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Immune Changes in Peripheral Blood Resulting From Locally Directed Interleukin-2 Therapy in Squamous Cell Carcinoma of the Head and Neck

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Interleukin-2 (IL-2) was administered locally by constant intra-arterial infusion in four escalating doses from 3×10^4–3×10^7 IU/day to 12 patients with squamous cell carcinoma of the head and neck (SCCHN) in a phase I trial. Lymphocyte phenotypic markers and serum cytokine concentrations were measured over the course of treatment. Serum IL-1-alpha, -beta and IL-6 were not induced at any dose level. Tumour necrosis factor (TNF)-alpha was induced in the 2 patients who showed a clinical response (at the lowest dose) as well as in 4/10 of the non-responders. In addition TNF-beta was induced in 3/10 and IFN-gamma in 5/10 non-responders. Soluble IL-2 receptor concentrations were increased at the two higher doses. The highest dose of IL-2 produced a lymphocytosis after day 5 until the end of administration reflected by a general rise in lymphocyte phenotypic markers. CD25, CD3/HLA-DR and CD56 showed an additional upregulation not accounted for by the lymphocytosis with a suggestion of a bell-shaped dose–response curve for CD25 and CD3/HLA-DR. Administration of IL-2 in this manner has been shown to be well tolerated and has some anti-tumour activity at low doses, with little toxicity.
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INTRODUCTION

THE TREATMENTS available for squamous cell carcinoma of the head and neck (SCCHN) are surgery and/or radiotherapy. Primary medical treatment with chemotherapy is associated with high response rates (78%) [1] but this does not influence survival and adjuvant chemotherapy is similarly ineffective [2]. SCCHN that recurs after surgery and radiotherapy has a poor prognosis (median survival, 6–10 months) and response rates to chemotherapy vary between 12 and 63% [3].

Patients with SCCHN demonstrate a variety of immunological phenomena: non-specific immunosuppression [4], tumour cell surface-bound autologous antitumour antibodies [5] and circulating serum antibodies specific to squamous cell carcinoma cell lines [6]. In addition, rises in circulating immune complexes have been correlated with patients' subsequent response to chemotherapy [7]. Therefore manipulations of the immune system could be used to treat SCCHN.

Interleukin-2 (IL-2) is an important immune modulator *in vivo*, expanding and activating T cell subsets and NK cells. When lymphocytes are incubated with IL-2 *in vitro*, cells capable of lysing fresh tumour cells are generated and are referred to as lymphokine-activated killer (LAK) cells. They have been identified as non-B, non-T cells that can recognise cancer cells in a non-major histocompatability complex (MHC) restricted way [8]. Tumour-infiltrating lymphocytes (TIL) can infiltrate growing tumours and can be grown *in vitro* under the influence of IL-2 [8]. *In vivo* studies have shown that TIL are 50–100 times more effective in treating established metastases than LAK cells [8].

IL-2 has been found to have a therapeutic effect in certain tumour types particularly those where escape from normal immune-surveillance has been proposed as a mechanism of recurrence or metastasis. Renal cell carcinoma and melanoma show the greatest responsiveness to IL-2 [9] but when delivered systemically, IL-2 is associated with significant side effects such as fever, anaemia, renal failure, hepatic and cardiac dysfunction [10]. A more physiological approach might be to deliver IL-2 locally to sites where potentially tumoricidal lymphocytes may be concentrated [11]. Such local therapy has the potential of reducing the concentration of cytokine in the circulation with a resulting reduction in the severity and frequency of toxicity. Murine data has shown that interleukin-2 (IL-2) can elicit significant anti-tumour reactions when injected locally at the tumour site [12] and this approach has already been used in patients with recurrent SCCHN. A study conducted by Cortesina and colleagues [13] demonstrated that IL-2 has activity in this tumour type when injected perilymphatically, with an overall response rate of 65%.

IL-2 can induce other cytokines such as IFN-gamma, tumour necrosis factor (TNF)-alpha, -beta, IL1-alpha, -beta and IL-6 which may act directly on the tumour or induce antitumour activity of other cell types [14–17]. IFNs can inhibit

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tumour cell growth with IFN-gamma being a more potent immunomodulator than IFN-alpha or -beta and interacting more closely with other cytokines [18]. TNF alone is cytotoxic *in vitro* for a number of tumour cell lines and primary tumour cell cultures, e.g. L929 and WeHi, although its activity is extended or enhanced by IFN-alpha or -gamma [19]. IL1-alpha and -beta are induced by TNF and IFN, have overlapping biological activities with TNF, [20] and may indirectly have potential in cancer therapy. IL6, like IL1 and TNF, has many biological effects and may be an important secondary signal mediating some of the biological activities ascribed to such regulators as TNF and IL1 [21].

Patients with tumours of the head and neck were selected for local intra-arterial infusion of IL-2 in this trial because the arterial supply to these tumours is frequently accessible to cannulation and the tumours are often easily biopsied. This therefore provided a unique opportunity to study the effects of IL-2 on serum concentration of IL1, IL6, TNF and IFN-gamma and soluble IL-2 receptor and peripheral blood lymphocyte (PBL) phenotypes in response to escalating doses of IL-2 starting at a lower dose than generally used in other trials.

MATERIALS AND METHODS

Treatment and response

The protocol was approved by the ethics committee at the Royal Marsden Hospital, London and all patients gave informed, written and witnessed consent. 12 patients (9 male, 3 female, age range of 31–73 years) with incurable SCCHN were treated with increasing doses of intra-arterial IL-2 via a cannula inserted into the feed artery. The schedule has been previously reported in detail [11]. Tumour response was defined according to standard criteria: Complete response (CR) was defined as the disappearance of all clinical, radiological and biochemical evidence of disease for at least one month; partial response (PR) was defined as a reduction in the product of two diameters of measurable disease by at least 50% for at least 1 month [11]. 2 patients who received the lowest dose of IL-2 (3×10^4 IU/day) had partial responses and no responses were seen at doses above this level. The dose administration protocol in patients receiving escalating doses of IL-2 is shown in Table 1.

Table 1. Dose administration protocol and outcome in patients receiving escalating doses of intra-arterial IL-2

Patient no.	Planned schedule (actual schedule) Days	Dose (IU/Day)
1*	5 (5)	
2	5–2–5 (1.5)	3×10^4
3*	5–2–5	(Dose 1)
4	5–2–5 (5–2–2.5)	
5	10	3×10^5
6	10	(Dose 2)
7	10	
8	10	
9	10	3×10^6
10	10	(Dose 3)
11	5–2–5	3×10^7
12	5–2–5	(Dose 4)

5–2–5 = 5 days of IL-2, 2 days rest, followed by a further 5 days of IL-2.5, 10 = 5 or 10 days of continuous IL-2 given. *Responders.

Blood collection

Venepunctures were performed daily and the blood collected into sterile vacutainers (Becton Dickinson, Oxford) containing either no anticoagulant (for serum), lithium heparin (for plasma) or EDTA (for full blood counts and flow cytometry).

Cytokine assays

IL1-alpha, -beta and IL6. IL1-alpha, -beta and IL6 were assayed by radioimmunoassay (RIA) using antibodies and standards kindly supplied by Dr S. Poole (National Institute for Biological Standards and Control (NIBSC), South Mimms, Herts). The assays were performed as previously described [22, 23]. The international standards used were 86/632 for IL1-alpha, 86/552 for IL1-beta and the interim reference preparation for IL-6 was 88/514. All were diluted in RIA buffer. The antiserum concentrations used were 1/2000 for IL1-alpha and IL6 and 1/500 for IL1-beta. The limits of detection for the assays were 0.21 ng/ml for IL1-alpha, 0.51 ng/ml for IL1-beta and 0.26 ng/ml for IL-6.

TNF and IFN-gamma. TNF-alpha, -beta and IFN-gamma were measured by enzyme-linked immunosorbant assays (ELISA) using paired monoclonal and polyclonal antibodies supplied by Dr A. Meager (NIBSC, South Mimms, Herts). The method was as described previously [24]. The standards (diluted in phosphate buffered saline /0.05% Tween 20 and 2.25% human serum albumin) were: 86/659 for TNF-alpha; 87/640 for TNF-beta and 82/587 for IFN-gamma. The limits of detection of the assays were 30 U/ml for both TNF-alpha and -beta and 5 U/ml for IFN-gamma.

Serum IL-2. Serum IL-2 was measured using the IL-2-dependent cell line, CTLL (a gift from C. Bird, NIBSC). CTLL cells were maintained in RPMI 1640 medium with 5% (v/v) foetal calf serum (ICN Flow, Bucks), 2 mmol/l glutamine (ICN Flow), 50 IU/ml penicillin/streptomycin (ICN Flow), 50 pg/ml gentamicin solution (Sigma Ltd, Poole, Dorset) and human recombinant IL-2 (50 U/ml) from Eurocetus UK Ltd. The cells were washed three times by centifugation at 300 *g* for 9 min in RPMI 1640, resuspended to a concentration of 1×10^5/ml in IL-2 free culture medium and 100 µl of cells were then added to each well of a 96 well flat-bottomed microtitre plate (ICN Flow, Bucks). An IL-2 standard curve and patients' plasma at various dilutions between 1/10–1/100 were also added to the plate at 100 µl/well. The plate was then incubated for 48 h at 37°C in 5% CO_2 and pulsed with 18.5 kBq/20 µl/well of tritiated thymidine (Amersham, Bucks), with specific activity 1.07 TBq mol/l, for the final 24 h of culture. The wells were then harvested onto glass fibre filters and thymidine incorporation measured using a beta counter (1205 Betaplate, Wallac, Pharmacia). The limit of detection of this assay was 0.2 U/ml. Soluble IL2 receptor was detected using ELISA kits (T Cell Sciences, Laboratory Impex, Middlesex) according to the manufacturers instructions. The lowest limit of detection was 50 U/ml and the upper normal limit was 919 U/ml.

Flow cytometry

Monoclonal antibodies (Coulter Electronics, Beds) against the following lymphocyte surface markers were used: CD2 (pan T cell, reference range 1215–2532 counts/µl); CD3 (pan T cell, reference range 929–2471 counts/µl); CD7 (early T cell, reference range 632–2093 counts/µl); CD8 (cytotoxic/

suppressor T cell, reference range 243–1013 counts/μl); CD4 (helper/inducer T cell, reference range 564–1476 counts/μl); CD19 (pan B cell, reference range 89–691 counts/μl); CD25 (IL-2 receptor, IL-2R, p55, reference range 18–180 counts/μl); CD56 (natural killer cell, NK, reference range 28–682 counts/μl); and class II HLA-DR (reference range 5–112 counts/μl). Laboratory reference ranges were derived from a maximum of 28 normal laboratory volunteers (age range 21–50 years) and are expressed as 2 significant differences (S.D.) from the mean.

The monoclonal antibody required (10 μl) was added to 100 μl of whole blood (EDTA) and vortexed. After 10 min at room temperature the red blood cells were lysed, and the sample buffered and fixed using the Coulter Q-Prep system. The cell surface markers were then analysed on a Coulter Epics Profile II flow cytometer. A model T-540 haematology analyser (Coulter) was used to assess total white blood cell and lymphocyte counts. The reference ranges were 4.8–10.8 $\times 10^9$/l and 1.2–3.4 $\times 10^9$/l, respectively and were supplied by Coulter.

Statistical analysis

A paired non-parametric two-tailed Wilcoxon signed rank test was used where possible to determine significance between preinfusion values and values on various days of treatment. An inter-dose comparison was not possible due to the small number of patients at each dose level.

RESULTS

Effects of IL-2 on serum cytokines and soluble IL-2 receptor

Serum IL-2 was detected in only 1 patient with both the pre-treatment sample and that taken 24 h after commencement of the infusion demonstrating the presence of IL-2. Concentrations of 15.05 U/ml and 13.05 U/ml, respectively, did not suggest detection of administered IL-2 despite the patient being treated with 3×10^7 IU/day.

The number of patients at each dose level showing induction of TNF-alpha, TNF-beta, IFN-gamma and soluble IL-2 receptor is shown in Table 2. With the exception of TNF-beta and soluble IL-2 receptor, induced concentrations were generally low and showed no dose-dependent or time-dependent relationship to administered IL-2.

Serum TNF-alpha concentrations were increased within 24–72 h of starting each 5 day infusion in the 2 patients at dose 1, with concentrations ranging from 45–131 U/ml. The concentration in 1 patient at dose 2 increased from 59.3 U/ml pretreatment to 75.2 U/ml on day 12, while 1 patient at dose 3 showed an isolated raised concentration of 236 U/ml 24 h after start of treatment. At dose 4, increased concentrations of 39.2 and 32.4 U/ml were found in each of the 2 patients within 72 h of commencing the second 5 day cycle.

Table 2. Number of patients in whom cytokine induction was found, at each dose level of IL-2

Dose	TNF-alpha	TNF-beta	IFN-gamma
1	2/3*	0/3	1/3
2	1/4	0/4	1/4
3	1/3	1/3	2/3
4	2/2	2/2	1/2

*Responders

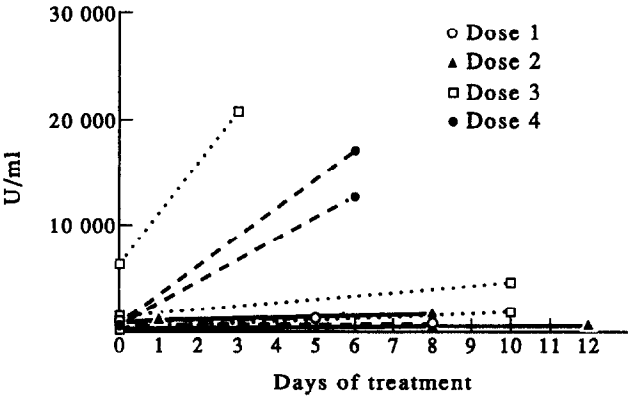

Fig. 1. Soluble IL-2 receptor values for each of the 12 SCCHN patients pre treatment and on the days of infusion at which the highest value was attained.

IFN-gamma was induced in 4 patients, one at each dose. The concentration increase for each of these patients was as follows: 35.2–48.8 U/ml; 9.9–13.7 U/ml; 58.6–66 U/ml, and <5–122.8 U/ml for doses 1–4, respectively.

Induction of TNF-beta appeared to be dose-dependent in that it was not detected in patients at the two lowest doses of IL-2 whereas concentrations ranging from 30 to 118 U/ml were detected in 2 of the patients at dose 3 and 35–217 U/ml in both patients at dose 4.

Soluble serum IL-2 receptor concentrations also appeared to be induced in a dose-dependent manner. The values ranged from 446.4 to 1361 U/ml at dose 1, 441.4 to 1986 U/ml at dose 2, 320.3 to 20 723 U/ml at dose 3, and 725 to 17 141 U/ml at dose 4 (Fig. 1).

IL-1-alpha, IL-1-beta and IL-6 were not significantly altered from pretreatment values at any dose level.

Cellular responses to IL-2

The white count rose in a dose-dependent manner with leucocytosis occurring only in the 2 patients at the highest dose level where the absolute lymphocyte counts were raised on day 8 (4600 and 6300 $\times 10^6$/l) and day 12 (5600 and 10 900 $\times 10^6$/l). Table 3 shows the mean pretreatment values together with the mean of the highest values reached during treatment for total white blood cell count, lymphocyte count and the phenotypes CD2, CD3, CD7, CD4, CD8 and CD19 in patients treated at doses 1–4. At dose level 4, there was a rise in the absolute number of PBLs bearing these phenotypes between days 5 and 12. However, when these counts were analysed as a percentage of total lymphocyte count no relative changes were apparent.

An increase in the number of PBLs bearing the CD25 phenotype (IL-2R) was seen in all patients treated at doses 3 and 4 (Fig. 2). There was a steady rise throughout the treatment period, with the number of CD25 positive cell numbers being significantly higher on day 12 than either 24 h after infusion or on day 5 ($P<0.005$ and <0.05, respectively). A similar pattern to that seen for CD25 positive cells was observed for activated T cells (CD3/HLA-DR positive) in response to IL-2 at both dose 3 and 4 (Fig. 3). Interestingly at dose level 4, the number of cells positive for CD3/HLA-DR fell between days 8 and 12, despite the fact that IL-2 was still being administered. An increase in the number of PBLs bearing the CD56 phenotype was only evident at dose 4 (Fig. 4) and

Table 3. The mean pretreatment values of the white blood cell counts, lymphocyte counts and T cell and B cell phenotypes investigated, together with the mean of the highest values reached during treatment.

	Dose 1		Dose 2		Dose 3		Dose 4	
	Px	Hx	Px	Hx	Px	Hx	Px	Hx
Lymphocyte Phenotypes	(Counts/μl)							
CD2	968	683	1 097	1 251	1 075	2 108	1 258	7 185
CD3	886	535	1 261	1 014	843	1 722	867	4 906
CD4	568	375	618	602	527	1 353	654	4 218
CD7	295	360	536	715	606	1 175	791	6 724
CD8	302	262	536	568	463	574	395	2 136
CD19	254	160	113	77	73	249	193	476
Lymphocyte number	1 400	1 000	1 300	1 500	1 300	2 400	1 200	8 600
White blood cell count	11 600	9 700	9 400	10 400	11 600	12 600	13 100	24 900

Px = Mean of prevalues. Hx = Mean of highest values reached during treatment.

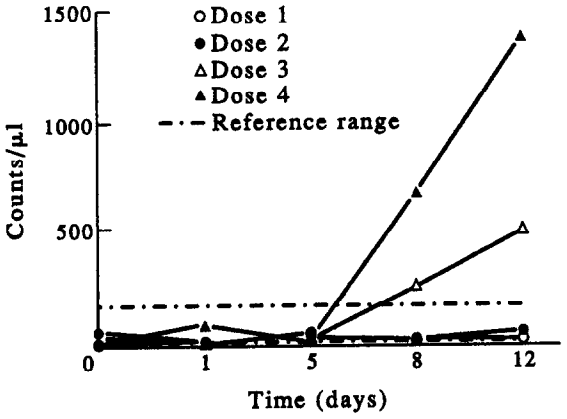

Fig. 2. Mean cell count of CD25 positive lymphocytes at each dose of IL-2 on days after the start of infusion as indicated.

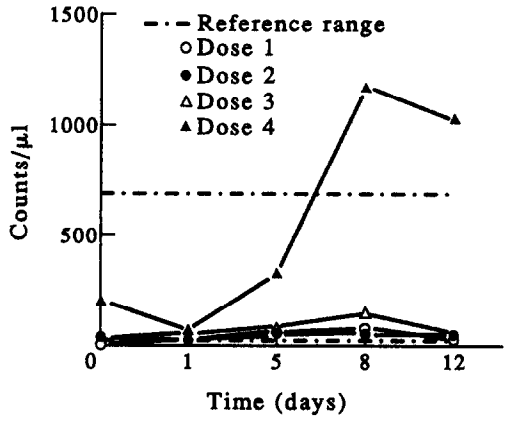

Fig. 4. Mean cell count of CD56 positive lymphocytes at each dose of IL-2 on days after the start of infusion as indicated.

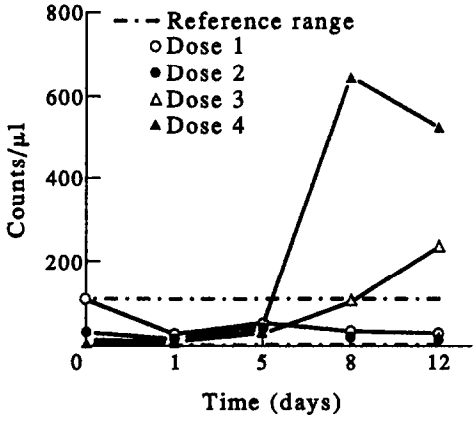

Fig. 3. Mean cell count of CD3/HLA-DR positive lymphocytes at each dose of IL-2 on days after the start of infusion as indicated.

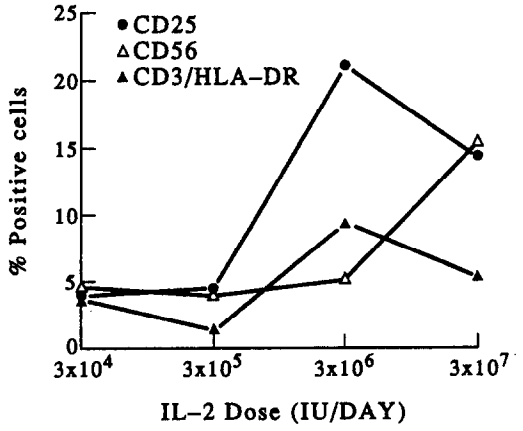

Fig. 5. Percentage positive lymphocytes for CD25, CD56 and CD3/HLA-DR meaned at each dose of IL-2 on day 12 of infusion.

as with the CD3/HLA-DR phenotype, numbers decreased between days 8 and 12 of treatment.

We have calculated the changes in CD25, CD3/HLA-DR and CD56 relative to the total lymphocyte count at the end of treatment on day 12 (Fig. 5). There seem to be two patterns present, the first is a dose-dependent rise in CD56 positive cells; the second is an initial dose-dependent rise, followed by a fall despite the increased dose of IL-2. There is thus the suggestion of a bell-shaped dose-response curve for both CD3/HLA-DR and CD25 positive cells.

DISCUSSION

The mechanism by which IL-2 mediates regression of tumours as well as the pathogenesis of the toxic side effects are not well understood although it seems likely that both are related to widespread immune activation and release of cytokines such as TNF, IL-6 and gamma-interferon.

Induction of cytokines and changes in lymphocyte numbers and phenotypic expression have been studied by a number

of groups, however, comparative assessment of the results is complicated by the variations of dose and route of administration of the IL-2. In general high dose regimens (e.g 10 000–100 000 U/kg) involving intra-venous bolus injections, used in the earliest trials, have been associated with the most severe side effects, even resulting in some deaths [9]. High dose regimens have been associated with an induced lymphopaenia during IL-2 administration followed by a rebound lymphocytosis after treatment [25, 26] and with the most consistent induction of cytokines and activation of peripheral blood lymphocytes [17].

Administration schedules using continuous intravenous infusion, or sub-cutaneous, peritumoral or perilymphatic injection, designed to limit the toxicity of IL-2, have indeed been associated with less systemic toxicity [26, 27, 28, 13] but also with less consistent induction of cytokine and peripheral blood lymphocyte activation [14].

The novel aspects of the present study included not only the intra-arterial route of administration in SCCHN patients, but also a very low starting dose. At doses of IL-2 between 3×10^4–3×10^6 IU/day, patients experienced few systemic side effects although some local toxicity occurred; at these doses therefore IL-2 was well tolerated confirming other studies using local administration. More severe toxicity was seen at the highest dose of 3×10^7 IU/day and confirmed the toxicities experienced in other high dose regimes of IL-2 [9]. The 2 patients who showed partial responses were both treated with the lowest dose schedule.

In the present study there were only sporadic findings of detectable concentrations of serum cytokines induced by IL-2 treatment. These results are consistent with other studies [14, 17]. Gemlo *et al.* [14] conducted a study in which 10 patients received IL-2 priming prior to IL-2/LAK therapy (intravenous bolus 100 000 units/kg every 8 h for 5 days, amounting to a total daily dose of 2.1×10^7 U/day for a 70 kg person). There was no detectable IFN-γ, TNF-α or TNF-β after the initial dose of IL-2, elevated TNF-β was seen in only 1 patient after 3 days and IFN-γ in 3 patients after 5 days treatment and there was no IFN-γ induction in patients primed for only 3 days. These same workers found more consistent induction of cytokines during the IL-2/LAK phase of treatment; bolus rIL-2 in conjunction with the infusion of LAK cells generated more frequent and higher cytokine levels than did continuous infusion of IL-2 suggesting that transient high levels of rIL-2 associated with bolus administration, and not achieved by continuous infusion, are necessary to elicit cytokine production. IFN is known to induce "influenza-like" symptoms [18] such as fever, headache, and fatigue common in IL-2 toxicity and the lack of IFN-gamma induction could explain the low toxicities experienced by our patients.

TNF has been shown to have anti-tumour activity, acting directly on the tumour, or indirectly, by stimulating monocyte cytotoxicity [29]. In the present study serum TNF-alpha was detected in both responders following IL-2 treatment, however, similar findings in 4 non-responders indicates that if TNF is involved in tumour killing other factors must also be important.

The detection of circulating IL-2 at only the highest dose of IL-2 in this study could be related to mode of administration. Other studies have shown barely detectable levels of circulating IL-2 during continuous infusion of IL-2 at doses up to 3000 units/kg/h [30] (amounting to 5×10^6 U/day for a 70 kg person) but transient detectable levels following bolus intravenous administration. Alternatively failure to detect IL-2 could result from an increase in soluble IL-2 receptor binding, sequestration at the tumour site, or insensitivity of the assay system. Biological activity was evidenced by the increase in lymphocyte activation markers and soluble IL-2 receptor, especially at the two higher doses.

The pattern of phenotypic expression induced by IL-2 in the present study was consistent with previous studies [13, 28] and showed that activation markers such as CD56, CD25 and HLA Class II increased both absolutely and as a percentage of the total count whereas the relative proportion of other markers remained constant.

The typically described IL-2-induced lymphopaenia followed by a rebound lymphocytosis was not seen at any dose in this study, as seen previously. This again could be related to mode of administration and peak serum IL-2 concentrations attained as these effects do seem to be dose related [25].

The systemic effects of intra-arterial administration of IL-2 reported in this study appear to be related to the dose of administered IL-2 and were unable to predict or characterise the responding patients. Indeed, responses were seen at a low dose where minimal systemic effects were apparent. There has been considerable interest in the role of both LAK and TIL cells in IL-2-induced tumour killing and therefore both cytokine induction and cell activation at the tumour level may be more important than systemic changes. During the present study biopsy specimens were taken pre and post treatment and these will be used for a further study on tumour localised effects.

In conclusion therefore, this study has shown that IL-2 can be administered locally at low doses with little toxicity resulting in activation of the immune system, however, there was no correlation between systemic immune activation and local response. It therefore seems possible that systemically, the expanded activated lymphocytes have arisen from pre-existing clones whose repertoire had on the whole permitted the patients' tumours to progress, i.e. there has been lymphoproliferation and activation of irrelevant clones.

At the local tumour level it is likely that the higher dose infusions have been at the far end of the "bell-shaped" curve and therefore possibly inhibitory. Certainly there were no significant reductions in tumour mass. However, at the lowest dose (where the two responses occurred) it is likely that induction of various receptors and adhesion molecules have increased more relevant activated cells without the disadvantage of high dose inhibition.

There is therefore sufficient evidence from this study to abandon local high dose therapy and to further explore low dose treatment.

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