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Original Paper

Interleukin-2 and Interleukin-4 Display Potent Antitumour Activity on Rat Medullary Thyroid Carcinoma Cells

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Currently, surgical resection is the only treatment used for human medullary thyroid carcinoma (MTC). However, as metastases are commonly observed, we investigated the potential of adjuvant therapy with interleukin-2 (IL-2) and interleukin-4 (IL-4) in a rat model. The interleukins were delivered by means of xenogeneic tumour cells engineered to secrete IL-2 and IL-4. We found that when a mixture of MTC cells and IL-2 or IL-4 secreting cells were implanted in rats, the growth of the resulting tumours was inhibited as a function of the number of interleukin-secreting cells in the inoculum. Moreover, association of the two interleukins exerted a synergistic antitumour effect. These experimental results, showing thyroid C cell tumour rejection, are of major interest, as they show the potential therapeutic application for these two interleukins, which could be used, in particular, as postsurgical adjuvants.

Key words: IL-2, IL-4, MTC cells, transduced xenogeneic cells, tumour growth inhibition
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INTRODUCTION

THE MEDULLARY thyroid carcinoma (MTC) is a tumour which develops from the C cells of the thyroid. In humans, it exists in both familial and sporadic forms [1]. The Wag/Rij rat (a Wistar-derived strain), which spontaneously develops with high frequency C cell carcinoma upon aging [2], is a useful model system for studying this tumour and its early development [3].

Currently, surgical resection is the only treatment used for human MTC. However, as this tumour commonly presents metastases, the use of adjuvant therapies is under investigation. The therapeutic potential of several cytokines has undergone clinical evaluation, and immunotherapies that employ interleukin-2 (IL-2) have given encouraging results with different human cancers, in particular with melanoma [4]. For these treatments, interleukin action was either obtained directly through systemic injection, or indirectly through *in vitro* interleukin activation of tumour infiltrating lymphocytes, which were subsequently re-injected into the patient [4]. However, major problems are encountered with the systemic use of IL-2 due to the short half life of the molecule and to the toxic side-effects which occur in patients treated with high doses.

In animal models, the inhibitory effects of IL-2 and interleukin-4 (IL-4) (both of which act via mobilisation of the host defences) on tumour growth are well documented [5–10]. In these experiments, local production of interleukins by engineered cells, which might locally stimulate the immune system, was developed. The authors used, as vectors for the interleukins, either syngeneic tumour cells [5–9] or tumour cells which were allogeneic or xenogeneic to the recipient host [10]. This latter approach was employed to circumvent ethical problems encountered in manipulating tumour cells of the host.

In this study, experiments were performed to determine the inhibition efficiency of IL-2 and of IL-4 on MTC cell growth, using the Wag-Rij rat as an experimental model. Interleukin delivery was achieved through xenogeneic IL-2 and IL-4 producing cells. Our results demonstrate an inhibitory action of these two interleukins on the growth of the rat tumour C cells.

MATERIALS AND METHODS

Animals

Wag/Rij rats were either bred in our laboratory or purchased from TNO (Rijswijk, The Netherlands) as the breeding stock. Two-month-old female rats were used in the experiments. Animals were randomly allocated into different experimental groups 1 day before injection of tumour cells.

Cell culture and transfections

P815 mastocytoma tumour cells are derived from DBA/2 mice. Rat MTC 6-23 cells are derived from a medullary thyroid

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carcinoma of Wag/Rij rat; they were kindly provided by Dr Raue (Heidelberg). Rat MTC (rMTC) and P815 cells were grown *in vitro* as previously described [10].

P815 tumour cells were transfected by the calcium phosphate technique with the BMG neo-vector containing either the murine IL-2 or IL-4 cDNAs [11]. Selection of stably transfected cells was established using the G418 antibiotic and cloned as previously described [7]. Supernatants were tested for IL-2 or IL-4 activity using, respectively, the CTL-L (IL-2 dependent T cell line) or the IL-4 dependent CT4 cell line. Activities are expressed as U/ml/10^6 cells/24 h and correspond to the supernatant dilution able to induce 50% of the maximal proliferation of the responding cells. These activities are 3500 U/ml/10^6 cells/24 h for IL-2 and 4000 U/ml/10^6 cells/24 h for IL-4.

MHC class I determination

Expression of class I MHC molecules in rMTC tumour cells was detected by immunohistochemical analysis. The cell line was cultured on special glass slides (Labtech), fixed in paraformaldehyde and sequentially incubated with a specific monoclonal mouse antibody to rat class I MHC molecules an a fluorescein-labelled donkey antimouse IgG antibody (Interchim).

Tumour cell inoculations

To evaluate the effects of the secretion of IL-2 and IL-4 by xenogeneic transduced P815 cells on the growth of rMTC cells, experiments were performed with increased amounts of xenogeneic transduced cells mixed with the rat tumour cells.

5×10^5 rMTC cells were implanted subcutaneously (s.c.) into the flank of the animals (5–6 per group). The rMTC cells were injected either alone or mixed with a given proportion of xenogeneic P815 cells transfected with the IL-2 or IL-4 gene. The number of xenogeneic cells in the inocula for each experiment is specified in the Results.

Tumour size estimation

Growth of the tumours was monitored by palpation in the earliest stages of tumour growth and then by measurement with callipers of the largest biperpendicular diameters of the tumours at least twice weekly.

Evaluation of the long-term protection of the animals from tumour growth

Systemic immunity to rMTC cells was tested by a new s.c. challenge of these cells (5×10^5 cells per inoculation) in the contralateral flank of animals that had previously rejected the rMTC cells, two or three months earlier.

Effect of a previous challenge with rMTC cells and IL-2 and IL-4 secreting P815 cells on a distant rMTC cell challenge

We investigated whether systemic inhibition of tumour growth could be observed after IL-2 or IL-4 treatment. Animals previously challenged with rMTC cells mixed with P815 (IL-2) and P815 (IL-4) cells were subjected, on the opposite flank, to a new challenge with rMTC cells alone. The animals initially received 5×10^5 rMTC cells with 2.5×10^6 P815 (IL-2 and IL-4) cells, present in equal proportions. Seven days later, a contralateral injection with rMTC cells alone (5×10^5 cells) was administered.

RESULTS

Detection of MHC class I molecules

A moderate positive signal on the rMTC tumour cells was observed with an antibody directed against MHC class I molecules (Figure 1). Membrane expression of MHC class I molecules on tumoural rMTC cells indicate that tumours may present antigens and so could be recognised by the immune system.

Inhibition of tumour growth induced by the presence of IL-2 and IL-4 secreting xenogeneic cells

Injection of rMTC cells (5×10^5 cells) induced tumours in all treated animals. Animals were systematically killed as soon as necrosis appeared, whatever the size of the tumour, for ethical reasons.

Addition of P815 (IL-2) or P815 (IL-4) cells at a ratio of 5:1 with rMTC cells resulted in striking inhibition of tumour growth, with only 1/5 animals developing a tumour (Figure 2c,d). When both the IL-2- and the IL-4-producing P815 cells were present in the inoculum (in the same final ratio of 5:1 P815 to rMTC cells), the tumour inhibition observed was even greater as all animals failed to develop tumours. Control animals which received rMTC cells mixed 1:5 with P815 cells transfected with the BMG vector had partial protection against tumour growth, with 3/5 animals developing a tumour, while all animals implanted with rMTC cells alone developed tumours (Figure 2a,b). Levels of protection of the animals after the different treatments are summarised in Table 1.

In preliminary experiments, we observed that when smaller numbers of P815 (IL-2) or P815 (IL-4) cells were mixed with rMTC cells, a less striking effect on the incidence of tumour development was observed. The tumour inhibition rate went from 16% for a ratio of 1:1 between the IL-2 transfected P815 cells and the rMTC cells, to 50% for a ratio of 2:1, and reached 80% for a ratio of 5:1, as described above. For animals co-injected with the IL-4 transfected P815 cells and the rMTC cells, the rate of tumour inhibition increased to 66% for a ratio of 2:1 between the xenogeneic and syngeneic cells, and reached 80% for the ratio of 5:1, as previously reported. A progressively higher inhibitory effect was observed as the amount of IL-2 or IL-4 producing cells was increased, and consequently the local concentration of secreted IL-2 and IL-4 increased.

When tumour inhibition was only partial, the tumours that emerged were delayed and grew more slowly. In addition, tumours that developed after co-injection of P815 (IL-4) cells and rMTC cells became rapidly necrotic.

A palpable inflammatory reaction of various extents developed

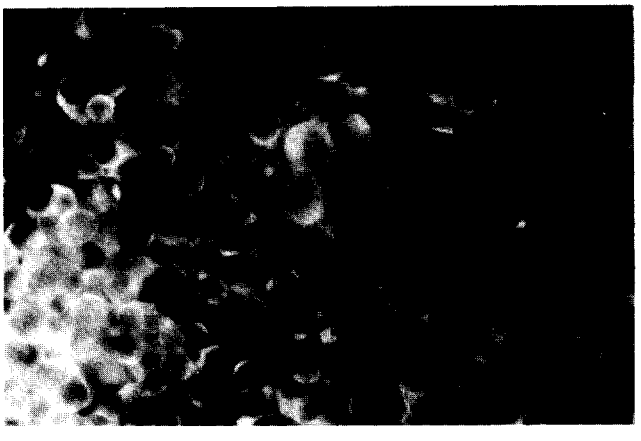

Figure 1. Immunohistochemical analysis of MHC class I antigen on rMTC cells showed a positive immunofluorescent reaction, indicating potential antigenicity of the cells.

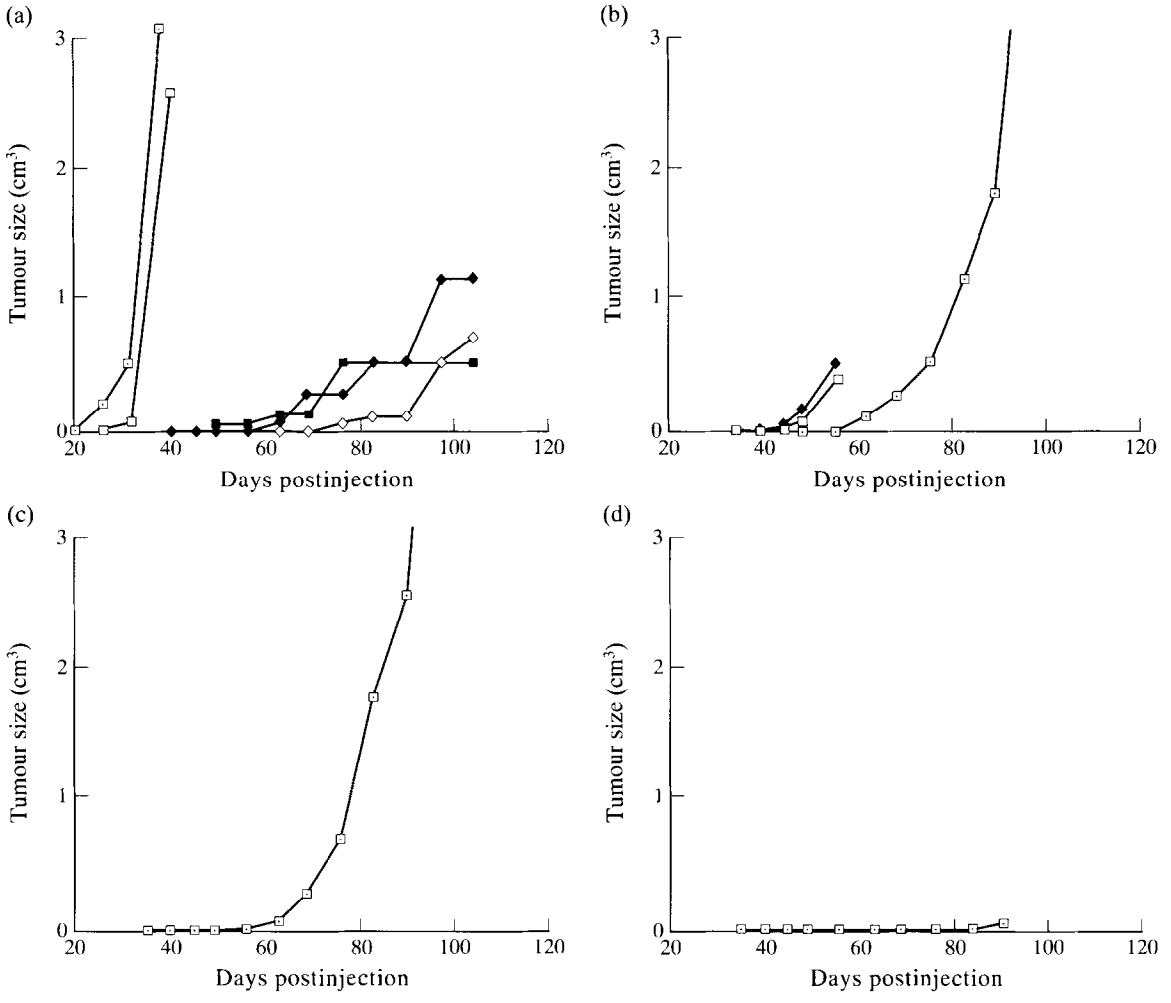

Figure 2. Tumour growth after s.c. inoculation on the right flank of rMTC cells (5×10^5) alone (a), in combination with P815 cells transfected with the vector alone (b), with P815 cells transfected with IL-2 or IL-4 (c and d, respectively), or with the combination of the two types of P815 transfected cells (not represented, as no tumour development occurred). Five-fold more P815 cells were used than rMTC cells. Animals were killed as soon as the tumour became haemorrhagic, whatever the size of the tumour, or when the tumour became too large ($n = 5$ animals for each group, except for the group injected with both P815 (IL-2) and P815 (IL-4) cells, wherer $n = 4$). Each line represents an individual tumour.

Table 1. Protection against tumour growth after inoculation of IL-2 and/or IL-4 secreting xenogeneic tumour cells

Inoculated cells	Fraction of animals with tumour growth inhibition	Percentage of protected animals (%)
rMTC*	0/5	0
rMTC + P815†	2/5	40
rMTC + P815 IL-2	4/5	80
rMTC + P815 IL-4	4/5	80
rMTC + P815 IL-2 + P815 IL-4	4/4	100

*5×10^5 rMTC cells; †2.5×10^6 P815.

at the site of injection, regardless of the type of transfected xenogeneic P815 cells added to the rMTC cells. This was even more striking with the injection of higher amounts of xenogeneic cells. When the syngeneic rMTC cells alone were injected, no local inflammatory response was detected.

Lack of long-term protection of the tumour implanted animals

Another injection of rMTC cells on the contralateral flank of protected rats induced a tumour regardless of whether IL-2- or IL-4-secreting cells were previously injected. In the case of tumour implants that contained the higher ratio of mixed P815

(IL-2) and P815 (IL-4) cells, partial protection was observed, as one animal developed resistance to the new challenge. Thus, these results indicate that, in the conditions tested, IL-2 or IL-4 treatment did not cause a sufficient immunological memory to develop.

Absence of systemic protection after a previous challenge with rMTC and P815 (IL-2) and (IL-4)

First, we observed that when two tumours were induced in the same rat (on each flank of the animal) after injection with rMTC cells, there was often a great disparity in the time of tumour appearance and, to a lesser extent, in their frequency of occurrence. Thus, in the control animals, development of the second tumour was slightly delayed or suppressed in these conditions (Figure 3b), while all the tumours that were induced first developed normally (Figure 3a). In animals subjected to concomitant injection with wild-type P815 cells, a partial inhibition of the development of the first tumour was observed, as in the experiment described above, and the emerging tumours rapidly became necrotic (Figure 3c). The contralateral rMTC challenge induced tumours in all the animals of this group (Figure 3d). IL-2 combined with IL-4 treatment (Figure 3e) did not completely prevent the appearance of a tumour in these conditions, as was previously the case. However, tumours grew more slowly and became rapidly necrotic, except for one that grew normally. Development of the contralateral tumours seemed not to be affected (Figure 3f). These different observations are summarised in Table 2, and they indicate that the simultaneous IL-2 and IL-4 secretion was not sufficient to protect animals against a subsequent contralateral challenge a few days later.

DISCUSSION

Our interest in human MTC led us to test the effects of interleukins in a rat MTC model of this tumour. The advantage of this model is the slow rate of tumour development, which allows survival of the animals for several weeks, and is in this way similar to human MTC. Indeed, tumour necrosis often requires sacrifice of the animals. Our present results establish that tumours caused by implanted MTC cells can be inhibited in their growth and even eliminated by a local IL-2 or IL-4 secretion. In addition, IL-2 and IL-4 were synergistic in their inhibitory effects, since when both interleukins were present, tumour growth was completely prevented. These results, achieved by use of xenogeneic cells that secrete IL-2 or IL-4, confirm the efficiency of this approach.

The necessity of using a high ratio (5:1) between these two types of cells to achieve protection may be explained both by the rapid elimination by the immune system of histoincompatible cells and by the necessity of a transient, high local concentration of interleukin to prevent growth of the MTC tumour cells. This can be clearly demonstrated by the increase in the protection level observed when the ratio between the interleukin-secreting xenogeneic cells and the rMTC cells was increased. This suggests that tumour growth inhibition correlates with the level of lymphokine production.

Inoculation with both the IL-2 and IL-4 producing cells resulted in a slightly greater effect on tumour rejection than when either P815 (IL-2) or P815 (IL-4) cells were used alone. Such a synergy between these two interleukins on tumour cells is an unusual phenomenon. As previously reported, it could be dependent on the type of tumour studies [10]. Although we did not identify the activated effectors that can lyse rMTC cells *in vitro*, the observed synergy between IL-2 and IL-4 argues in favour of similar or compatible effectors that have been implicated in tumour rejection. These two cytokines have been shown to be synergistic *in vitro* for optimal cytolytic activities of murine CTL and lymphokine activated killer cells [12]. Poor or even absent immunogenicity is relatively frequent in proliferative tumour cells, although in our case, expression of tumoral antigen by rMTC cells is highly suggested as it has been already described on another cell line of MTC, the TT cells [13]. In addition, detection of MHC class I antigen on these cells would be consistent with specific recognition of tumour antigens by the immune system.

When protected animals were submitted to a new but delayed challenge with rMTC cells, tumours developed, indicating an absent or insufficient immune memory. This assumes that tumour rejection is mediated by the recruitment of several cellular mechanisms, among which the non-specific one must predominate. There was no longer systemic tumour growth inhibition when rMTC cells were injected in the opposite flank of animals previously treated with a mixture of xenogeneic IL-2 and IL-4 transduced P815 cells and rMTC cells. These latter observations suggest that there was either an absence or an insufficient quantity of circulating effectors. This may be because the local delivery of interleukins at the first site was too small to generate a strong enough immune response.

Development of immune memory after IL-2 or IL-4 treatment using engineered syngeneic tumour cells depends both on the type of tumour treated and on the interleukin used. Thus, it has been shown that after IL-4 therapy, which causes the rejection of mastocytoma cells, there was no development of immune memory, as we have found to be the case in our experiments [8]. However, an effective memory has been observed after IL-4 induced renal tumour cell rejection [9]. With IL-2 therapy, an immune memory is developed more frequently [5–7].

In our interleukin delivery method, the immune system is stimulated by interleukins and also by the presence of xenogeneic cells. The strong response of the immune system to eliminate xenogeneic cells inoculated in high quantity (as indicated by the large inflammatory response) may participate in the elimination of the MTC tumour cells. This would explain the substantial tumour inhibition that develops with the injection of non-secreting xenogeneic cells. A partial inhibition of tumour growth was also reported to occur after co-inoculation of tumour cells and bacteria [14]. However, in the case of the combined inoculation of IL-2 and IL-4 secreting xenogeneic cells, the high increase in the level of rejection of the MTC tumour cells could be related to the synergistic effect of the two cytokines, through activation of a CTL compartment as previously discussed *in vivo* [10] and *in vitro* [12].

A great disparity in the rate of tumour growth was observed when rMTC cells were injected in the two flanks of animals. The growth of the second but also of the initial tumours was delayed or inhibited. This phenomenon of interaction between the two developing tumours has been previously reported by different authors [15, 16], who suggested that tumours may secrete facilitating or inhibiting factors that could affect the development of the second tumour. Our observations are consistent with a cytostatic effect of the first tumour on the second. This prevalence of circulating inhibitory factors may explain clinical observations of increased growth of metastases after excision of the primary tumour [15]. Thus, our results suggest that IL-2 and IL-4 therapy should be combined with surgical resection, as it may improve the elimination of any remaining tumour cells that

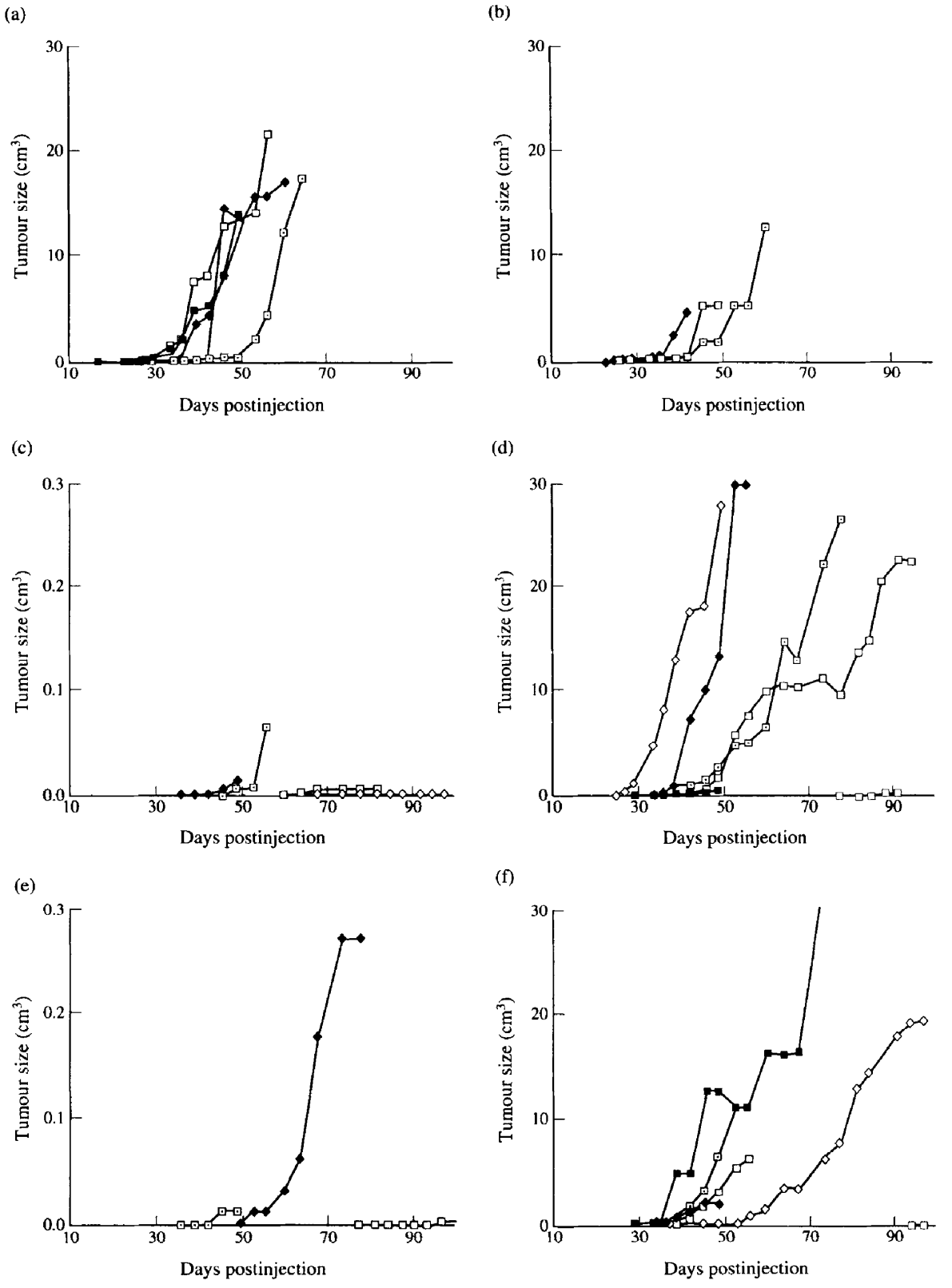

Figure 3. Tumour growth after initial s.c. inoculation in the right flank of animals, with rMTC cells alone (a), in combination with P815 cells (c), or with P815 (IL-2) and P815 (IL-4) cells (e), in identical quantities as on Figure 2, and a subsequent inoculation 7 days later in the left flank (b,d,f), with rMTC cells (5×10^5) for all animals ($n = 6$ animals for each group, except for group (a), where $n = 5$). Note the difference in the *y*-axis scale in figures (c) and (e). Each line represents an individual tumour.

Table 2. Effect of a primary challenge with IL-2 and IL-4 secreting xenogeneic cells (right flank) on development of tumours induced at a second site (left flank)

Inoculated cells		Percentage of tumour growth inhibition (%)	
On right flank*	On left flank†	On right flank	On left flank
rMTC	rMTC	0	40
rMTC +P815	rMTC	33	0
rMTC + P815 (IL-2)(IL-4)	rMTC	50	0

*5×10^5 rMTC cells and 2.5×10^6 P815 or P815 (IL-2)(IL-4) cells were injected. †5×10^5 rMTC cells were inoculated 7 days after the injection on the right flank.

could cause recurrence of the tumour. They may also decrease the risk of tumour cell dissemination at a time when the patients' immune defences are already attenuated by major surgery [17]. It has been reported [18] that peri-operative immunotherapy with low s.c. doses of IL-2 in patients undergoing surgery for colorectal cancer exerted profound effects on the cellular antitumour immune system, and thus may be able to deal with cells liberated around the time of surgery.

Attempts to cure animals of established tumours have been undertaken by several research groups employing mouse models. These studies have mainly consisted of vaccination with repeated injections of tumour cells transfected with the IL-2 gene [19, 20]. Our strategy to deliver interleukin is potentially interesting as it represents a simple and advantageous alternative to this vaccination protocol.

In conclusion, our results indicate that, in our rat tumour model, local production of IL-2 or IL-4 for a short period of time can trigger a potent antitumour reaction leading to major rejection of tumour cells. Concomitant local production of the two interleukins exerted a synergistic effect which completely abolished tumour development. The immune response generated by the xenogeneic producing cells is critically dependent on the amount of IL-2 and IL-4 produced and requires the presence of antigen at the site of interleukin secretion. These results are potentially important, as they could provide a rationale for the use of local interleukin therapy as an adjuvant of surgical MTC resection. In addition, we confirmed that xenogeneic cells can be a good vector for interleukin production; this may represent an alternative approach to the use of viral or retroviral vectors for local interleukin delivery.

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