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Local interleukin-2 therapy in bovine ocular squamous cell carcinoma

A pilot study

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Summary. Five cows bearing bovine ocular squamous cell carcinoma (BOSCC) were treated with low doses of recombinant human interleukin-2 (rhIL-2). A dose of 2500 U rhIL-2 was injected intralesionally and another 2500 U were injected into the subparotid regional lymph node once a day during a period of 5 consecutive days. This cycle of 5 days was repeated after an interval of 2 days. Total regression of the tumor was observed in three out of five animals. One cow showed tumor regression (>80%) accompanied by metastases to the regional lymph node that were observed from the fifth week after the beginning of the treatment. Growth of the tumor of the fifth animal was retarded after treatment. In vitro proliferation of peripheral blood lymphocytes was investigated in two animals and tumor-infiltrating lymphocytes in one animal during incubation in various rhIL-2 concentrations. Cytotoxic activity of both cell populations against P815, Yac-1 and BOSCC-derived cell lines increased during incubation with rhIL-2. Cultured BOSCC-infiltrating lymphocytes showed predominant killing of the BOSCC-derived autologous cell line after 4 weeks of culture. Preliminary phenotype analysis did not give conclusive results with respect to the types of cells responsible for killing.

Introduction

Lymphokine-activated killer (LAK) cell therapy combined with injections of large doses of interleukin-2 (IL-2) has been successful in some murine tumor models [16]. In various human tumors total or partial regression could be obtained with this therapy [18, 20]. However, in vitro activation and culture of very high numbers of cells is time-consuming and expensive and the systemic injection of high doses of IL-2 caused serious toxic side-effects in patients [18].

Rosenberg et al. [19] reported successful treatment of some established murine tumors by intravenous injection of large amounts (approx. 100000 U) of recombinant IL-2 without LAK cells. The beneficial effects of repeated injections of rather low doses of T-cell growth factor/IL-2 around the site of a s.c. inoculum of cells of a methylcholanthrene-induced fibrosarcoma in mice were reported by Bubenik et al. [2–4]. Recently, Vaage [24] reported the successful treatment of s.c. implantations of a murine mammary carcinoma in mice. A complete cure was achieved by 12 daily peritumoral injections of 1500–10000 U recombinant IL-2. Higher local doses of the interleukin caused some regression in contralateral tumors, but also toxic effects. After treatment of bladder carcinoma by repeated intralesional injections of only 2000–4000 units of IL-2, Pizza et al. [17] observed complete and partial tumor regression in three out of six, and two out of six patients, respectively. Partial regression was noticed with lower doses down to 300 units per day. Forni and Giovarelli [12] observed regression of five out of five inoperable human oral squamous cell carcinomas after daily intralesional injections of 200 U IL-2 accompanied by injections of 200 U IL-2 around the regional lymph node during one or more cycles of 10 days. All authors mentioned speculate on possible local activation of cells, like T cells or NK cells, by the exogenous IL-2.

Bovine ocular squamous cell carcinoma (BOSCC) is a spontaneously occurring tumor of veterinary importance because of its high frequency in some countries. It can also be considered as a good model for immunotherapy of comparable human epithelial tumors. In former and ongoing studies we investigated the effect of Bacillus Calmette-Guérin (BCG) immunotherapy in this model [14]. In the present pilot experiment low doses of recombinant human IL-2 (rhIL-2) were injected intralesionally in animals bearing BOSCC and into the draining lymph node. The effect of this local/regional therapy was recorded as well as proliferation, cytotoxicity and phenotype characteristics of peripheral blood lymphocytes and tumor-infiltrating lymphocytes during in vitro incubation with various doses of rhIL-2.

Materials and methods

Animals. Five animals (cows 1–5), two of the Dutch Frisian and three of the Maas-Rijn-IJssel breed, aged 5–11 years, with histologically confirmed BOSCC were used for IL-2 therapy. The animals were in good physical health. Tumor sizes varied between 1 × 1 and 4 × 4 cm. The regional lymph nodes were not enlarged.

Treatment protocol. Recombinant human IL-2 from Glaxo (Geneva, Switzerland) (*E. coli*-derived) was used. Each day 2500 U were injected into the tumor in 1 ml saline and

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another 2500 U in 1 ml into the draining lymph node. Therapy consisted of two cycles of 5 consecutive days with an interval of 2 days.

Monitoring. Before, during and after treatment tumor sizes were recorded. Total and differential blood cell counts were performed on days 0, 1, 3, 7, and 14. Alkaline phosphatase, γ-glutamyltransferase, total protein, and protein fractions were determined on days 0, 7, and 14. Clinical examination, with special attention to signs of metastasis, was done weekly throughout the experiment. After slaughter (cows 4 and 5) pathological examination was performed.

Peripheral blood lymphocytes. Peripheral blood lymphocytes (PBL 5×10^6 cells) from a control and a tumor-bearing animal were incubated in 5 ml Iscoves medium, supplemented with 10% fetal calf serum, glutamine, penicillin and streptomycin, containing 500 U rhIL-2/ml at 37° C, in 5% CO_2 and a humidified atmosphere. The culture medium was refreshed every 3–4 days. Cell numbers were recorded during a period of 14 days. Cytotoxic activity of the cells was determined at days 0, 7, and 14.

Tumor-infiltrating lymphocytes. Tumor-infiltrating lymphocytes (animal 3) were isolated by mincing a tumor biopsy to pieces of approximately 1 mm^3. Pieces and single cells were incubated during 7 days in the above-mentioned medium supplemented with 100 U or 1000 U rhIL-2. After 7 days non-adherent cells were washed and again incubated in medium with rhIL-2. The culture medium was refreshed every 3–4 days. Cell numbers were recorded and the cytotoxic activity of the cells was determined at days 14 and 28. Preliminary analysis of phenotypes was carried out.

Cytotoxicity assay. Cytotoxic activity of PBL (days 0, 7, and 14) and tumor-infiltrating lymphocytes cultured in rhIL-2 (days 14 and 28) was determined in a 4-h ^{51}Cr-release assay. Target cells, either Yac-1 and P815 mouse cell lines or cell lines grown from BOSCC of various cows, were labelled by incubation of 1×10^6 cells with 100 μCi $Na_2{}^{51}CrO_4$ in 0.5 ml complete Iscoves medium (see above) for 1 h. After washing, 5000 target cells were incubated with effector cells, E:T ratio (50:1), for a period of 4 h. The percentage specific release was calculated as follows: (experimental release − spontaneous release) × 100 / (total release − spontaneous release).

Immunofluorescence. Cells were stained with mouse monoclonal antibodies designated BoT2, BoT4, and BoT8, recognizing bovine CD2, CD4, and CD8 analogues, respectively. The antibodies were obtained from ILRAD Nairobi [1, 7, 8]. The second step in this procedure was an antibody to mouse immunoglobulin labelled with fluorescein isothiocyanate. Stained cell populations were analysed with a FACScan.

Results

In vivo

Figure 1 shows tumor sizes as a function of time after the IL-2 treatment. Two tumors (cows 1 and 2), initially the smallest in size, regressed within 2–3 months. A third

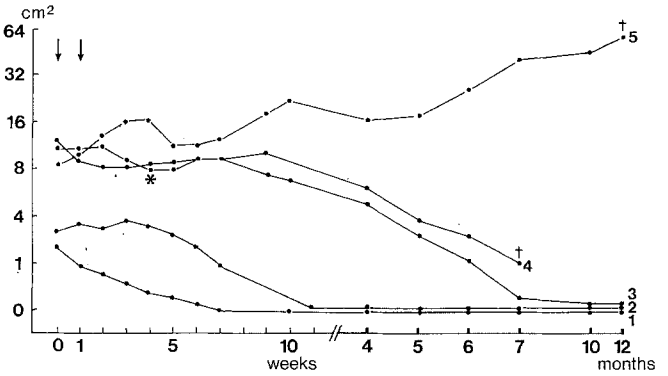

Fig. 1. Tumor sizes before, during, and after local/regional recombinant human interleukin-2 (rhIL-2) therapy in five animals with bovine ocular squamous cell carcinoma (BOSCC). Tumor sizes (cm^2) in cows 1–5 as a function of time after initiation of rhIL-2 treatment. ↓, Start of treatment cycle of 5 days. †, Animals 4 and 5 were slaughtered, ∗. Animal 4 showed metastasis starting at week 5

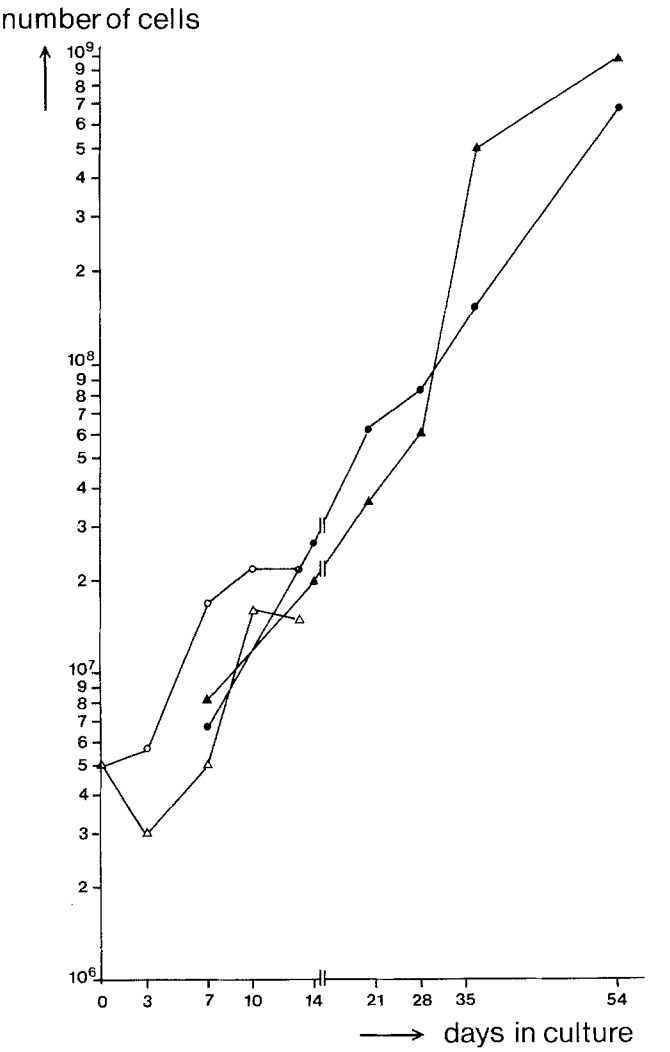

Fig. 2. Cumulative growth curves of peripheral blood lymphocytes (PBL) and tumor-infiltrating lymphocytes (TIL) during in vitro incubation with rhIL-2. ○, PBL of a normal animal incubated with 500 U rhIL-2/ml. △, PBL of a tumor-bearing animal incubated with 500 U rhIL-2/ml. ●, TIL of animal 3 incubated with 1000 U rhIL-2/ml. ▲, TIL of animal 3 incubated with 100 U rhIL-2/ml

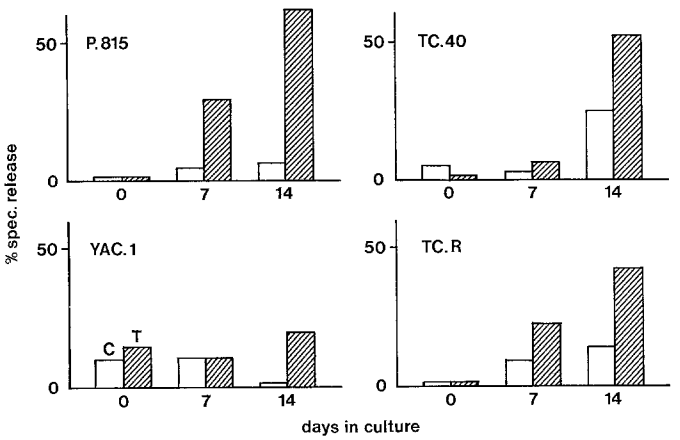

Fig. 3. Lymphokine(rhIL-2)-activated killer activity of PBL. Cytotoxic activity, induced in vitro by recombinant human IL-2 (500 U rhIL-2/ml), of PBL of a BOSCC-bearing animal (*T*) and a control animal (*C*) in course of time. Effector:target ratio 50:1. *TC.40* is a BOSCC-derived target cell line autologous to animal T. *TC.R* is a BOSCC-derived target cell line allogeneic to animals C and T *Yac-1* and *P.815* are natural-killer(NK)-sensitive and NK-resistent mouse tumor cell lines, respectively

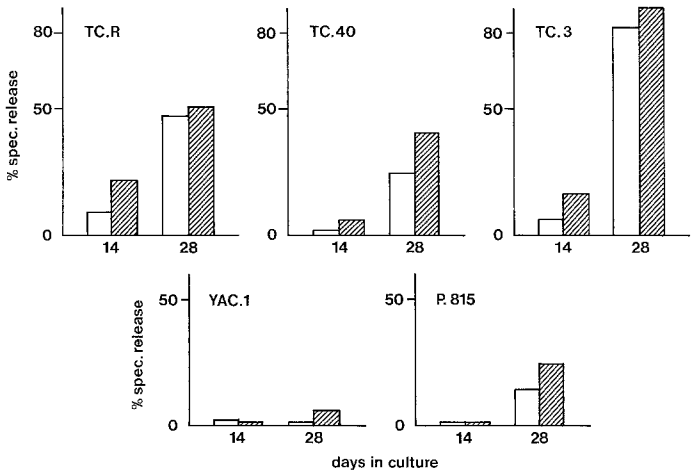

Fig. 4. Tumor-infiltrating lymphocytes from BOSCC. Cytotoxic activity, induced in vitro by recombinant human IL-2 (100 U/ml), *open bars*; 1000 U/ml, *shaded bars*, of tumor-infiltrating lymphocytes from animal 3. Effector:target ratio 50:1. *TC.40* and *TC.R* are allogeneic BOSCC-derived target cell lines. *TC.3* is an autologous BOSCC-derived target cell line. *Yac-1* and *P.815* are NK-sensitive and NK-resistent mouse tumor cell lines, respectively

tumor (cow 3), larger in size, regressed more slowly but steadily until it had disappeared between 6 and 7 months after treatment. One tumor (cow 4) regressed to approximately 20% of its original size within 7 months, after which the animal had to be slaughtered because of fast-growing lymph node metastases, which were first observed 5 weeks after the beginning of the IL-2 therapy. The fifth tumor (cow 5) was rapidly growing before treatment and retarded in growth after IL-2 application. After 12 months the animal had to be slaughtered because of tumor burden.

Clinical examination of the animals did not show abnormalities except for the increase in size of the regional lymph node of cow 4.

No changes were noticed in total and differential blood cell counts, alkaline phosphatase, γ-glutamyltransferase in blood and serum protein levels during and after IL-2 therapy.

Pathological examination was performed on animals 4 and 5 after slaughter. In the animal with the large primary tumor (cow 5) metastases were present in the subparotid lymph node. In cow 4, which showed tumor regression but lymph node enlargement, metastases were present in subparotid and retropharyngeal lymph nodes. No lung metastases were found.

In vitro

In vitro cell growth curves (Fig. 2) showed that stimulation of PBL from a normal and a tumor-bearing animal (not incorporated in the rhIL-2 trial) with rhIL-2 resulted in an increase in cell number of about 3–5-fold within 13 days (500 U/ml). Tumor-infiltrating lymphocytes obtained from animal 3 at day 0 of treatment showed 150-fold (1000 U IL-2/ml) to 200-fold (100 U IL-2/ml) increase in number during culture from day 7 to day 54. The curves shown are representative for growth of tumor-infiltrating lymphocytes from a number of BOSCC.

Cytotoxic activity of PBL generated during incubation in rhIL-2 is shown in Fig. 3. The killing of the natural-killer-sensitive Yac-1 cell line by cells of the tumor-bearing animal increased to a limited extent. However, ^{51}Cr-release from Yac-1, induced by PBL from the control animal, decreased. Killing of the natural-killer-resistant P815 and the two cell lines obtained from BOSCC increased gradually during incubation of PBL of the tumor-bearing animal in rhIL-2. The cells of the control animals showed, to a lesser extent, increased killing of both bovine cell lines. Increase of killing of P815 cells was marginal. Tumor-infiltrating lymphocytes (animal 3) showed an increase in cytotoxic activity upon incubation in rhIL-2 (Fig. 4). The effect is strongest at day 28 against BOSCC-derived cell lines and especially against the autologous cell line (killing >80%). Yac-1 target cells are hardly killed, while some cytotoxic activity against P815 cells seems to be generated. At day 28 differences in cytotoxicity between cells incubated in 100 U rhIL-2/ml and 1000 U rhIL-2/ml are only small.

Preliminary results of phenotype analysis at day 14 showed that of the tumor-infiltrating lymphocytes from animal 3, incubated in 100 U rhIL-2, 40% were BoT2$^+$, 2% BoT4$^+$, and 15% BoT8$^+$; of those incubated in 1000 U rhIL-2, 60% were BoT2$^+$, 9% BoT4$^+$, and 20% BoT8$^+$.

Discussion

Local regional therapy of cows bearing ocular squamous cell carcinoma with low doses of rhIL-2 resulted in total tumor regression in three out of five animals, partial tumor regression (to 20% of the original size) accompanied by lymph node metastasis in one out of five cows and retarded tumor growth in the fifth animal. After a period of 12 months animals 1–3 are still alive and tumor-free. The results were achieved although the treatment was limited to two cycles of 5 days of daily intratumoral and intranodular injections of 2500 U rhIL-2. Repeated cycles might have even better effects [11, 12]. The present results are surprisingly good considering the low doses of rhIL-2 used, the limited number of treatment cycles, the high percentage of complete remission, and the duration of remis-

sion. In former studies [14] spontaneous regression was observed in less than 5% of the BOSCC-bearing animals.

No signs of toxicity were noticed of the sort described in cases of application of high doses of rhIL-2 in sheep [13], mice [19, 24], and humans [18]. Increase of the number of circulating lymphocytes and changes in serum protein levels, observed by Sondel et al. [21], who used a high dosage of rhIL-2, were not seen in our low-dose regimen (data not shown). This is the first report of the successful treatment of large animal tumors with rhIL-2. In vitro bovine peripheral blood lymphocytes proliferated upon contact with various doses of rhIL-2 for a period of at least 2 weeks. This was shown before by Fong et al. [10], who used lectin-activated lymphocytes, and by Stott et al. [22] in short-term cultures. Fenwick et al. [9] published similar observations for equine, caprine, ovine, canine, and feline PBL.

Lymphocytes obtained from the tumor showed comparable growth characteristics and continued growing for at least 8 weeks. In the cultures of tumor-infiltrating lymphocytes, in contrast to PBL cultures, (tumor)antigen is present at least initially. Thus, different activating mechanisms may be involved in the proliferative responses of tumor-infiltrating lymphocytes and PBL.

In vitro incubation with rhIL-2 leads to an increase in toxicity of PBL and tumor-infiltrating lymphocyte cells to a variety of tumor cells (Figs. 3 and 4). Cytotoxic activity, generated by in vitro incubation of bovine PBL in rhIL-2, is most likely LAK activity [15, 16] as allogeneic BOSCC-derived cell lines as well as the mouse P815 cell line are killed. In vitro incubation of tumor-infiltrating lymphocytes with rhIL-2 shows, especially at day 28 of culture, predominant killing of the autologous BOSCC-derived cell line. Because killing of allogeneic cell lines and P815 was observed as well, it must be assumed that at least part of the killing was the result of LAK activity. The high specific release from the autologous cell line can be due to additional specific activity of cytotoxic T cell, as described for human melanoma [6]. On the other hand differences in sensitivity for LAK-mediated killing between the BOSCC-derived lines cannot be excluded. Preliminary phenotype analysis of tumor-infiltrating lymphocytes cultured in the presence of rhIL-2 does not give conclusive results concerning the types of cells involved in killing. Of these, 40%–60% are T cells ($BoT2^+$) and the number of $BoT8^+$ cells, 15%–20%, is higher than that of $BoT4^+$ cells, 2%–9%. These findings seem to be representative for BOSCC-derived tumor-infiltrating lymphocytes in general. However, possible activity of $BoT2^-$ cells or even $BoT2^+T4^-T8^-$ cells can not be excluded for the time being.

The mechanism of tumor regression in vivo during local rhIL-2 therapy of BOSCC is not clear. On the one hand natural killer cells can be stimulated to LAK activity [4, 5] especially when high doses of IL-2 are used [23]. On the other hand tumor-specific cytotoxic T cell activity may be generated preferentially when low doses of IL-2 are used [23]. Future experiments in vitro will be directed at the identification of mechanisms and cells involved in this form of therapy. In vivo experiments will be extended as well. As a follow-up of our previous studies on intratumoral injection of BCG [14] and the present pilot study, cows will be treated with (repeated cycles of) rhIL-2 and BCG, and a possible synergism of IL-2 and BCG will be determined.

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