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ORIGINAL ARTICLE

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The mechanism of regression of solid SL2 lymphosarcoma after local IL-2 therapy

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Abstract Treatment of cancer by the administration of interleukin-2 (IL-2) at the tumour site is a very effective approach. The mechanism of this tumour regression is not clear, although it is generally assumed that it involves an IL-2-stimulated immune reaction. There are, however, no immune parameters that consistently correlate with the therapeutic effect. We have studied the histopathological events in a subcutaneously (s.c.) growing SL2 lymphosarcoma (transplantation of tumour cells at day 0) treated with peritumoural IL-2 injections at days 10–14. Most IL-2-treated tumours had already begun to regress from day 12 onwards, showing that local IL-2 therapy was also effective in the present study. The immediate reaction after local IL-2 administration is vascular leakage from the surrounding circulation, causing oedema within the tumour and in a broad zone surrounding it. The presence of oedema is always accompanied by markedly increased tumour necrosis. After a few days extensive angiogenesis occurs at the border between the oedematous area and the healthy connective tissue. Leucocytes, mainly macrophages, migrate via the newly formed blood vessels to gain access to the necrotising tumour site, where they form a granuloma. These macrophages phagocytose the dead tumour material. During growth, the SL-2 tumours infiltrate the surrounding tissue. The infiltrating tumour strands are apparently attacked by macrophages, as the tumour cells in close proximity to the latter are progressively destroyed. Therefore, the body of the tumour and the infiltrating tumour strands are attacked in different ways. The primary effect of IL-2 administration at the tumour site is vascular leakage that causes oedema in and around the tumour. This is followed by extensive angiogenesis, with the resulting migration of white cells from the circulation, which form a granuloma around the tumour. Both the oedema and the granuloma cause tumour regression.

Keywords Cancer immunotherapy · IL-2 mechanism · Interleukin-2 · Tumour regression mechanism

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Introduction

Recombinant human interleukin-2 (IL-2) is a powerful anticancer drug if it is administered locally. This has been demonstrated in various experimental models [1, 6, 8, 9, 10] and in some human [3, 7] and animal [4, 5, 12] tumours. However, the mechanism of tumour regression following locally administered IL-2 is still not fully understood because the clinical features of IL-2-induced tumour regression vary considerably. In some cases, the tumour regresses within a few days of IL-2 administration – this is the immediate type of tumour regression – an example is that of transplanted SL2 lymphosarcoma in DBA/2 mice. In other cases, complete tumour regression requires up to 9 months, and over – a typical example of this delayed-type tumour regression is bovine ocular squamous cell carcinoma (BOSCC) [5].

In vivo IL-2-induced tumour regression may depend on a host of factors such as endothelial cell injury, intratumoural vascular leakage, and activation of cytotoxic T lymphocytes (CTL) [2]. The sequence and relative influence of the specific vascular and cellular mechanisms involved in locally administered IL-2 are not clear.

To investigate the immediate type of tumour regression induced by local IL-2 administration, we used SL2 lymphosarcoma in DBA/2 mice. After subcutaneous (s.c.) transplantation, the tumour expands by deep invasion of the surrounding tissues and by metastasising.

The untreated primary tumour and its metastases usually lead to death in about 3–4 weeks. Histopathological findings show a densely packed lymphoblastic tumour, consisting of cells with a scanty cytoplasm and irregular round to oval strongly basophilic nuclei, often with a conspicuous nucleolus. The mitotic index is high, often reaching 5–10 mitoses per high-power field. Tumour vascularity is poor, and areas of necrosis are common. We performed extensive immuno-histochemical studies to determine the involvement of T-helper lymphocytes, CTL, suppressor T cells, neutrophils, eosinophils, and macrophages. We did not find any correlation with tumour regression (unpublished observations) except for an extensive accumulation of macrophages at the tumour site. Therefore we decided to focus on a histopathological analysis of the events induced by the local administration of IL-2 at the tumour site. The results show that the primary effect of injection of IL-2 at the tumour site is the induction of oedema.

Materials and methods

Mice

Inbred 5–7-week old female DBA/2 mice were obtained from Iffa Credo, France. They were kept under standard laboratory conditions and used after an acclimatisation period of at least 10 days.

Tumour cells

SL2 lymphosarcoma cells are derived from a spontaneous tumour in a DBA/2 mouse. Tumour cells were harvested and kept frozen in vials until use. After thawing, they were transplanted at least twice before being utilised. Transplantations at weekly intervals were performed by intraperitoneal (i.p.) inoculation of 10^5 tumour cells. For experimental use, tumour cells were harvested on day 7 after transplantation by peritoneal lavage with 5 ml RPMI 1640, prepared from powder stock (Life Technologies, Paisley, U.K.). Two grammes of $NaHCO_3$, 0.25 g penicillin, 0.1 g neomycin, 0.05 g streptomycin and 4 ml 1 N HCl were added to 1 litre of RPMI medium.

Interleukin-2

IL-2 was a gift from Chiron, and is produced by *Escherichia coli* into which the human IL-2 gene has been inserted. Bacterial IL-2 is not glycosylated. The contents of one vial of lyophilised IL-2 (specific activity 18×10^6 IU/mg) was diluted in 1 ml sterile water according to the manufacturer's instructions. This solution was diluted using phosphate-buffered saline (PBS; Life Technologies), supplemented with 0.1% bovine serum albumin (BSA; Sigma, St. Louis, Mo.) and 5% dextrose.

Experimental design

The experimental design is shown in Table 1. On day 0, we inoculated 34 mice s.c. with 2×10^5 tumour cells in 0.1 ml RPMI in the dorso-caudal region of the right flank. One day prior to the first IL-2 treatment (day 9), the animals were divided into 6 groups (A–F), as shown in Table 1. On days 10–14, 2×10^5 IU IL-2 in 0.1 ml PBS/0.1% BSA was injected peritumourally once a day. Mice from groups A, B and C were killed on day 15 (one day after the last IL-2 injection) and mice from groups D, E and F on day 22 (8 days after the last IL-2 injection) by ether narcosis.

Table 1. Experimental design

Group	No. of animals	Treatment (D10–D14)[a]	Autopsy (day)
A	6	No treatment	15
B	6	PBS/BSA/dextrose	15
C	6	IL-2	15
D	4	No treatment	22
E	6	PBS/BSA/dextrose	22
F	6	IL-2	22

[a]D0: day of s.c. SL2 inoculation

The growth of the s.c. tumours was established as the product of the longest and the smallest diameter. At autopsy, the size of these tumours was measured in three dimensions with vernier calipers, and the tumour volume was calculated as $V = 1/6\ \pi\times L\times W\times H$.

Histopathology and immunohistochemistry

At days 15 and 22, the s.c. tumours and their surrounding tissue were excised and fairly large samples were fixed in phosphate-buffered 4% formalin solution for histopathological examination. Small fragments were frozen in liquid nitrogen and stored at –80°C for additional immunohistochemical and enzyme histochemical studies.

Histopathology

The central area of the tumour was always included for paraffin embedding and, if the tumours were not too small, slices of the peripheral region were also examined. Paraffin sections (5 μm) were prepared. Three sections with a 25-μm interspace were stained with haematoxylin-eosin (HE). In addition, sections were stained with phosphotungstic acid haematoxylin (PTAH; Mallory's method) to study blood vessels. Luna's stain for eosinophilic granules was also employed.

Enzyme histochemistry

Cryostat sections (7 μm) were stained for acid phosphatase (Barka's method) to identify macrophages, and to discriminate between macrophages and tumour cells.

Immunohistochemistry

Cryostat sections were exposed to rat anti-mouse F4/80 antibody (a gift from Jan De Groot, Academic Medical Centre, Amsterdam) to identify mature macrophages. A two-step immunoperoxidase staining method was employed. After acetone fixation and washing in Tris-buffered saline (TBS; Merck, Germany), the sections were preincubated with normal goat serum (diluted 1:10 in TBS) for 20 min. Sections were then incubated overnight with rat anti-mouse F4/80. After washing with TBS, polyclonal goat anti-rat horseradish peroxidase-conjugated antibody (Serotek, Oxford, U.K.) was applied for 45 min. The antibody was diluted 1:50 in TBS/0.1% BSA, and contained 2% mouse serum. Sections were washed with TBS and Tris-HCl. The peroxidase was then developed for 10 min with DAB solution containing 0.001% H_2O_2. Finally, the sections were counterstained with haematoxylin, dehydrated and mounted.

Histopathological evaluation

All sections were independently evaluated by three pathologists. Following several panel discussions, consensus was obtained.

Table 2. Tumour volume at autopsy

Group No.	A[b] 15D control	B 15D solution	C[b] 15D IL-2	D[c] 22D control	E 22D solution	F 22D IL-2
1	272.6[a]	142.6	268.6	3,139.7	639.9	508.8
2	389.6	125.5	114.2	2,449.6	965.5	38.5
3	208.3	573.7	117.5	1,809.7	3,464.7	35.6
4	255.6	57.7	138.0	2,079.2	858.1	69.2
5	198.6	578.9	157.3	–	987.6	19.4
6	688.0	189.5	343.0	–	123.1	14.4
Mean	352.1	308.0	189.8	2,370	1173.2	114.3

[a]Volume of s.c. tumor (mm^3)
[b]Comparison of tumour volumes in groups A and C: $P = 0.06$
[c]Comparison of tumour volumes in groups D and F: $P < 0.002$
P was calculated by the Mann-Whitney U-test

Statistical analysis

GraphPad InStat software was used for statistical analysis. For the comparison of the median of two unpaired columns, the Mann-Whitney U-test was applied. This is a non-parametric test. If the two-tailed P-values are low, it is unlikely that the data have been sampled from groups with equal medians.

Approval of the ethical committee

The research protocol was approved by the Ethical Committee for Experimental Animal Research of the Faculty of Veterinary Medicine, Utrecht.

Results

Clinical findings

Table 2 shows that 2 mice died before they could be killed on day 22: E4 on day 18 and D3 on day 21. In addition, mouse E3 was moribund on day 21 and therefore killed. All the other mice did not show any typical clinical symptoms such as fatigue, apathy, or exhaustion.

Fig. 1 shows the growth of the s.c. tumours. In the IL-2-treated groups, a reduction of tumour growth could already be observed between days 12 and 15. This was very early, as the tumours had been treated with IL-2 on days 10–14.

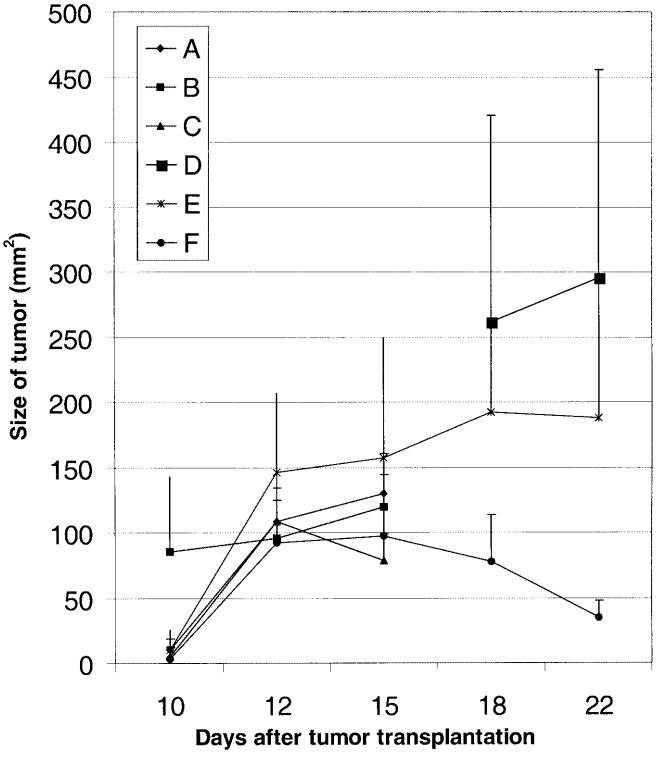

Fig. 1A–F. Graph of tumour growth and regression. **A–C** Autopsy at day 15. **A** No treatment; **B** PBS/BSA/dextrose; **C** IL-2; **D–F** autopsy at day 22; **D** no treatment; **E** PBS, BSA/dextrose; **F** IL-2 (mouse F1 was excluded from this calculation)

Therapeutic effects

The tumour sizes at autopsy are presented in Table 2. The tumour volume was considerably less in the IL-2-treated animals than in the controls. At day 15, the difference between the IL-2-treated groups and the control groups almost reached significance (A vs C; $P = 0.06$), whereas at day 22 the differences between IL-2-treated and both control groups were highly significant ($P < 0.002$). This implies that tumour regression markedly progressed between the first and the eighth day after the last IL-2 injection. There seemed to be a slight difference in tumour growth between the untreated groups and the PBS/BSA/dextrose treated groups. Though statistically not significant, this suggests that the injection of this solution interferes with tumour growth. Fluid injection will disrupt capillaries and small blood vessels surrounding the tumour, leading to stagnation of the blood circulation. This may enhance the tumour necrosis provoked by IL-2.

Histopathological findings in non-IL-2-treated tumours

The histopathological findings in the tumour samples from all control groups (A, B, D, E) were almost identical. The following observations were made. (1) In most cases, the necrosis was quite extensive. The transition from vital to necrotic tumour tissue was abrupt, and not

accompanied by an inflammatory response. Some tumour cells showed vacuolar degeneration. The necrosis was of the ischaemic type. (2) At the subepidermal margins of the tumours, some inflammatory response was apparent, consisting of both acute inflammatory and mononuclear inflammatory cells. In some cases in the subdermal zone the inflammation had a more granulomatous aspect. (3) In general, There was usually a small capsular zone surrounding the tumor. This zone contained angioblastic fibrous tissue.

Histopathological findings in IL-2-treated tumours at day 15

All s.c. tumours in this group showed signs of necrosis, which was of the same type as in the controls, but far more extensive. The tumour cells were interspersed with inflammatory cells, i.e. neutrophilic and eosinophilic granulocytes, lymphocytes, and macrophages. The necrosis in the border areas of the tumour consisted largely of an eosinophilic homogeneously stained zone with only indistinct remnants of tumour tissue. It appeared to be liquefaction-type necrosis (Fig. 2A, zone 3). There were also some congested blood vessels in this area, but neither vascular thrombosis nor intravascular fibrin deposition was observed. This was further confirmed by PTAH sections; this staining method reveals fibrin deposits. At some distance from the necrotic tumour, there was a highly vascular fibro-angioblastic layer displaying marked angiogenesis (Fig. 2A, zones 1 and 2B, zone 1). The new blood vessels were surrounded by a dense inflammatory cell infiltrate that apparently arose from these vessels. There was a large zone of pale stained

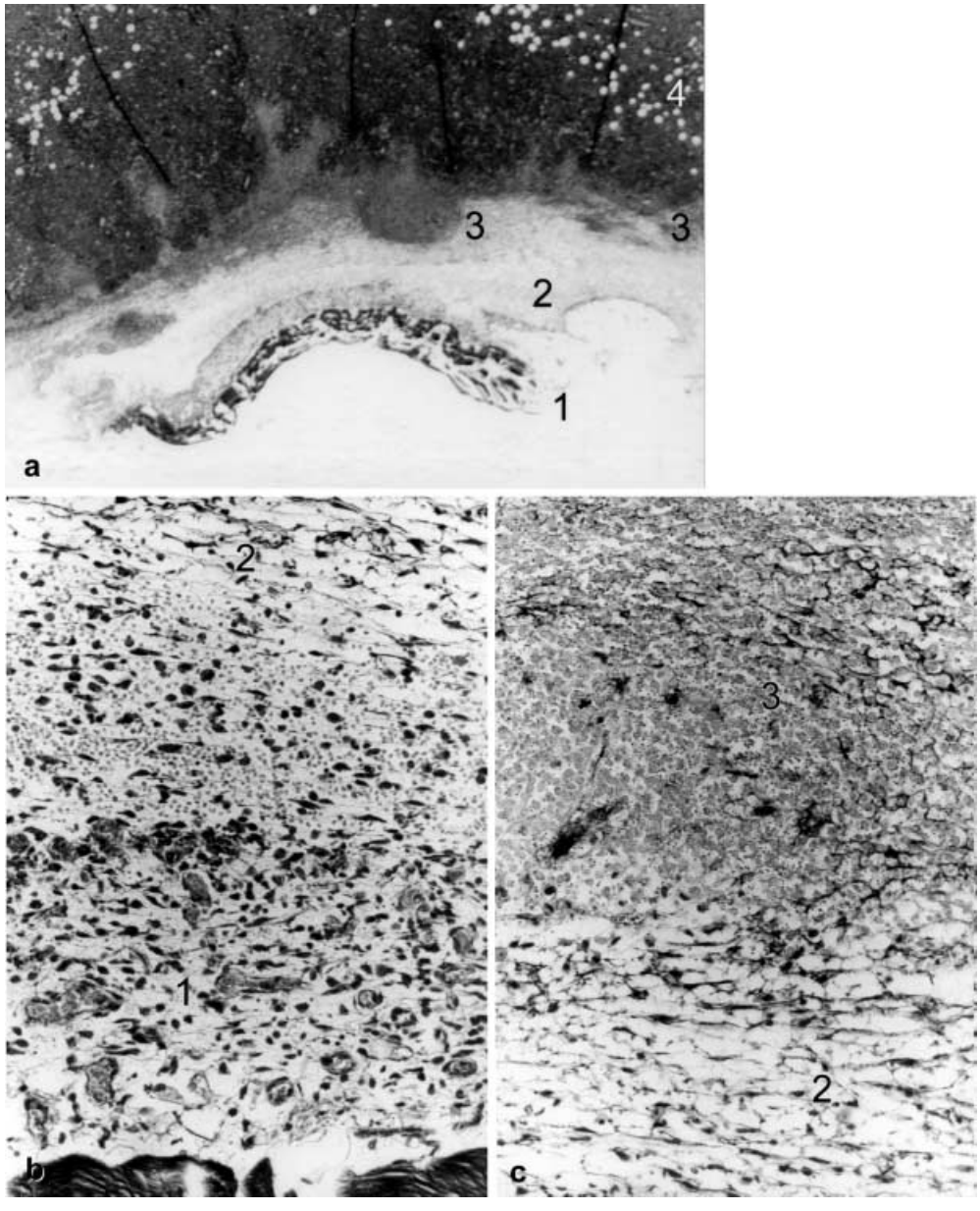

Fig. 2. A Regressing IL-2-treated tumour at day 15. Note 4 zones: (1) angiogenesis surrounded by inflammation; (2) an oedematous zone; (3) a semi-necrotic tumour zone; (4) a zone with viable tumour (PTAH; 20×). **B** High-power view of zone 1 in previous section. Note the zone with angiogenesis (1) surrounded by an inflammatory infiltrate. In the upper part: the oedematous zone (2) (PTAH; 200×). **C** High-power view of zones 2 and 3 in **A** (PTAH; 200×)

oedematous tissue between the angioblastic inflammatory zone and the area displaying tumour necrosis (Fig. 2A–C, zone 2). This zone was much larger than in the control groups.

Histopathologic findings in IL-2-treated tumours at day 22

Only one IL-2-treated mouse (F1 in Table 2) still bore a tumour that did not respond (or responded poorly) to IL-2 therapy. All the other tumours had regressed to 1% to 3% of the size of the control tumours. Small foci with some remaining degenerated or necrotic tumour tissue were present (Fig. 3A, 4A). Immediately adjacent to the tumour, there was a broad zone of dense granulomatous tissue containing numerous macrophages (Fig. 3B, C). The mononuclear infiltrate was particularly prominent in the tumour border areas. The foci of remaining ''viable'' tumour cells were often surrounded by a chronic inflammatory response, containing macrophages (Fig. 3B, C; Fig. 4B, C). Macrophages were identified by both F4/80 antibody and acid phosphatase staining. In the border areas of the tumour, there was a change in staining intensity of the tumour cells in addition to other degenerative features such as the ground glass appearance of the cytoplasm, vacuolisation of the cytoplasm, and swollen nuclei with a rather translucent aspect (Fig. 5). In one treated case, the tumour had disappeared completely, leaving an active keloidal tissue reaction, with occasional foci of mononuclear cell infiltrate (Fig. 6).

In the granulomatous area angiogenesis occurred, but there were also blood vessels with marked endothelial proliferation (Fig. 4B, C) occluding part of or even the whole of the blood vessel lumen. This was also observed in the tumour tissue (Fig. 7).

Discussion

Fig. 1 shows that IL-2-treated s.c. tumours already started to regress between days 12 and 15. This is very early, as the IL-2 injections were only given on days 10–14. Local IL-2 injections led to a cascade of pathological mechanisms, which reduced the tumour volume to 1% to 3% of the size of untreated tumours 8 days after the last IL-2 injection. One of the first phenomena in this cascade is extensive IL-2-induced oedema in the tumour and in a broad zone around the tumour – this oedema is superposed on that induced by the untreated SL2 tumours – and is accompanied by massive tumour necrosis. The latter might result from interstitial pressure due to the oedema and/or to reduced food and oxygen supply. It is clear that tumour tissue is vulnerable, as it is fast-growing and its vascularisation is not well established.

This induction of oedema is followed by a strong angiogenic response in the vascular region surrounding the oedematous area. The new blood vessels allow the infiltration of white blood cells that cause a granulomatous reaction, with more individualized tumour cell destruction by the adjacent inflammatory cells. This leads to a cellular non-specific and specific immune

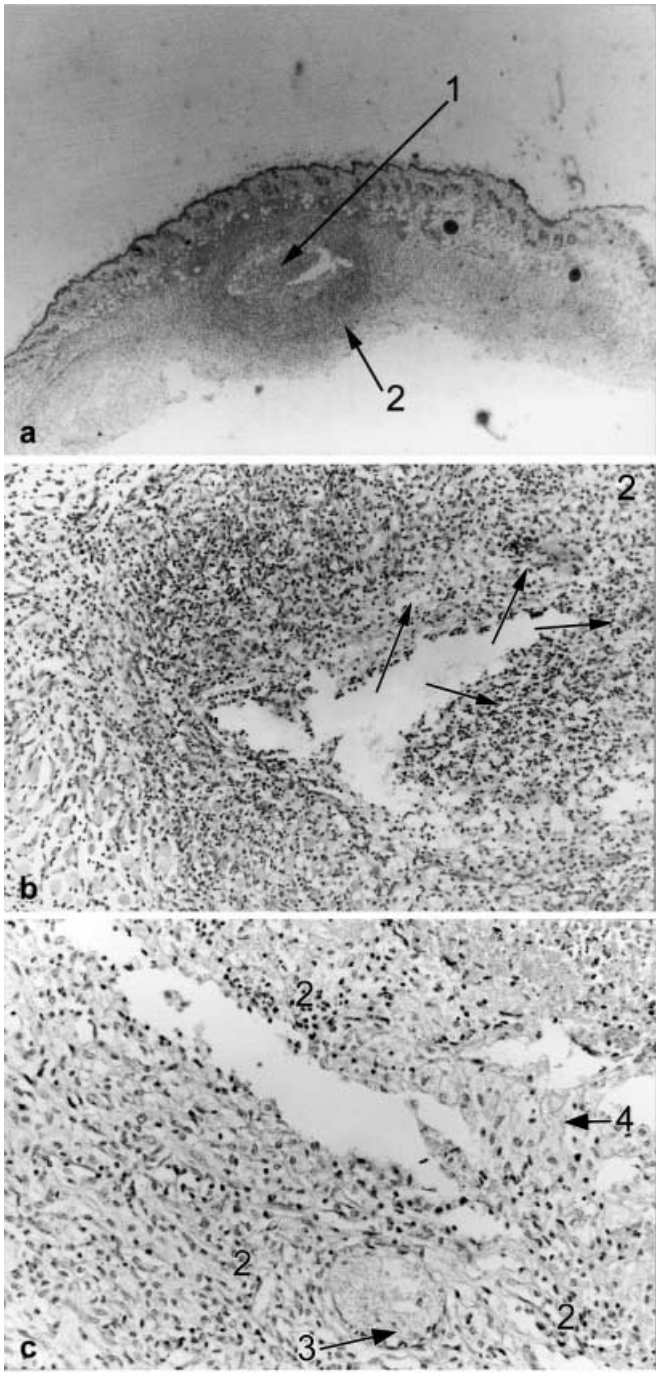

Fig. 3. **A** Regressing IL-2-treated tumour at day 22. Small semi necrotic tumour focus (1) is still present. This is surrounded by a dense inflammatory zone (2). Some semi-necrotic degenerated tumour cells are presumably still present (HE; 20×). **B** Detail of **A**. Dense inflammation consisting of macrophages and lymphocytes (2) and semi-necrotic tumour debris (*arrows*) (HE; 200×). **C** Detail of **A** showing chronic inflammation (2), an occluded blood vessel (3), and some remaining degenerated tumour cells (4) (HE; 200×)

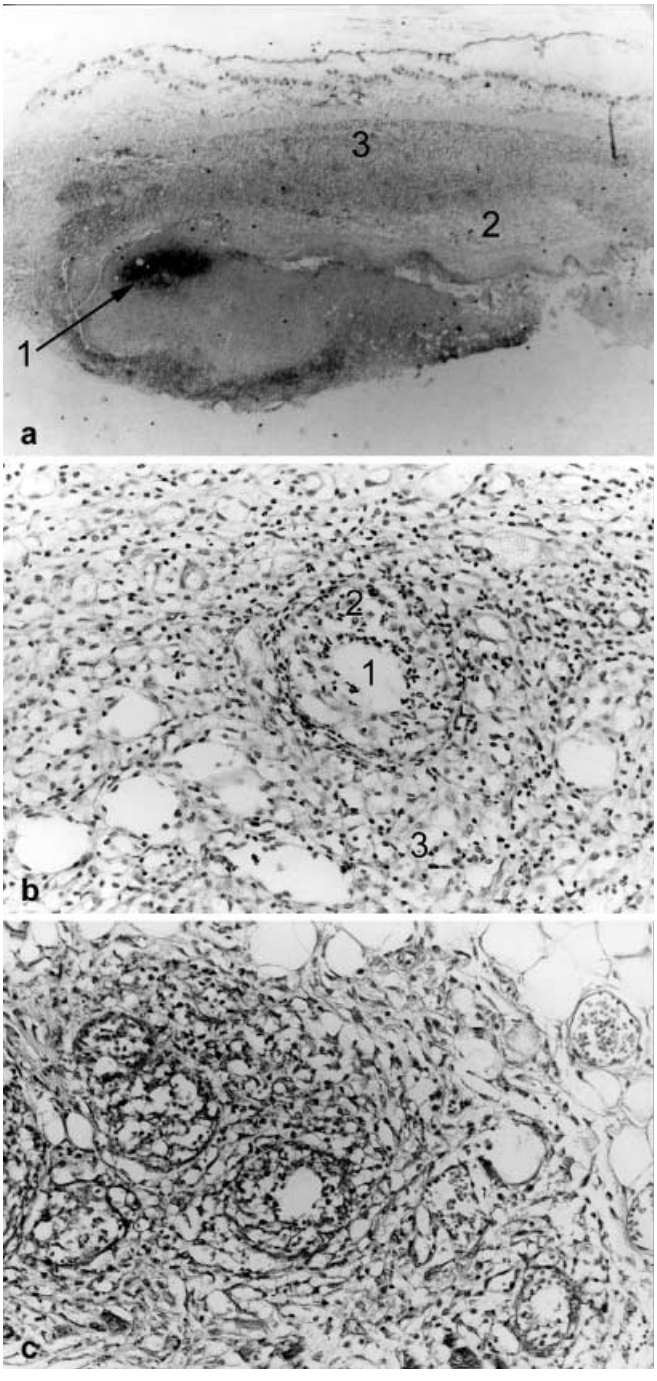

Fig. 4. **A** Regressing IL-2 treated tumour at day 22. Inflammatory area (3) surrounding a dense haemorrhagic, semi-necrotic area (1). Dense haemorrhagic necrosis in the centre (1). Some oedematous tissue is still present (2) near the tumour (HE; 20×). **B** Magnification of **A**. In the granulomatous area, a dilated blood vessel (1) showing endothelial proliferation (2). The surrounding granulomatous area (3) predominantly shows macrophages and some lymphocytes (HE; 400×). **C** Magnification of **A** showing a peritumoural chronic inflammatory zone (HE; 100×)

response, which may result in further tumour regression. It is well known that cured mice are specifically immune [8, 9] and that metastases are destroyed [8, 9, 11].

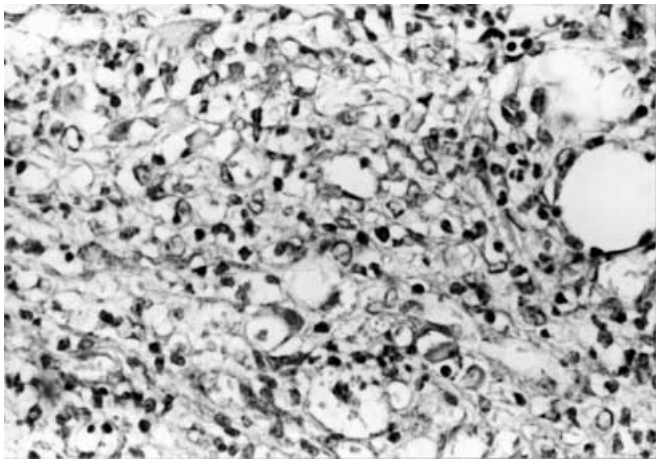

Fig. 5. Regressing IL-2-treated tumour at day 22. Degenerating tumour cells. Tumour cells are pale and swollen (1). Scanty lymphocytic infiltrate is present (HE; 400×)

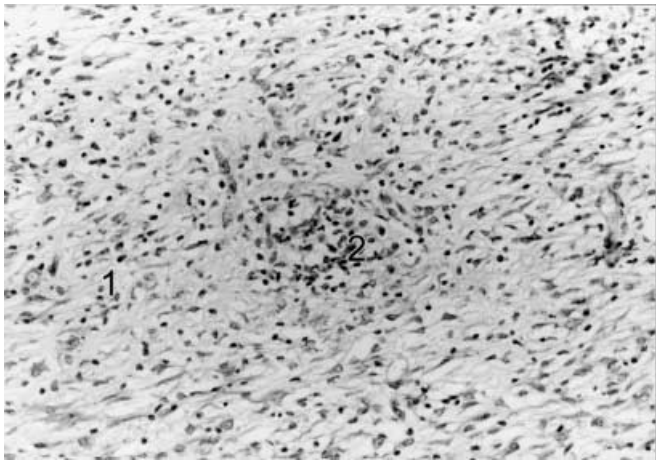

Fig. 6. IL-2-treated tumour that has regressed completely at 22 days. A small focus of inflammatory cells (2) surrounded by active fibromatous connective tissue (1) (HE; 200×)

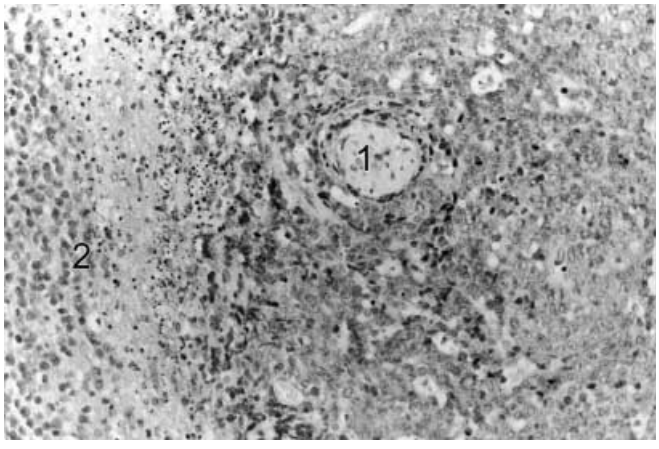

Fig. 7. IL-2 treated tumour at 22 days. Occluded blood vessel (1) in necrotising tumour tissue (2) (HE; 200×)

In comparison with immediately regressing solid SL-2 tumours, the mechanism of delayed regressing tumours is quite different. The prototype of these tumours is BOSCC [2, 3] and equine sarcoids. Complete tumour regression usually requires more than 3 months, and occasionally even between 9 and 21 months. In BOSCC the vascular effect of IL-2 is less clear than in the case of SL2. On the other hand, BOSCC usually contains a dense lymphocytic infiltrate. We have the impression that IL-2 strongly stimulates these lymphocytes. Lymphocytes penetrate strands of the remaining tumour tissue, and may contribute toward tumour regression. In addition, IL-2 may stimulate macrophages that – very slowly – induce degenerative changes in the adjacent tumour strands. Tumour cell necrosis is not massive, but individual cells degenerate and are killed due to the close contact with macrophages (Stewart et al., unpublished results). So the tumour regression after local treatment of BOSCC appears to be a stimulated immune reaction against tumour-associated antigens.

Conclusions

IL-2 is a molecule with a variety of functions. The most striking early effect after local IL-2 treatment of solid SL-2 tumours is the immediate induction of oedema at the site of the IL-2-treated tumour and the accompanying severe tumour necrosis. Somewhat later, angiogenesis is induced at some distance from the tumour – this enables white blood cells to reach the tumour area, and leads to a granulomatous reaction that kills tumour cells and removes the tumour debris.

The course of events induced by IL-2 depends on the composition of the tumour. In the case of a tumour that is fast-growing and poorly vascularised (solid SL-2), the oedema-inducing IL-2 effect is dramatic, as massive death of the tumour cells is induced. The tumours may regress and even disappear completely within a few days. In the case of a tumour (BOSCC) that is slow-growing, well-vascularised and infiltrated with lymphocytes and macrophages, this oedematous/necrotic reaction is less obvious. In this case, IL-2-induced tumour regression is mainly caused by stimulation of macrophages. These macrophages kill adjacent tumour cells. Cell death is the final stage of the cellular degenerative process, with vacuolisation of the cytoplasm and altered staining intensity. This is a slow process that may require months, which is surprising, as IL-2 is removed from the body within a few hours.

In all cases of regressed tumours, the dead tumour cells will lead to an immune reaction, provided that the tumour has tumour-associated antigens. Hence it is not surprising that cured animals become immune to re-challenge.

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