RESEARCH ARTICLE

Local immunotherapy of spontaneous feline fibrosarcomas using recombinant poxviruses expressing interleukin 2 (IL2)

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We tested the canarypox virus vector ALVAC and the genetically attenuated vaccinia virus vector NYVAC as vehicles for achieving local immunomodulation in domestic animals bearing spontaneous tumours. Following intra-tumoral administration of ALVAC-, or NYVAC-luciferase in dogs with melanoma, it was demonstrated that viral recombinants remained localized along the needle track, with no virus detectable in the periphery of the tumour. Given these distribution characteristics and their well-documented safety profile, ALVAC- or NYVAC-based recombinants expressing feline or human IL2, respectively, were administered to domestic cats, in order to prevent the recurrence of spontaneous fibrosarcomas. In the absence of immuno-

therapy, tumour recurrence was observed in 61% of animals within a 12-month follow-up period after treatment with surgery and iridium-based radiotherapy. In contrast, only 39 and 28% of cats receiving either NYVAC-human IL2 or ALVAC-feline IL2, respectively, exhibited tumour recurrences. Based on such results, and in the context of ongoing clinical studies conducted in humans, we discuss the utilization of ALVAC- or NYVAC-based recombinants as viable therapeutic modalities for local immunotherapy or therapeutic vaccination against cancer, both in humans and companion animals.

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Introduction

Viral vectors have been frequently used in cancer patients to express genes encoding immunostimulatory cytokines or tumour-associated antigens, thereby aiming to recruit locally and/or systemically, tumour-specific immune effector mechanisms.^{1–6} In particular, highly attenuated poxvirus vector systems have been developed to address safety concerns linked to the administration *in vivo* of replicative viruses to cancer patients.

For example, the NYVAC vector was derived from the Copenhagen vaccinia virus strain, following deletion of 18 open reading frames whose products had been implicated in the virulence of poxviruses.^{7–9} Similarly, the ALVAC viral vector was derived as a plaque-cloned isolate from an existing canarypoxvirus vaccine strain, Kanapox. As an avipoxvirus, ALVAC does not replicate in mammalian cells but can readily infect human cells.^{9,10} A number of animal models have established the attenuation profile of both ALVAC and NYVAC, without any evidence for reversion to virulence. In addition, ALVAC and NYVAC recombinant viruses have been, as of today, administered without any major adverse effects,

to more than 2000 and 100 human volunteers, respectively.^{11–16} The safety profile of these viruses was essentially documented in healthy volunteers, but only to a limited extent in cancer patients.^{17,18} A number of preclinical studies have established the safety and biological activity of recombinant ALVAC viruses expressing cytokines or tumour-associated antigens in murine tumour models.^{19–21} Such models relying upon established, as opposed to spontaneous tumours, are however often criticized for not recapitulating the complexity of biological factors involved in tumorigenesis or in the tumour–host interface.²² Also, when considering the utilization of viral vectors for cancer immunotherapy, local delivery and expression of the inserted gene product is desired in order to minimize the risk of dissemination of recombinant viruses to the environment, and in order to reduce the risk of toxicity to the patient (for example, by achieving local release of a toxic gene product).

Thus, this study was undertaken to document the biodistribution and efficacy profile of ALVAC- and NYVAC-based vectors in companion animals bearing spontaneous tumours. Using these models, we demonstrate that following intratumoral administration in dogs with melanoma, the distribution of both ALVAC- and NYVAC-based recombinants is restricted to the injection site. We also demonstrate that ALVAC- and NYVAC-

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cytokine recombinants expressing IL2 decrease local recurrence rates of spontaneous feline fibrosarcomas.

Results

Biodistribution studies following intratumoral administration of pox-luciferase recombinants

ALVAC- or NYVAC-luciferase were administered intratumorally to two dogs (one dog per recombinant) bearing spontaneous melanomas. We assessed the biodistribution of ALVAC or NYVAC within the tumour itself, and also in normal tissue surrounding the tumour, using as a control a noninjected tumour. All tissue samples were cut into thin sections and processed as described in Materials and methods to probe for luciferase expression (Figure 1).

Following administration of the ALVAC-luciferase recombinant, luciferase expression was only detected in three out of five tumour fragments, suggesting a limited virus spread following injection (Figure 2, left panels

Figure 1 Schematic representation of ALVAC-luciferase biodistribution following intratumoral administration. The tumour was divided into five pieces, each one being subsequently cut into 70–90 thin sections of 5 μ m width along the inoculation path (left panel). Luciferase activity was found in a number of thin sections from pieces 1, 2 and 3, but not within tumour fragments 4 and 5, allowing to map the distribution of ALVAC-luciferase along the needle tract (right panel, grey area).

Figure 2 Intratumoral expression of luciferase following administration of ALVAC-luciferase into a spontaneous dog melanoma. ALVAC-luciferase was administered intratumorally and luciferase activity was subsequently analysed inside the injected tumour (left panel, a–c) and within peritumoral tissue (right panel, d–f). (a,d) Macroscopic view of the thin section across the tissue considered. The area examined for luciferase expression is shown under arrow. (b,e) Luciferase expression shown by enhanced DAB peroxidase reaction. (c,f) Luciferase expression detected by indirect immunofluorescence.

a–c). As a control, we failed to detect any luciferase activity in surrounding tissues (Figure 2, right panels d–f). Further, ALVAC-luciferase was not detectable in proximal lymph nodes draining the injected tumour, nor in the dog melanoma that had not been injected (data not shown).

In order to map precisely the diffusion of the ALVAC vector within the tumour, we compiled data obtained from the various tumour sections. Some luciferase activity was found within the tumour in fragments number 1 (17 slides were found positive, representing a diffusion of approximately 2.7 mm), number 2 (16 slides found positive, with a diffusion of 2.6 mm) and number 3 (25 positive slides, representing a diffusion of 4 mm). No luciferase activity was detectable in fragments number 4 and 5. Altogether, these results established that the distribution of luciferase and therefore of the ALVAC vector was restricted to the needle track (Figure 1, right panel, schematized as grey area). Similar to the ALVAC vector, the analysis of multiple slides suggested that NYVAC-luciferase was also distributed along the needle track but not in the periphery of the tumour, nor in draining lymph nodes (data not shown).

Biodistribution studies in healthy cats and dogs were also performed by administration of either ALVAC parental virus, NYVAC parental virus, ALVAC-fIL2 or NYVAC-hIL2 through the subcutaneous route (cats) or, submucosally, in the upper lips facing the superior canines (dogs). Swab samples taken from the injection site and other biological samplings were assessed for ALVAC and NYVAC at various time points, ranging from 10 min to up to 6 days after administration. The presence of infectious viral particles was assessed using either QT35 cells (for ALVAC) or Vero cells (for NYVAC)

as cell substrates. ALVAC or NYVAC virus could only be recovered from the inoculation site 10 min after injection, but were undetectable by 1 h postadministration. Further, ALVAC or NYVAC virus was not detectable in saliva, faeces or blood at any time point (data not shown). Collectively, these results suggest a localized biodistribution of ALVAC/NYVAC following intratumoral, subcutaneous or submucosal injection.

Prevention of local recurrence of spontaneous feline fibrosarcomas

Having established a localized biodistribution profile of both ALVAC and NYVAC vectors, we proceeded to evaluate the efficacy of viral recombinants carrying cytokine genes in an appropriate tumour model. Rather than testing the recombinants in the highly metastatic melanoma model, we elected to focus on spontaneous fibrosarcomas frequently observed in domestic cats. In such animals, the reference treatment consists of surgical removal of the tumour, and adjunct brachytherapy with iridium (1 week exposure with delivery and removal of the implants performed under general anaesthesia).^{23,24} While such a treatment is efficacious, the recurrence rate is still very high, with tumour recurrences typically observed at the site of the primary tumour.

An ALVAC recombinant virus expressing the feline IL2 gene (ALVAC-fIL2) was engineered and used to treat cats as described in Materials and methods. Furthermore, in the context of a study having established some bioactivity of human IL2 – expressed by a transfected xenogeneic cell line – in this feline spontaneous tumour model,²⁴ we tested an NYVAC-hIL2 (ie NYVAC expressing the human IL2 gene) recombinant in parallel. As described in Material and methods and in Table 1, three groups of cats without evidence of metastasis were randomly assigned to receive either the reference treatment only (control group), or the same supplemented with ALVAC-fIL2 or NYVAC-hIL2. A total of seven injections of poxvirus-IL2 recombinants were administered within the tumour bed, in order to achieve a sustained expression of IL2 locally for up to 8 weeks. This scheme of administration was based on the observation that infection of tumour cells *in vitro* with such viral recombinants allows for a peak expression of the cytokine gene 4 days after injection or infection, with a progressive decline thereafter.^{17,20} Results obtained during the 12-month observation period for recurrences are shown in Figure 3. Treatments were well tolerated in all groups (with only self-resolving signs of local inflammation observed). The rate of recurrence was however greater in control animals when compared to either of treatment groups (χ^2 test, $P < 0.01$) with 11, 7 and 5 cases (ie recurrence rates of 61, 39 and 28%) observed in controls, or in groups receiving NYVAC-hIL2 or ALVAC-fIL2, respectively. Differences in recurrence rates observed in animals receiving either ALVAC-fIL2 or NYVAC-hIL2 were not statistically significant, and could potentially be explained by the initial higher tumour burden observed in animals belonging to the latter group, as suggested by differences found in initial tumour extension indexes (Table 1, Materials and methods). The mean time observed between inclusion and documented recurrence was 6–7 months in all groups. Despite this effect on tumour recurrence, no

significant differences were found in survival between the three groups during the follow-up period considered (Figure 4).

Discussion

Local gene expression profiles are clearly desired for cancer immunotherapy based on immunostimulatory cytokines, in order to trigger immune effector mechanisms or biological responses in the absence of systemic toxicity. Importantly, when relying upon viral vectors, achieving local distribution also reduces the risk of dissemination of the virus to the environment. In the present study, we document the local distribution characteristics of ALVAC and NYVAC viral recombinants administered through the intratumoral route in dogs bearing spontaneous melanomas. Such spontaneous cancers in domestic animals likely represent more applicable experimental models than conventional murine models of established tumours, for the design and testing of immunotherapeutic approaches for humans. Our results in the dog melanoma model may imply the necessity in the future of multiple injections for achieving a more relevant tissue expression profile for ALVAC- or NYVAC-borne foreign gene expression. A local pattern of distribution was also observed *in vivo* following injection of normal dogs and cats through either the submucosal or subcutaneous routes, respectively. It is noteworthy that these results are in agreement with recent studies demonstrating a local distribution of either ALVAC-luciferase or ALVAC-LacZ in mice following either intramuscular injection¹⁹ or intravesicular instillation.²⁵ Only in mice injected intravenously, some ALVAC virus is detected in the lungs, the liver and the spleen.¹⁹ This unique association of a highly attenuated profile and local distribution characteristics suggests that ALVAC- and NYVAC-based recombinants expressing genes encoding immunostimulatory cytokines could be useful for therapeutic purposes in patients or animals with cancer, with the aim to elicit tumour-specific immune responses.

As a consequence, ALVAC- and NYVAC-cytokines, which have been successfully used to eradicate established tumours in mice,^{20,26} were tested for their capacity to prevent recurrence of fibrosarcomas, a tumour frequently encountered in domestic cats.

We elected to focus on the immunostimulatory cytokine IL2, since it has been shown to induce tumour regression in both murine^{1,20} and also humans.²⁷ Also, human IL2 was shown to affect the recurrence of feline fibrosarcomas following expression by transfected xenogeneic cells.²⁴ In this context, ALVAC- and NYVAC-based IL2 recombinants were chosen to assess their capacity to supplement current therapeutic options in a highly recurrent feline fibrosarcoma model. The reference therapy for feline fibrosarcoma is based on surgical excision and local radiotherapy with implantation of iridium needles in the tumour bed.^{23,24} Despite the fact that such a treatment has considerably improved the prognosis of fibrosarcomas in cats, local recurrence is frequently observed, and additional therapies are needed to complement current treatment options. Of significance, data presented in this study demonstrate a decrease in tumour recurrence rates from 61% in control

Table 1 Cats with spontaneous fibrosarcomas, enrolled to evaluate ALVAC- or NYVAC IL2 as an adjunct therapy

No	Sex	Age (years)	Tumour localization	Tumour extension index	Evidence for recurrence within 12 months after treatment (time to recurrence in months)
<i>Control group</i>					
1	M	6	Interscapular	26	No (12)
2	MC	9	Interscapular	2	Excudative, recurrent (2)
3	FO	5	Inguinal	3	Recurrent (3)
4	MC	9	Chest	4	Recurrent on site (4)
5	MC	7	Interscapular	13	No
6	MC	10	Interscapular	5	Recurrent in deeper layer (5)
7	MC	13	Lumbar	3	Recurrent (3)
8	MC	10	Chest	10	Recurrent (10)
9	FO	8	Interscapular	15	No
10	MC	12	Interscapular	6	Multiple recurrences (6)
11	MC	11	Chest	2.5	Recurrent (2.5)
12	MC	10	Interscapular	15	No
13	MC	5	Interscapular	16	No
14	MC	9	Interscapular	6	Recurrent (6)
15	MC	11	Chest + thigh	16	No
16	MC	13	Interscapular	3	Recurrent (3)
17	MC	14	Interscapular/thigh	6	Recurrent in deeper layer (6)
18	MC	5	interscapular	15	No
<i>NYVAC-hIL2 group</i>					
1	F	8	Interscapular	10	Recurrent (10)
2	FO	7	Interscapular	9	No
3	MC	11	Thigh	7	No
4	M	12	Chest	4	Recurrent (4)
5	FO	7	Interscapular	6	No
6	FO	10	Interscapular	3	Recurrent (3)
7	FO	10	Interscapular	11	No
8	FO	13	Interscapular	12	No
9	MC	12	Chest	12	No
10	FO	8	Chest/interscapular	13	No
11	F	9	Chest	10	Recurrent (10)
12	FO	5	Interscapular	13	No
13	F	10	Chest	12	Recurrent (12)
14	F	15	Chest	3	Recurrent (3)
15	FO	7	Interscapular	14	No
16	MC	8	Thigh	14	No
17	F	13	Interscapular	5	Recurrent (5)
18	FO	8	Interscapular	14	No
<i>ALVAC-fIL2 group</i>					
1	FO	5	Interscapular	18	No
2	MC	10	Interscapular	18	No
3	MC	9	Chest	14	No (NB: recurrent at 14 months)
4	FO	10	Chest	18	No
5	MC	11	Chest	18	No
6	FO	7	Chest	19	No
7	FO	7	Lumbar	19	No
8	FO	14	Interscapular	5	Recurrent (5)
9	FO	10	Lumbar	19	No
10	MC	10	Neck	8	Recurrent (8)
11	FO	9	Interscapular	18	No
12	FO	9	Interscapular	6	Recurrent (6)
13	FO	10	Interscapular	19	No
14	FO	8	Interscapular	21	No
15	MC	9	Chest	21	No
16	FO	11	Interscapular	5	Multiple recurrences(5)
17	MC	10	Interscapular	7	Multiple recurrences (7)
18	MC	7	Chest	17	No

M, F, MC, FO refer to male, female, castrated male, castrated female, respectively. Tumour extension indexes were calculated as specified in Materials and methods, based on the initial size of the tumour and the presence or not of recurrence.

animals to 39 and 28% in domestic cats receiving NYVAC-hIL2 or ALVAC-fIL2, respectively, in addition to conventional therapy. Further, our results do not provide evidence for major differences between ALVAC

or NYVAC, nor between feline – and human-IL2, in controlling tumour recurrence. Thus, ALVAC- or NYVAC-IL2 recombinants can be used as an adjunctive therapeutic approach to this very frequent tumour

Figure 3 Prevention of recurrences of feline fibrosarcomas, using ALVAC- or NYVAC-IL2 as an adjunct therapy. After surgical removal of spontaneous fibrosarcomas, and at the onset of iridium-based radiotherapy, cats were randomly assigned to either one of the three groups (with 18 animals per group): controls (which did not receive any immunotherapy), and two groups receiving seven subcutaneous injections in the tumour bed of either ALVAC-fIL2 or NYVAC-hIL2. Recurrences were evaluated by clinical examination.

Figure 4 Survival over time of cats with spontaneous fibrosarcomas. Survival curves are shown for cats treated with the reference treatment, with or without additional immunotherapy. The last time point compiles data obtained at month 14 or above. Numbers include animals that died either naturally or as a consequence of their disease, as well as animals that had to be euthanized as a consequence of disease progression.

in felines. Intratumoral administration of ALVAC-cytokines (expressing either IL2, GM-CSF or IL12) has also been evaluated in a series of phase I/II human clinical trials conducted in patients with metastatic melanoma.¹⁷ Interestingly, in these studies, intratumoral injection of recombinant ALVAC viruses resulted in a strong expression of the inserted cytokine gene for at least 4 days, as demonstrated by RT-PCR performed on biopsies from injected tumours. Regression of the injected nodule was observed in 50% of patients receiving ALVAC-IL2. This was associated with infiltrates of immune cells, production of IFN- γ and, in a few patients, with the induction of tumour-specific T lymphocytes.¹⁷ Together, these results further support the idea of using ALVAC- or NYVAC-cytokine recombinants for local immunotherapy. One advantage of using a viral vector, as opposed to relying upon a bolus administration of the cytokine, is that high levels of the cytokine can be produced locally (ie intratumorally) over several days without any risk of inducing systemic toxicity.

Such viral vectors could also potentially be used to prevent recurrence of melanomas in dogs after surgery (using the submucosal route) or to slow down the progression of advanced tumours no longer removable with surgery (following intratumoral administration). The efficacy of pox-cytokine immunotherapy on metastatic disease remains however to be demonstrated.

In addition, there is obviously a major interest in using such viral vectors as antigen presentation platforms, to express genes encoding tumour-associated antigens, with the aim of eliciting a systemic tumour-specific immune response. Therapeutic vaccination against selected tumour-associated antigens has been conducted using ALVAC vectors in murine models, with encouraging results: mice immunized with ALVAC-p53 recombinants were found to be protected from challenge with tumour cells transfected with the wild-type human p53 gene.²⁸ Similarly, an ALVAC CEA recombinant was shown to elicit both humoral and proliferative as well as cytotoxic T-cell responses in immunized mice. Such animals were protected against challenge with a murine tumour cell line expressing the human CEA gene.²⁹ Based on these preclinical results, several clinical studies evaluating ALVAC-tumour-associated antigen vaccines have been initiated in humans.^{17,18} In a first phase I study, patients with CEA, expressing colorectal carcinomas, were administered with ALVAC CEA via the intramuscular route. Treatments were well tolerated, with no major vaccine-related toxicities observed, beyond mild local reactions. An increase in CEA-specific T-cell precursor frequencies was noted in at least some patients.^{30,31} Similarly, patients with colorectal cancer receiving ALVAC p53 as a vaccine through the intravenous route mounted some p53-specific antibody and lymphoproliferative T-cell responses.³² With the aim of enhancing tumour-specific immune responses, most particularly T-cell responses, future experimental protocols are likely to rely upon ALVAC vectors carrying within a single recombinant, multiple genes encoding tumour-associated antigens, costimulatory accessory molecules such as B7.1³³ and/or cytokines such as GM-CSF and IL2.

Material and methods

Recombinant poxviruses

The ALVAC (viral strain CPpp) and NYVAC (strain vP866) viruses and all viral recombinants were produced on chick embryo fibroblasts maintained in DMEM-Ham F12 medium. Viral suspensions were clarified and diluted in 25% lactoglutamate. ALVAC-based recombinants were generated as described elsewhere³⁴ by cotransfection of ALVAC-infected primary chick embryo fibroblasts with an insertion plasmid and noninfectious purified ALVAC genomic DNA, leading to the integration of the foreign gene expression cassette into the viral genome via homologous recombination at either the C3 or C6 locus. ALVAC-fIL2 (vCP1338) is a recombinant canarypox virus containing the gene expressing feline interleukin-2. The ALVAC-luciferase (vCP297) recombinant expresses the luciferase gene from *Photinus pyralis*. NYVAC-based recombinants were similarly produced by homologous recombination at the TK locus as described elsewhere.³⁵ NYVAC-hIL2 (strain vP1241) is a vaccinia

recombinant virus expressing human interleukin-2. In each vector, inserted genes are under the transcriptional control of early/late vaccinia virus H6 promoter.³⁴ The expression of bioactive IL2 by the two poxviral vectors was confirmed by both ELISA and lymphoproliferation assays (data not shown). ALVAC-IL2 recombinants have been successfully used to eradicate established tumours in mice.^{20,26}

Approval for in vivo studies in companion animals

Detailed protocols describing the animal experiments have been submitted to and approved by the French national regulatory committee 'Comit de Gnie Biomolculaire' (CGB) and by the ethical committee from the Maisons-Alfort veterinarian University (Maisons-Alfort, France). All administrations of recombinant viruses have been performed by the same certified veterinarian at the radiotherapy centre in Maisons-Alfort, France.

Intratumoral distribution of ALVAC- and NYVAC-luciferase recombinants

Intratumoral injections of either ALVAC- or NYVAC-luciferase were performed in two separate dogs bearing spontaneous melanomas in the oral cavity: animals were anaesthetized using Tiletamine and Zolazepam i.v., and doses of 10⁸ DICC50 of ALVAC- or NYVAC-luciferase were administered within the tumour in a single injection of 0.5 ml. The sites of administration were disinfected post-injection with a swab soaked with an antiseptic solution (Betadine, Sarget Lab, or Biseptine, Wiclocks Lab). At 10 h after intratumoral administration of either ALVAC- or NYVAC-luciferase, the tumour and draining lymph nodes were surgically removed. A melanoma tumour and a draining lymph node from another dog, which had not been injected with any recombinant virus, were used as negative controls in these experiments. Each tumour was cut into five pieces (approximately 0.5 cm wide), and each section was frozen in liquid nitrogen for 20 s and stored in freezing vials at -80C until luciferase expression was analysed. To this aim, three samples of tissue surrounding the tumour, as well as draining lymph nodes were processed in parallel with the tumour. Transversal sections, 5 thick, of these various tissues were cut every 75 m using a cryostat (cryocut 3000, Leica, France), to assess luciferase expression. Tissue samples were fixed for 15 min on a slide using methanol. Slides were dried and frozen at -20C prior to assessing luciferase activity by indirect immunofluorescence (kit RPN 1232, Amersham). For this purpose, slides prepared from serial sections of a tumour injected with ALVAC- or NYVAC-luciferase were thawed at room temperature, washed with PBS pH 7.2, 0.025% Tween 20, 0.005% BSA four times and incubated in PBS pH 7.2 and 1% BSA for 30 min at 37C. Slides were subsequently incubated for 1 h 30 min at 37C with a 1/100 dilution of rabbit antiluciferase immunoglobulins (Promega). After four washes, a second-step biotinylated antiserum specific for rabbit IgGs (Amersham) was added for 1 h at 37C. Slides were washed and incubated with a 1/300 dilution of streptavidin-FITC (Amersham). After four washes with PBS pH 7.2, slides were dried, mounted with DABCO media (1,4 diazabicyclo -[2,2,2]-octane, Sigma) and analysed with a microscope under UV light. Luciferase expression was

confirmed using a method based on enhanced DAB peroxidase detection: slides were treated with a suppressor of endogenous peroxidase (Pierce 35000) for 30 min at lab temperature. After four washes, slides were incubated for 90 min at 37C with a 1/100 dilution of rabbit antiluciferase IgGs. After four additional washes, a 1/250 dilution of a biotinylated anti-rabbit IgG antiserum was added. Slides were washed and incubated with an avidin/biotinylated peroxidase mixture for 30 min at 37C. Slides were washed and incubated with a 1/10 dilution of a metal enhanced DAB substrate for 10 min. After washing and drying, slides were mounted in Mowiol 4-88 medium (Calbiochem, France) and analysed under a microscope.

ALVAC-fIL2 and NYVAC-hIL2 administration in cats with spontaneous fibrosarcoma

The study was conducted in the Radiotherapy Center of Alfort Veterinary School (France). A total of 54 European cats were included in the study (Table 1). The sex ratio was 27 males for 27 females, most of them (ie 25 males and 22 females) castrated. Upon inclusion, cats were on average 9 years old in the three groups and all were diagnosed with a fibrosarcoma confirmed by histopathology after resection. None of the animals included in the study had any evidence of metastasis. As expected, tumours were predominantly located between the shoulders (31 cases), suggesting that they were associated with a local trauma (eg previous injection, bite). Other locations included the chest (16 cases), lumbar area (three cases), thigh (two cases) and neck (one case).

After surgical removal of the tumour and at the onset of iridium-based radiotherapy,^{23,24} cats were randomly assigned to either one of three groups (with 18 animals included per group): a group of control animals that did not receive any immunotherapy, a group receiving ALVAC-fIL2 and a group administered with NYVAC-hIL2. The investigator was not masked to the treatment groups. Poxvirus-based immunotherapy consisted of seven consecutive subcutaneous injections with either 5 10⁶ DICC₅₀ of ALVAC-fIL2 or 5 10^{6.7} PFU of NYVAC-hIL2 in 0.5 ml of buffer (one vial per cat per injection). Test products were injected twice during the first week (on day 1 and day 5), then once a week on the first day of week 2, 3, 4, 6 and 8. This scheme of administration was based on the observation that following infection *in vitro* of various cultured cell lines with ALVAC- or NYVAC-IL2 recombinants, significant levels of IL2 were found to be produced in culture supernatants for at least 4 days.

A tumour extension index was calculated as per the WHO TNM classification of tumours in domestic animals³⁶ for each animal as $i = \text{tumour diameter (cm)} \times (1 + 2 \text{ (if recurrence)} + 3 \text{ (if metastasis)})$. It is noteworthy that animals included in our study had no detectable metastases and presented a single tumour site at the time of enrolment. Cumulated indexes for the three groups were 181 (for both the control group as well as the group receiving ALVAC-fIL2) and 275 (for the group receiving NYVAC-hIL2), suggesting an overall larger tumour burden in this latter group. More males (13/18) were included in the control group, and more females (11/18 and 13/18) were included in the ALVAC-fIL2 and NYVAC-hIL2 groups, respectively. Other

characteristics of animals included in the study, as summarized in Table 1, were not found to differ significantly among the three groups. Recurrences have been evaluated by clinical examination during at least two planned routine visits (every 6 months) or on an *ad hoc* basis during consultation, when there was evidence of disease progression.

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