PERGAMON

European Journal of Cancer 37 (2001) 1053–1060

European Journal of Cancer

www.ejconline.com

A combination of interleukin-2 and 60 nm cationic supramolecular biovectors for the treatment of established tumours by subcutaneous or intranasal administration

S. El mir [a], A. Casanova [b], D. Betbeder [b], F. Triebel [c,*]

[a] *Laboratoire d'Immunologie des Tumeurs, Faculté de Pharmacie, 5, rue J.B. Clément, 92296 Chatenay-Malabry, France*
[b] *Biovector Therapeutics, Chemin du Chêne Vert, B.P. 169, 31676 Labège Cedex, France*
[c] *Laboratoire d'Immunologie Cellulaire, Institut Gustave-Roussy, 39, rue Camille Desmoulins, 94805 Villejuif Cedex, France*

Received 13 October 2000; received in revised form 29 January 2001; accepted 20 February 2001

Abstract

The Supramolecular Biovector (SMBV^{TM}) KY is a drug delivery nanocarrier which consists of a discretely sized, ionically charged, cross-linked polysaccharide core surrounded by a lipid membrane. We used the non-immunogenic spontaneous mammary adenocarcinoma TS/A tumour to test the efficacy on tumour growth of low (10^4 IU) or ultra-low (10^3 IU) doses of interleukin-2 (IL-2) adsorbed to these 60 nm cationic synthetic particles. In comparison with the progressive growth of TS/A cells in syngeneic mice, KY/IL-2 particles coinjected with TS/A cells or administered at a distance from the tumour, inhibited tumour growth while free IL-2, even at 10–100 times the dose used in the KY/IL-2 formulations, had no effect. Studies performed on implanted tumours (treatment at day 6 (D6)) showed that KY/IL-2 administered subcutaneously (s.c.) at five sites distant from the tumour (10^3 IL-2 IU per site) induced rejection of the implanted tumours. Six out of 10 mice were cured while the other four had residual tumours only. In the same experiment, free IL-2 induced only tumoral growth reduction. Protection induced by KY/IL-2 administered s.c. at five sites involved recruitment of a $CD8^+$ T cell response since nu/nu mice and CD8-depleted mice did not reject the tumours. Mice cured were protected significantly to completely against a rechallenge with TS/A tumour cells, and a systemic tumour-specific CTL activity was induced. Finally, we showed that repeated intranasal (i.n.) administration of KY/IL-2 (low-dose) also led to complete regression of pre-established tumours and partial protection from tumour rechallenge. We therefore suggest that, in contrast to free IL-2, a KY/IL-2 formulation could be used as a systemic immunostimulant leading to the eradication of non-immunogenic, established tumours.

Keywords: Cytotoxicity; Interleukin-2; Formulation; Tumour immunity; Tumour model; Nanoparticle

1. Introduction

The administration of cytokines to augment the functioning of the immune system can be accomplished safely, and without cytotoxicity, provided that a rational approach is used. The modest therapeutic performances of interleukin-2 (IL-2) in most clinical trials to date can be attributed to either rapid plasma clearance or a distribution to irrelevant tissues, or both. It should be possible to overcome these problems by encapsulation of the cytokine in a delivery system in order to obtain a reduction in dosage, a slow release and a modified biodistribution. The use of viral vectors to express low doses of IL-2 for several days at the site of the infected cells can not be widely applied while the safety and economic problems of such procedures remain to be solved. However, the use of nanoparticles offers several distinct advantages as an efficient, controlled delivery system, including protection of the entrapped cytokine from the deleterious effects of tissues or plasma enzymes, and the feasibility of pharmaceutical companies producing dosage formulations as required in an industrial setting. In any event, the endpoint of IL-2 therapy is to enhance the functioning of the immune system with, hopefully, an increased therapeutic index in the treatment of several diseases,

* Corresponding author. Tel.: +33-1-4211-4068; fax: +33-1-4211-5268.
E-mail address: ftriebel@igr.fr (F. Triebel).

0959-8049/01/$ - see front matter
PII: S0959-8049(01)00084-3

including tumours, immunodeficiency syndromes and infection, and also for vaccination [1,2].

$CD8^+$ T cells specific for tumour antigens have often been identified in tumour-bearing hosts, and effector cytotoxic T lymphocytes (CTLs) are thought to be the major component of an immune response to most tumours. $CD4^+$ T cells can also be necessary for supporting tumour-specific $CD8^+$ T cell responses [3,4], either by 'conditioning' antigen presenting cells (APCs) to more effectively present the antigen (Ag) to the $CD8^+$ T cells [5,6], or by producing IL-2 or other cytokines to support the $CD8^+$ T cell responses. Early induction of tolerance in tumour-specific $CD4^+$ T cells may limit the immune response that can occur in the tumour [7]. IL-2 appears necessary, and sufficient, to mediate the help provided by the $CD4^+$ T cells which are usually required for a sustained, effective $CD8^+$ T cell response. Administration of low-dose IL-2 alone has been shown to reverse tolerance [8]. The activation-induced non-responsiveness (AINR) observed in $CD8^+$ T cells within 3 or 4 days of responding appears to be an aspect of the normal differentiation of fully stimulated $CD8^+$ T cells and, in the absence of T cell help (or exogenous IL-2), there is no further expansion of $CD8^+$ T cells and tolerance to the Ag occurs [9]. These recent results led us to reassess the potential of formulated IL-2 to reactivate tumour-specific CTLs for tumour immunotherapy.

A first generation of nanoparticles made of an anionic polysaccharide core covalently acylated with palmitic acid was used to entrap IL-2 in a formulation process based on the aggregation of freeze-dried nanoparticles rehydrated with the solution [10]. This formulation was found to stabilise IL-2 biological activity [10]. However, the large amounts of particles necessary to entrap IL-2 prevented these formulations from being tested in animal studies. We have developed a new family of 60 nm particles, termed the Supramolecular Biovector ($SMBV^{TM}$) composed of an internal cross-linked polysaccharide core (PSC), which may be substituted with charged groups surrounded by a lipid membrane [11]. The lipid membrane is composed of dipalmitoyl phosphatidyl choline (DPPC) and cholesterol (70:30 w/w). Due to their bi-compartmental structure, $SMBV^{TM}$ are able to load a wide variety of molecules. The internal PSC acts as an ion-exchange hydrogel matrix capable of including ionic compounds, and the external phospholipid layer can adsorb amphiphilic or hydrophobic compounds. This new family of protein nanocarriers has been successfully tested in vivo in investigations into the immunogenicity of human cytomegalovirus Ags [12] and influenza virus Ags (unpublished data).

In the present study, we demonstrate that the formulation of IL-2 into a delivery system can be achieved using a simple mixture of pre-made 60 nm cationic synthetic particles, termed KY, and IL-2 without any aggregation. The resulting low KY/IL-2 ratio of 10/1 (w/w) necessitates the administration of only minute amounts of the formulation to mice. We then compare the efficacy of low or ultra-low doses of IL-2 encapsulated into these nanoparticles on tumour growth in mice bearing the non-immunogenic TS/A mammary adenocarcinoma.

2. Materials and methods

2.1. Biovectors and IL-2

DPPC was purchased from Lipoid KG (Mannheim, Germany). Cholesterol was obtained from Sigma (St Louis, MI, USA). Cationic PSCs of 60 ± 15 nm, and having a charge density of 2 mmol/g of quaternary ammonium functions, were prepared as an aqueous suspension at 20 mg/ml. $SMBV^{TM}$ composed of a PSC (77% w) and a mixture (23% w) of DPPC and cholesterol (70:30 w/w) were prepared as described elsewhere [11,12].

Proleukin (human recombinant (r) IL-2 with a specific activity of 18×10^6 IU/mg) was purchased from Chiron (Suresnes, France), and vials were reconstituted with sterile water and stored at $-80^\circ C$. Formulations were prepared by simple mixing of a suspension of pre-made $SMBV^{TM}$ and IL-2 at a 10/1 KY/IL-2 ratio (w/w). Formulations were stable in a solution of phosphate-buffered solution (PBS) for at least 2 months at $+4^\circ C$ (retention of at least 95% of the biological activity of fresh free IL-2 as assessed by $[^3H]$-thymidine incorporation in CTLL-2 murine cells).

Surface plasmon resonance was used to analyse interactions between IL-2 and KY and to quantify the level of their association. KY at 0.1 g/l in PBS, (50 mM NaPO4) was injected in a Biacore X system (Uppsala, Sweden) and immobilised on an HPA sensor chip. Free IL-2 was injected and a standard curve of IL-2 adsorption (expressed as resonance units or RU) versus injected protein concentration was plotted. A formulation made at a 10/1 KY/IL-2 ratio (w/w) was then injected and the percentage of free protein in the formulation adsorbed on $SMBV^{TM}$ was determined from the sensorgram using the formula: total protein binding = $100 - (100 \times$ free protein in the formulation (UR)/total bound (UR)).

2.2. Tumour cell lines

The MHC class I^+, class II^-, $B7.1^-$, ICAM-1^- tumour cell line used was the non-immunogenic, undifferentiated, spontaneous mammary adenocarcinoma TS/A cell line (syngeneic from BALB/c H-2^d mice). Cells were maintained at $+37^\circ C$ in a humidified 5% CO_2 atmosphere in air, in complete medium (Roswell

Park Memorial Institute (RPMI)) 1640 culture medium supplemented with L-glutamine, sodium pyruvate, penicillin/streptomycin and 10% endotoxin-free fetal calf serum). For immunostaining experiments and *in vivo* experiments, cells were removed from their culture vessels with 0.1% trypsin. Before subcutaneous (s.c.) injection, the cells were washed three times in cold PBS (1×) and resuspended in the same buffer. Cells were not cultured for longer than 2 weeks.

2.3. In vivo *evaluation of tumour growth*

Female BALB/c (H-2^d) mice, 6–8 weeks old, were purchased from Janvier Laboratories (Le Genest, St Isle, France) and raised in specific pathogen-free conditions. The intranasal (i.n.) administrations (20 μl) were performed on non-anaesthesised mice.

Tumour cell lines were established by s.c. injection using 1×MTD (minimal tumorigenic dose, 5×10^4 cells for TS/A), or 5×MTD where indicated. Mice which were tumour-free 46 days after injection were rechallenged s.c. in the contralateral flank with the parental tumour cell line (5×MTD). Tumor growth was monitored twice per week by measuring two perpendicular tumour diameters using calipers.

For lymphocyte depletion, mice received intraperitoneally (i.p.) 200 μg of purified anti-CD4 (YTS 191.1.2) or anti-CD8 (YTS 169.4.2.1) mAb at day −3, −2, −1, +4 and +8. TS/A tumour cells were inoculated s.c. at day 0 (D0) (MTD). Cytofluorimetric analysis of control mice receiving these doses of mAb showed more than 95% reduction of the target population in the spleen (data not shown).

2.4. *Studies* in vitro

For cytotoxicity assays, tumour-specific short term CTL were generated using mixed lymphocyte tumour cell culture. Briefly, 3×10^7 spleen cells were collected at day 30 from mice that had rejected established tumours (that is days 75 or 79 from the start of the experiment in Fig. 7). These cells were stimulated with 3×10^6 TS/A tumour cells in complete medium for 4 days and then supplemented with 50 IU/ml of IL-2 for 2 days. The effector functions of splenocytes were tested at day 6 in a standard 4 h [^{51}Cr] release assay (effector-to-target ratios from 25/1 to 200/1) against labelled target cells: autologous tumour cells, a H-2^d irrelevant sarcoma WEHI 164, and a NK-sensitive YAC cell line. The lysis percentage of triplicates was calculated as ((average experimental counts per min (cpm) — average spontaneous cpm)/(average maximum cpm — average spontaneous cpm))×100. We defined specific lysis as lysis mediated by splenocytes from mice that rejected tumour cells minus the lysis mediated by splenocytes from naïve mice.

2.5. *Statistical analysis*

The significance of differences in tumour growth among the different groups of mice was determined by the Mann–Whitney U test using Statistica™ software.

3. Results

A cationic (QAE) SMBV™, termed KY, was first selected over uncharged or anionic (phosphates) SMBV™ for its enhanced capacity to (i) stably load rIL-2 when mixed at a 10/1 KY/IL-2 ratio (w/w), (ii) stabilise the IL-2 protein (as determined by enzyme-linked immunosorbent assay (ELISA)) or IL-2 activity ([^{3}H]-thymidine incorporation in the murine CTLL-2 cell line) at different temperatures, and (iii) increase the growth of human T cells activated with phytohemagglutinin (PHA) over a 2-week period, compared with free IL-2 (data not shown). Using plasmon resonance experiments, the percentage of IL-2 binding to KY adsorbed on sensorchips at a 10/1 KY/IL-2 ratio (w/w) was found to be 85% (see Fig. 1 for the sensorgram).

3.1. *Effect of co-injection of tumour cells with either free IL-2 or KY/IL-2*

We investigated whether co-administration *in vivo* of KY/IL-2 with tumour cells stimulated enough host immune effector cells to reduce tumour growth. Reduced growth rates were observed when TS/A cells were co-injected (i.e. mixed) s.c. at D0 with 10^3 IU of KY/IL-2. Free IL-2 even at higher doses of IL-2 (10^4 IU) had no antitumoral effect (Fig. 2), as assessed at the end of the experiment (days 31, 35).

3.2. *Effect of injection at day 0 (D0) of free IL-2 or KY/IL-2 at a distance from the tumour implantation*

In order to investigate whether the antitumoral effect was due to the activation of systemic or local immunity, experiments were performed with IL-2 injected s.c. at D0 in the flank contralateral to the injected tumour site (Fig. 3). In this experimental setting, even high doses of IL-2 (10^5 IU) did not reduce tumour growth, while low and ultra-low doses of KY/IL-2 led to significant differences in tumour growth ($P<0.01$ at days 23, 27 and 30).

3.3. *Effect of s.c. KY/IL-2 administration on implanted tumours*

We tested whether ultra-low doses of IL-2 complexed to KY might have a therapeutic effect on established tumours. BALB/c mice were s.c. injected with 1×MTD of TS/A cells. These mice were then treated at day 6 (D6) (Fig. 4a) by injection of 5×10^3 IU IL-2 complexed

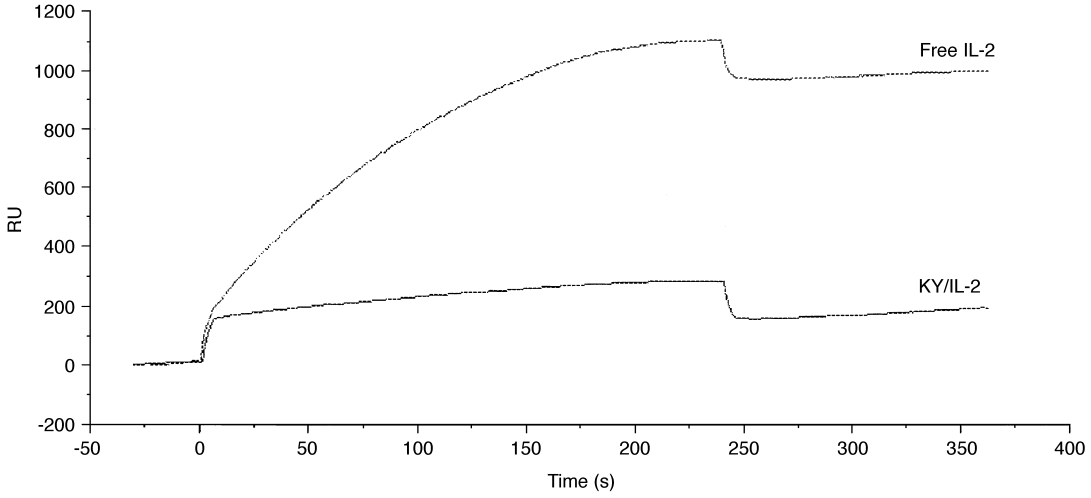

Fig. 1. Biacore analysis of IL-2 binding to KY. The KY formulation was injected at 0.05 g/l in 45 mM phosphate buffered solution (PBS) and, after 2 min, either free IL-2 or the KY/IL-2 (10/1, w/w) formulation was injected. The data are expressed as numbers of resonance units (RU) as a function of time. Free protein in the formulation is quantified as the amount of protein, not bound to KY in the formulation, adsorbed to the sensor chip.

to KY at one s.c. site on the contralateral flank or at five sites (i.e. 10^3 IU IL-2 per site) spread over the contralateral flank at intervals of at least 1 cm. Reduction of tumour growth was observed when the KY/IL-2 formulation was administered as a single injection (D6), compared with administration of either PBS or KY alone ($P<0.01$ at days 25, 27, 32 and 35). Remarkably, six out of 10 of the tumour-bearing mice treated at D6 with five injections at distant s.c. sites were scored as tumour-negative at the end of the experiment and the others four had only residual tumours. Mice which had rejected the tumour were rechallenged at day 46 (D46) in the contralateral flank to test their antitumoral immunity memory. Protection of mice rechallenged with 5×MTD tumour cells was observed against TS/A ($P<0.01$), with four out of nine mice showing no evidence of tumour growth (Fig. 4b). Mice first inoculated with irradiated (100 Gy) TS/A cells (negative controls) have been previously shown [13] not to be protected from this non-immunogenic tumour, with no difference between these mice and naive animals.

Similar experiments were performed in nude mice and no difference was ever observed in these mice between the growth of TS/A cells in untreated animals or animals treated with 5×10^3 IU IL-2 complexed to KY injected s.c. at either one or five sites (data not shown). Altogether, these results suggest that activating tumour-specific T cells by a single s.c. delivery of the KY/IL-2 formulation, preferably as multiple depots, may lead to tumour disappearance and protection from tumour rechallenge.

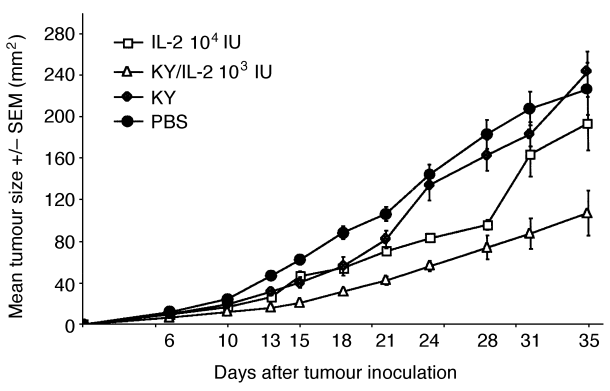

Fig. 2. Effect of KY/IL-2 administration at day 0 (D0) on TS/A tumour growth. BALB/c mice were injected subcutaneous (s.c.) with 1×minimal tumorigenic dose (MTD) of TS/A cells (5×10^4 cells) with either PBS (the untreated group),or with KY, IL-2 10^4 IU, or KY/IL-2 10^3 IU. Data are means±standard error of the mean (SEM) of five mice in each group from a single, representative experiment.

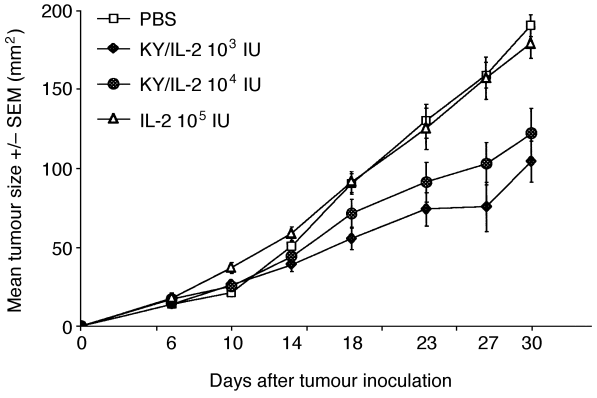

Fig. 3. Effect of KY/IL-2 administration at day 0 (D0), at a distant site, on tumour growth. BALB/c mice were injected s.c. in the contralateral flank with 1×MTD of TS/A cells with PBS, IL-2 10^5 IU, KY/IL-2 10^3 IU or KY/IL-2 10^4 IU. Data are means ± standard error of the mean (SEM) of five mice in each group from a single, representative experiment.

3.4. CD8$^+$ T cells mediate the primary rejection

Rejection of TS/A tumours was dependent on T-lymphocytes, as no rejection was observed in T cell-deficient *nu/nu* mice. The respective contribution of CD4$^+$ and CD8$^+$ T cells to the antitumour response was examined by depleting mice of these cell subsets. As shown in Fig. 5, administration of the CD8-specific mAb at the time of TS/A MTD inoculation abrogates the rejection of TS/A tumour cells induced by treating mice (such as in Fig. 4a) at D6 with 5×10^3 IU IL-2 complexed to KY at five sites (i.e. 10^3 IU IL-2 per site) on the contralateral flank ($P<0.01$ at days 23, 25, 27 and 29 for CD8-depleted versus non-depleted animals). Depletion of CD4$^+$ cells led to a partial loss of tumour protection, which may be the result of inhibition of the tumour-specific CTL response due to the lack of T-helper activity (Fig. 5).

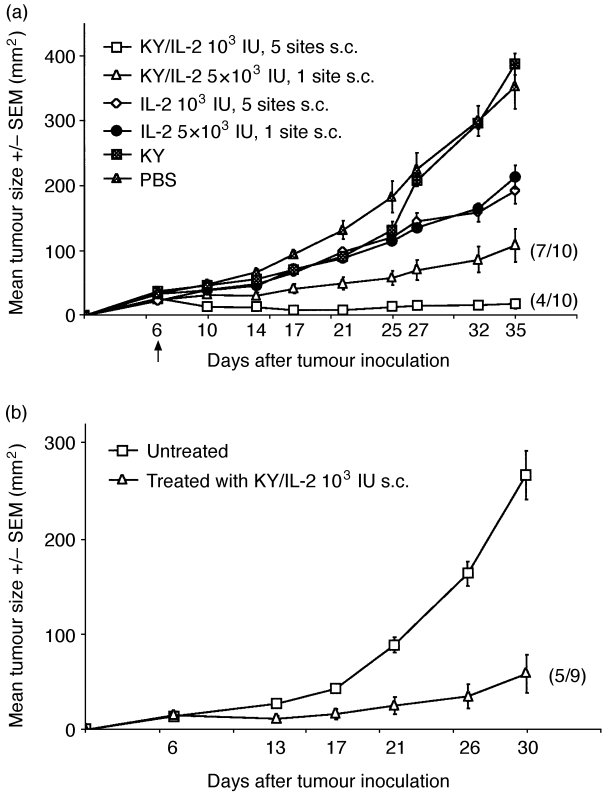

Fig. 4. Subcutaneous (s.c.) administration of KY plus ultra-low doses of IL-2 at five different sites leads to total regression of 6-day established tumours. Mice with TS/A tumours were treated at day 6 (D6) (arrow) by injecting PBS, KY, KY/IL-2 5×10^3 IU at one site or KY/IL-2 10^3 IU at five different sites in the contralateral flank (10 mice/group, panel a). Tumour growth is depicted for each group. The numbers of mice bearing tumours at day 35 for groups with tumour-free mice are shown in parentheses. Mice which were tumour-free at day 46 after s.c. administration of ultra-low doses of IL-2 complexed to KY at five sites at D6 and naïve mice were rechallenged with 5×MTD of TS/A cells (25×10^4 cells) injected s.c. in the controlateral flank (panel b). The mean size of the tumour as well as the number of mice with a palpable tumour are shown. These experiments were performed twice with similar results.

3.5. Effect of repeated intranasal (i.n.) administration of KY/IL-2 on implanted tumours

To determine whether i.n. administration of KY/IL-2 nanoparticles affected tumour growth, we first treated mice with a single i.n. administration of KY/IL-2 at D6 and observed reduced tumour growth rate, but no tumour rejection (data not shown). Since it was possible that the formulation was being rapidly degraded in the upper airway epithelium, syngeneic animals in a second series of experiments were injected s.c. with 1×MTD TS/A cells and then treated once or twice daily between days 6 and 10 with 10^4 IU IL-2 complexed to KY. Mice instillated with the same dose of free IL-2 were used as a comparison. None of the mice showed any local or systemic toxicity due to repeated IL-2 or KY/IL-2 instillation (data not shown). In this experimental setting, repeated i.n. administration of free IL-2 twice daily for 5 days led to significant reduction in tumour growth ($P<0.01$ at days 24, 27, 31 and 35) (Fig. 6a). The same dose and administration schedule with KY/IL-2 produced an increased therapeutic effect, with six out of the 10 mice being tumour-free at the end of the experiment, the other four having residual tumours. A single, daily i.n. KY/IL-2 administration did not significantly change the results, with four mice out of 10 being tumour-free at day 35 (Fig. 6a).

Mice that were tumour-free at day 48 were rechallenged with 5×MTD TS/A tumour cells and partial protection was observed, with four mice out of 10 showing no evidence of tumour growth (Fig. 6b). Overall, our findings demonstrate that IL-2 complexed to nanoparticules may be given i.n. to achieve complete tumour regression, thus providing a powerful non-invasive approach to influencing antitumoral immunity.

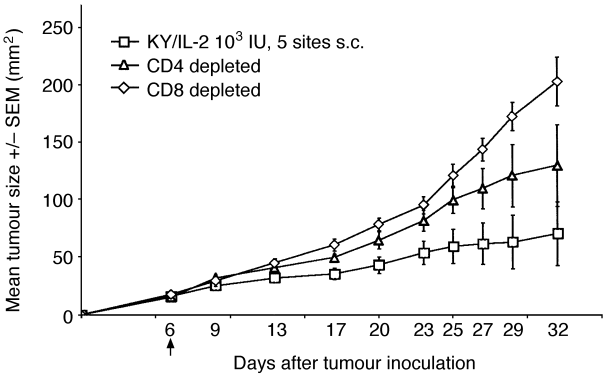

Fig. 5. CD8$^+$ lymphocytes mediate the rejection of TS/A tumour cells induced byKY/IL-2. Mice received intraperitoneally (i.p.) 200 μg of purified CD4 or CD8-specific mAb at day −3, −2, −1, +4 and +8. TS/A tumour cells (MTD) were inoculated subcutaneously (s.c.) at day 0 (D0). Mice with TS/A tumors were treated at day 6 (D6) (arrow) by injecting KY/IL-2 10^3 IU at five different sites in the contralateral flank, as in Fig. 4a. Data are means ± standard error of the mean (SEM) of five mice in each group from a single experiment.

3.6. Tumour-specific CTL response is enhanced by KY/IL-2

To further address the mechanism behind the antitumour activity of KY/IL-2, we evaluated its effect on the generation of CTL-killing of the non-immunogenic TS/A cells. Splenocytes were harvested at day 75 from four KY/IL-2 s.c. treated mice that were cured and able to reject 5×MTD TS/A tumour cells in rechallenge experiments. Splenocytes were restimulated *in vitro* for 6 days with TS/A cells. CTL activity against TS/A cells was detected in splenocytes of KY/IL-2 treated mice (Fig. 7a), while splenocytes from naïve animals displayed no cytotoxic activity [13]. CTL activity appears to be selective for the TS/A cells, since syngeneic WEHI 164 sarcoma cells, as well as NK-sensitive YAC cells, were not lysed (Fig. 7a).

Similarly, four mice treated i.n. with KY/IL-2 that were cured and able to reject 5×MTD TS/A tumour cells in rechallenge experiments were tested at day 79 for the capacity of their splenocytes to lyse TS/A cells. A selective CTL activity was detected in those mice (Fig. 7b).

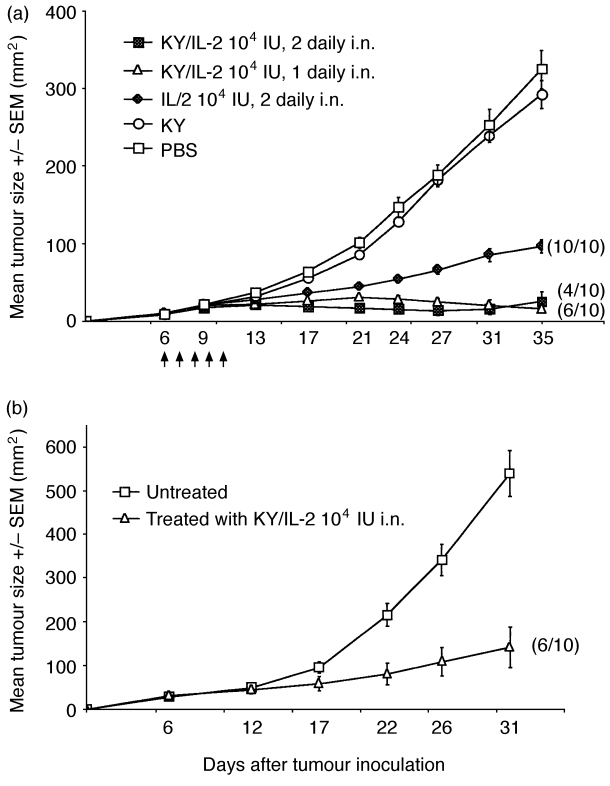

Fig. 6. Intranasal (i.n.) administration of KY plus low doses of IL-2 leads to total regression of 6-day established tumours. Mice with TS/A tumours were treated at days 6–10 (arrows) by once- or twice-daily i.n. administrations of PBS, KY, IL-2 10^4 IU or KY/IL-2 10^4 IU. Tumour growth is depicted for each group. The number of mice bearing tumours at day 35 are shown in brackets. These experiments were performed twice with similar results. The mice treated with KY/IL-2 10^4 IU that did not show evidence of tumour growth at day 48 were rechallenged with 5×MTD of TS/A cells (panel b).

4. Discussion

Because of IL-2's rapid clearance, its use as an effective *in vivo* immunostimulant has been limited. Current experimental and clinical protocols generally have to utilise local or multiple injections in order to achieve a significant antitumour effect. High-affinity IL-2 receptors (IL-2R) (Kd = 10 pmol) are constitutively expressed on 10% of human NK cells [14], as well as on some patients' T cells already primed to tumour antigens *in vivo*. In the case of advanced tumours or residual tumour cells, persistence of Ag may be combined with infusion of low-dose IL-2 to increase the continued reactivation and proliferation of tumour- or tissue-reactive T cells, thereby inducing an auto-immunity to treat cancer [15]. In fact, s.c. low-dose IL-2 given for 3 months as a treatment for cancer patients may lead, in some instances, to the appearance of dominant T-cell

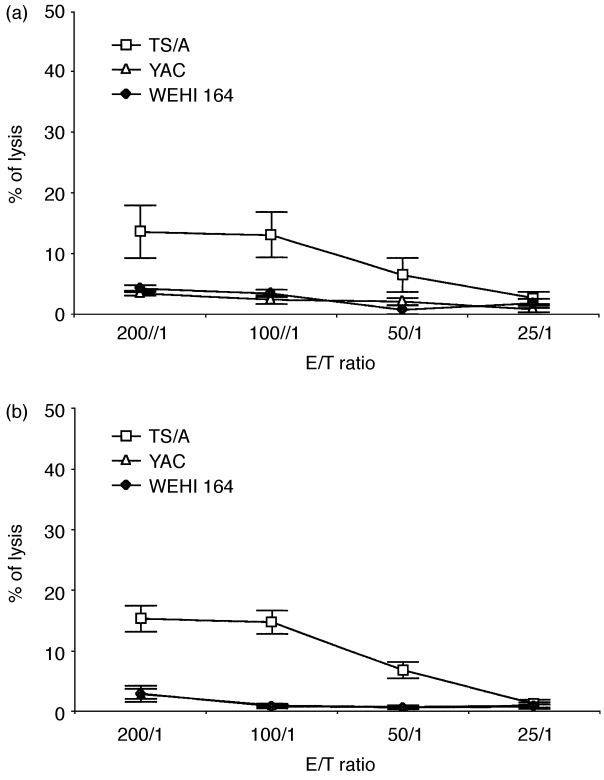

Fig. 7. Increased activity of antitumour cytotoxic T lymphocytes (CTLs) in mice which had rejected TS/A cells following administration of KY/IL-2. Mice received subcutaneous (s.c.) transplants of 1×MTD (5×10^4) TS/A cells and rejected the tumour following treatment at day 6 (D6) with a s.c. injection of KY/IL-2 (10^3 IU) at five different sites in the contralateral flank (panel a) or i.n. administration of KY/IL-2 10^4 IU at days 6–10 (panel b). These tumour-free mice were rechallenged at day 46 (panel a) or 48 (panel b) with 5×MTD TS/A cells. Spleens were collected at day 75 (panel a) or day 79 (panel b) in tumour-free mice and the splenocytes were cocultured for 6 days with TS/A cells. Cytolytic activity against indicated target cells was examined in a standard 4 h [^{51}Cr] release assay with different effector-to-target ratios (E:T). Data are means±standard error of the mean (SEM) of counts per minute (cpm) values obtained in four mice analysed individually.

clonotypes in the blood, together with thyroid dysfunction resembling that described in auto-immune thyroiditis [16]. The idea that systemic IL-2 administration alone (i.e. not as a vaccine adjuvant) may represent an appropriate way of boosting antigen-specific immune responses has been supported by the finding of multiple dominant T-cell clonotypes in the tumour and peritumoral tissues of patients treated with IL-2 as a preoperative adjuvant therapy [17]. The relevance of tissue-specific antiself responses in cancer immunotherapy is supported by the finding that patients with melanoma whose tumours respond to IL-2-based immunotherapy occasionally develop vitiligo, whereas vitiligo is never seen among melanoma patients who fail to respond to IL-2 [18]. Experimentally, it has since been shown that TRP-1-expressing recombinant vaccinia can cause auto-immunity (vitiligo) in mice and concomitant antitumour immunity (protection against B16 melanoma challenge) [19]. Thus, interfering, through IL-2 administration, with immunological checkpoints in order to harness tissue-specific auto-immunity may represent a worthwhile approach to cancer immunotherapy.

We investigated whether IL-2 complexed to a SMBV^{TM}, termed KY, provided systemic *in vivo* immunostimulation and an antitumour effect superior to that produced by the free cytokine, as a result of different biodistribution and depot characteristics. Our approach was to evaluate this new IL-2 formulation based on the simplicity of the procedure. This formulation was shown to preserve IL-2 biological activity ($>95\%$ of the initial IL-2 biological activity) and to adsorb 85% of free IL-2 at a low KY/IL-2 w/w ratio (10/1, i.e. for a 10^3 IU IL-2 s.c. injection, 55 ng IL-2 associated with 0.55 μg KY).

We observed that, if KY/IL-2 was co-injected with the tumour cells (Fig. 2), even at a lower dose than free IL-2, an inhibition of tumour growth was observed. If the administration was performed at a distance from tumour implantation (Fig. 3), KY/IL-2 had a growth inhibitory effect on the tumour. It is interesting to note that free IL-2, even at 100 times the dose of KY/IL-2, had no antitumoral effect. This result might be explained by an improved activation of the T cells by the formulation, due for instance to a slow release process.

Experiments performed on implanted tumours (Fig. 4) showed that tumour rejection could be performed after administration of KY/IL-2 at a distance from tumour. The finding that splitting the injection of KY/IL-2 into five different s.c. sites increased the antitumour effects in comparison to a single injection may be related to an increased availability of IL-2 for circulating tumour-specific IL-2R$^+$ T cells, and especially CD8$^+$ T cells that are the main effector cells leading to tumour regression (Fig. 5). In other words, the spatial distribution of IL-2 to several areas of the mouse drained by different regional lymph nodes may lead to a greater number of lymphocytes with different specificities for the tumour cells being recruited with, consequently, a more complete and stronger immune response. It is, in fact, unlikely that the systemic IL-2 levels reached after s.c. injection of 10^3 IU IL-2 (55 ng, i.e. for a 25 g mouse, a dose of 1.2 μg per kg body weight) would be high enough to cause peripheral immune stimulation, especially when considering that SMBV^{TM} release their content slowly over time. Alternatively, this difference may be simply due to the different pharmacokinetics of the same dose of IL-2 injected at a single site versus at multiple sites. It has not been possible to determine the local release rate of IL-2 in vivo for the injected KY/IL-2 formulation.

I.n. administration of KY/IL-2, for 5 consecutive days, also allowed rejection of implanted tumours while free IL-2 had only a tumoral growth inhibition effect (Fig. 6). The nasal administration was performed by instillation of non-anaesthesised mice with a small volume (20 μl). In these conditions, lung delivery is not observed [20]. IL-2 delivery at mucosal surfaces can activate the immune system so efficiently that pre-implanted tumours can be rejected. Many clinical trials were performed in humans using inhalation administration (mainly through lung delivery). Inhalation of IL-2 has been reported to prevent somewhat the progression of pulmonary and mediastinal metastases of metastatic renal cell carcinoma, breast and ovarian carcinoma, and melanoma [21]. These results using free IL-2 are of interest for further development using improved formulations.

In mice, liposomal IL-2 or poly(lactic acid) microspheres showed improved pharmacokinetic performances and better antitumour activities than free IL-2 [22–24]. However, in most studies, significant tumour regression occurred only when the encapsulated IL-2 was administered at much higher doses (1–10 μg) locally or peritumorally, but not systemically. In addition, the clinical development of IL-2 in tumour immunotherapy requiring immunostimulation, such as vaccination and the enhancement of tumour immunity, has been plagued with a lack of activity against tumours of low immunogenicity and the failure to eradicate established tumours. The host antitumour immunity could be clearly enhanced by the introduction of genes encoding diverse types of immunostimulatory molecules into tumour cells but, in most models, the expression of these immunostimulatory molecules only rarely induces rejection of established tumours [25]. This is in contrast to the present results obtained with a number of animals treated with low dose KY/IL-2 given s.c. or i.n., and which produced animals that were free of disease. Although the mechanisms of the antitumour effect of KY/IL-2 remain to be elucidated, complete responses seen against established tumours when using ultra-low

dose IL-2 indicate that such a formulation may hold promise for clinical applications in the treatment of cancer.

Our approach represents a more practical alternative to the current IL-2-based gene therapy approaches, and we propose that a sustained *in vivo* activation of tumour-specific T cells may be achieved with multiple site s.c. depots or repeated i.n. administration of KY/IL-2 formulations. Further studies are needed to test whether this formulation, when combined with irradiated tumour cells or a vaccinal Ag, could also be used to improve a specific and systemic immune response associated with a potent protective antitumour activity against poorly immunogenic tumours. This is a critical goal in vaccine development that has recently been approached with some success using liposomal IL-2 [26,27].

References

1. Kaplan G, Cohn Z, Smith K. Rational immunotherapy with interleukin 2. *Biotechnology* 1992, **157**, 157.
2. Rosenberg S. New approaches to the immunotherapy of cancer using interleukin-2. *Ann Intern Med* 1998, **108**, 853.
3. Ossendorp F, Mengedé E, Camps M, Filius R, Melief CJM. Specific T helper cell requirement for optimal induction of cytotoxic T lymphocytes against major histocompatibility complex class II negative tumors. *J Exp Med* 1998, **187**, 693–702.
4. Pardoll DM. Cancer vaccines. *Nature Medicine* 1998, **4**, 525–531.
5. Ridge JP, Rosa FD, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature* 1998, **393**, 474–478.
6. Schoenberger SP, Toes RE, Voort EIVd, Offringa R, Melief CJ. T-cell help cytotoxic T lymphocytes is mediated by CD40–CD40L interactions. *Nature* 1998, **393**, 413–414.
7. Staveley-O'Carroll K, Sotomayor E, Montgomery J, *et al*. Induction of antigen-specific T cell anergy: an early event in the course of tumor progression. *Proc Natl Acad Sci USA* 1998, **95**, 1178–1183.
8. Shrikant P, Khoruts A, Mescher MF. CTLA-4 blockade reverses CD8+ T cell tolerance to tumor by a CD4+ T cell- and IL-2-dependent mechanism. *Immunity* 1999, **11**, 483–493.
9. Deeths MJ, Kedl RM, Mescher MF. CD8+ T cells become nonresponsive (anergic) following activation in the presence of costimulation. *J Immunol* 1999, **163**, 102–110.
10. Castignolles N, Betbeder D, Ioualalen K, *et al*. Stabilization and enhancement of interleukin-2 in vitro bioactivity by new carriers: supramolecular biovectors. *Vaccine* 1994, **12**, 1413–1418.
11. Major M, Prieur E, Tocanne JF, Betbeder D, Sautereau AM. Characterisation and phase behaviour of phospholipid bilayers adsorbed on spherical polysaccharide nanoparticles. *Biochim Biophys Acta* 1997, **1327**, 32–40.
12. Prieur E, Betbeder D, Niedergang F, Major M, Alcover A, Davignon JL, Davrinche C. Combination of human cytomegalovirus recombinant immediate-early protein (IE1) with 80 nm cationic biovectors: protection from proteolysis and potentiation of presentation to CD4+ T-cell clones in vitro. *Vaccine* 1996, **6**, 511–520.
13. Prigent P, Mir SE, Dreano M, Triebel F. LAG-3 induces tumor regression and antitumor immune responses in vivo. *Eur J Immunol* 1999, **29**, 3867–3876.
14. Caligiuri MA, Zmuidzinas A, Manley TJ, Levin H, Smith KA, Ritz J. Functional consequences of IL-2 receptor expression on resting human lymphocytes: identification of a novel NK cell subset with high affinity receptors. *J Exp Med* 1990, **171**, 1509.
15. Pardoll DM. Inducing autoimmune disease to treat cancer. *Proc Natl Acad USA* 1999, **96**, 5340–5342.
16. Farace F, Angevin E, Dietrich PY, *et al*. Low-dose IL-2 treatment: activation of discrete T- and NK-cell sub-populations in vivo. *Int J Cancer* 1995, **62**, 523–528.
17. Kumar A, Farace F, Gaudin C, Triebel F. Clonal T cell expansion induced by interleukin-2 therapy in blood and tumors. *J Clin Invest* 1996, **97**, 1219–1226.
18. Rosenberg SA, White DE. Vitiligo in patients with melanoma: normal tissue antigens can be targets for cancer immunotherapy. *J Immunother Emphasis Tumor Immunol* 1996, **19**, 81–84.
19. Overwijk WW, Lee DS, Surman DR, *et al*. Vaccination with a recombinant vaccinia virus encoding a "self" antigen induces autoimmune vitiligo and tumor cell destruction in mice: requirement for CD4(+) T lymphocytes. *Proc Natl Acad Sci USA* 1999, **16**, 2982–2987.
20. De Haan A, Renegar KB, Small PA, Wilschut J. Induction of secretory IgA response in the murine female urogenital tract by immunisation of the lungs with liposome-supplemented viral subunit antigen. *Vaccine* 1995, **13**, 613–616.
21. Huland E, Heinzer H, Huland H, Yung R. Overview of interleukin inhalation therapy. *Cancer J Sci Am* 2000, **6**(Suppl. 1), S104–S112.
22. Anderson PM, Katsanis E, Leonard AS. Increased local antitumor effects of interleukin-2 liposomes in mice with MCA-106 sarcoma pulmonary metastases. *Cancer Res* 1990, **50**, 1853–1856.
23. Anderson PM, Katsanis E, Sencer SF, Hasz D, Ochoa AC, Bostrom B. Depot characteristics and biodistribution of interleukin-2 liposomes: importance of route of administration. *J Immunother* 1992, **12**, 19–31.
24. Vaage J, Mayhew E. Immunotherapy of a mouse mammary carcinoma by sustained peritumor release of IL-2. *Int J Cancer* 1991, **47**, 582–585.
25. Pardoll DM. Paracrine cytokine adjuvants in cancer immunotherapy. *Ann Rev Immunol* 1995, **13**, 399–415.
26. Krup OC, Kroll I, Bose G, Falkenberg FW. Cytokine depot formulations as adjuvants for tumor vaccines. I. Liposome-encapsulated IL-2 as a depot formulation. *J Immunother* 1999, **22**, 525–538.
27. Kwak LW, Pennington R, Boni L, Ochoa AC, Robb RJ, Popescu MC. Liposomal formulation of a self lymphoma antigen induces potent protective antitumor immunity. *J Immunol* 1998, **160**, 3637–3641.