹ however, that patients suffering from advanced gastrointestinal cancer possess normal numbers of lymphocytes expressing the CD3 (pan T-cell) marker. Similarly there is no convincing evidence that circulating NK cell numbers are diminished in patients with advanced malignancies including colorectal cancer.^{19,20} Despite impaired basal NK activity we have found that activated cytotoxicity against the NK target cell K562 is normal

in the advanced cancer patients in this investigation. Overall, therefore, our data tend to the conclusion that it is a defect in the T-cell component of LAK precursors which is responsible for the impaired LAK activity which we report in the advanced cancer group. In view of the impaired basal NK activity in these patients, however, a proliferative and cytotoxic defect in this component of LAK activity cannot be excluded.

In conclusion, this investigation has revealed that LAK activity in patients with advanced gastrointestinal cancer is impaired compared with that observed in patients with localised disease and healthy control subjects. This impaired activity is associated with diminished proliferation of LAK precursors and may explain the failure of LAK/IL-2 therapy in patients with advanced gastrointestinal cancer. Further studies are required to dissect this defect in greater detail, with a view to identifying means of augmenting LAK activity in therapeutic protocols.

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