INTRALYMPHATIC INFUSION OF AUTOCHTHONOUS TUMOR CELLS IN CANINE LYMPHOMA

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The feasibility of repeated intralymphatic injections of cellular materials was investigated in dogs having spontaneous lymphomas. Forty-two administrations of enzymatically-modified irradiated autochthonous tumor cell preparations were performed with minimal difficulty and negligible deleterious side effects, indicating that the intralymphatic route of administration and these methods of cell vaccine preparation may be safely used for further pre-clinical investigations. Although the number of animals treated thus far is insufficient to realistically evaluate the immunotherapeutic potential of this method, all treated tumor-bearing dogs demonstrated marked reduction in tumor volume and significant clinical improvement.

Intralymphatic infusion, Autochthonous tumor cells, Canine lymphoma.

INTRODUCTION

The rationale of tumor immunotherapy is based upon the potential host recognition and response to tumor associated cell surface antigens distinguishable from normal cell surface structures which could ultimately lead to the rejection of the tumor in much the same way other foreign materials including cells are rejected. Thus, in designing an immunological approach to the treatment of malignant neoplasms, it seems likely that manipulations of the immune system which produce active and specific sensitization to the host’s own tumor would be the most effective in immunotherapeutic control of tumor growth. The most obvious approach would be to actively immunize a tumor-bearing host against its autochthonous tumor. Experimental animal models have clearly demonstrated the importance of several interrelated factors involved in the eliciting of responses to cellular antigens, among which are: The biochemical and steric status of cell surface immunogens,1,5 method of immunizing cell inactivation and viability, route of immunization, number of cells used for immunization, and vaccination schedule.1 Approaches to active immunotherapy should attempt to optimize the immuno-stimulatory effects of these factors while minimizing any clinically deleterious or tumor growth enhancing effects.

The following is a very preliminary assessment of the technical and clinical feasibility of an active immunization method which combines a number of manipulations in an effort to increase the efficacy of immunization. In this method, tumor tissue is removed and placed in tissue culture to provide an available source of autochthonous cells. Prior to each treatment,

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cells are removed from culture and treated with neuraminidase to remove cell surface sialic acid. After enzyme treatment, the cells are irradiated to prevent possible proliferation and immediately injected into the peripheral afferent lymphatic of tumor-bearing animals. The intralymphatic route has been used to concentrate the modified autochthonous tumor cells at foci of potentially immunoreactive lymph node cell populations in an effort to increase the probability and frequency of lymphoid cell stimulation without disruption of functional lymph node architecture.

Although the use of enzymatically modified cells is not new, in other studies the cells have been administered via more conventional routes. It was, therefore, of primary interest to determine the technical feasibility of multiple consecutive intralymphatic administrations of a cellular vaccine in the same animal and the possible side effects and management problems consequent to such treatment.

METHODS AND MATERIALS

Dogs

Dogs of various breeds with extremely advanced spontaneous lymphoma were referred by local veterinarians and used with consent of the owners.

Canine malignant lymphoma was chosen for investigation for several reasons: They are relatively common, a satisfactory amount of tumor tissue can be obtained and cultured, the intralymphangial route of administration can be applied to dogs, the palpable lymphadenopathies can be measured, and the gross effect of the treatment on tumor mass can be easily evaluated. These lymphomas, like human malignancies, are spontaneous.

A preliminary study has been conducted on five dogs having advanced malignant lymphoma (Table 1). The diagnosis was established by biopsy. To determine initial clinical status, a complete physical examination with palpation of the abdomen under sedation, radiographies of the chest and the abdomen, CBC, liver function studies, alkaline-phosphatase and uric acid levels were performed on all dogs. Lymphangiographic studies were purposely omitted because of possible interference with subsequent injections. Lymph node mensurations, clinical examination, CBC, and blood chemistry were repeated every three to eleven days as indicated.

Tissue for in vitro culture was obtained by excision of a peripheral lymph node under anesthesia by sterile techniques. All diagnoses were reconfirmed by examination of histological sections of the biopsy material.

Tissue culture

Tumor cell suspensions were prepared for culture from sterile biopsy material, which was minced into fine pieces in Hank's balanced salt solution and forced through a 200 mesh sterile stainless steel screen. The screened cells were then washed through a nylon monofilament screen; treated with Gey's solution to remove red blood cells; washed twice with HBSS; counted and resuspended in RPMI-1640 (Grand Island Biological) supplemented with 12% fetal calf serum (Reheis-Armour), 1% L-Glutamine, 15 mM HEPES buffer, 1% 0.1 M sodium pyruvate, 1% nonessential amino acids, 1% penicillin streptomycin and fungizone (Grand Island Biological), and gentamycin (Schering) and sodium bicarbonate to pH 7.2. Cell suspensions containing large numbers of dead cells were sedimented on Ficoll density gradients prior to

<table>
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<td>V Weimaraner</td>
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culture to remove dead cells. The cultures were then kept at 37°C in 5% CO₂ humidified atmosphere and given fresh medium as needed until use.

**Preparation of cells for vaccine**

Immediately prior to their use for intralymphatic injection, cells were removed from culture, washed twice in HBSS, and counted. Cells were then treated with *Vibrio cholera* neuraminidase (Calbiochem) according to a modification of the method of Simmons and Rios. Briefly, the cells were treated with 100 units/10^7 cells (one unit of neuraminidase is equivalent to the amount required to release 1 μg of N-acetyl neuraminic acid from a glycoprotein substrate at 37°C in 15 min at pH 5.5) at pH 6.0 for 1 hr at 37°C. After neuraminidase treatment, the cells are washed three times in 100 volumes of HBSS and resuspended to a final concentration of 2.5 x 10^⁸ viable cells per ml. Viability is assessed by trypan blue exclusion.

**Irradiation of the cell suspension**

Immediately prior to injection, the cells suspension was irradiated with the electron beam of a 6 MeV linear accelerator; 10,000 rad were given over 4 sec.

**Viability of cell suspension**

The viability of the tumor cells after neuraminidase treatment and irradiation as determined by trypan blue exclusion was within 20% of the original cell suspension obtained from culture, which varied from 40% to 90%. Ability to incorporate ^3^H-thymidine was used to assess the proliferative ability of treated and untreated cell preparations. Briefly, replicate samples of untreated and neuraminidase-treated irradiated cells were cultured in complete RPMI-1640 media containing ^3^H-thymidine (Schwarz-Mann: Specific activity: 14.8 Ci/mmol) at a final concentration of 2.0 μCi/ml. At various times, the cells were lysed, the acid insoluble fraction precipitated, solubilized, and counted in a Beckman LS-330 liquid scintillation spectrometer. Results showed insignificant levels of ^3^H-incorporation in treated cell samples as compared to untreated controls indicating that while the cells were intact and apparently metabolically active, they were not capable of DNA synthesis and subsequent replication.

**Intralymphatic injection**

Just prior to irradiation of the cell suspension, the lymphatic vessels of the four distal extremities were surgically exposed. The vessels were cannulated and 2–4 ml of the cell suspension containing 2.5 x 10^⁹ viable cells per ml were then injected into the afferent lymphatics of all four limbs. After each injection, the cannula was removed and the incision closed. Intralymphatic injection of each animal’s own treated cultured tumor cells was repeated every 3–10 days (see Results).

Initially, general anesthesia using sodium thiamylal was given for intralymphatic injections but was found to be excessively depressive for repeated use, especially in animals demonstrating abnormal liver function. Finally, the use of a tranquilizer (xylazine) combined with local anesthesia (lidocaine hydrochloride) at the site of incision gave adequate immobilization for the duration of the procedure and was well tolerated by the dogs.

**Skin tests**

Animals were skin tested by intradermal injections of primary and recall antigens before and during the courses of intralymphatic injections. The following antigens were used: Mantoux PPD (Connaught), Monilia (Holister-Stier), Coccidiodin (Bioproducts), Trichophyton (Holister-Stier), Varidase (Lederle) and DNCB. Skin tests were read at 24, 48 and 72 hr.

At the time of each intralymphatic injection, the animal was also skin tested with 0.1 ml of autochthonous vaccine containing 0.25 x 10⁸ viable treated cells and with 0.1 ml of a vaccine blank consisting of the suspending media minus cells.

**Precipitating antibody determination**

Blood was collected from animals before and after intralymphatic injections by venipuncture, allowed to clot at 4°C, and then centrifuged 15–20 min at 400 x g. The serum fraction was then decanted and heat inacti-
vated at 57°C for 30 min. All sera were stored frozen at 20°C until use.
Sera were tested for the presence of precipitating antibody to various antigen preparations by the Ouchterlony technique.

RESULTS

Technical feasibility of intralymphatic immunization

Lymphatic cannulation and infusion of autochthonous vaccine were performed 42 times in 4 dogs without major technical difficulties. The dogs appeared to tolerate the procedure well. Of the 42 injections, one case of local edema at the site of the incision was observed but subsided within 48 hr post injection. No other undesirable local reactions were observed. All incisions were approximately 1 cm in length, and care was taken to minimize damage to lymphatic vessels in the course of cannulation and infusion. It was found that intralymphatic injections could be repeated within 3 days using the same vessel without difficulty. One animal received 8 consecutive injections over 3 months in the same limb with no associated complications. These observations indicated that intralymphatic injection of cellular materials could be used repetitively for prolonged periods of time without technical restrictions or significant discomfort to the animals.

Immunologic side effects of intralymphatic immunization

Since the Vibrio cholerae neuraminidase preparations used to treat the cells contain considerable amounts of Vibrio cholera endotoxin, it was of interest to determine if the animals developed an immune response against components of this preparation which might have been residual in the vaccine preparation or which had adhered to the treated cells. To approach this question, sera from animals before and after intralymphatic immunization was tested for precipitating antibody against the neuraminidase preparation by the Ouchterlony technique, which is sufficiently sensitive to detect 40 μg/ml of serum antibody. None of the sera from treated animals demonstrated detectable precipitating antibody to the enzyme preparation at any dilution.

Similarly, these sera were tested for precipitating antibody against the vaccine blank and found to be negative by this method.

In an effort to assess cellular reactivity to soluble components of the vaccine, animals were skin tested at the time of intralymphatic injection with the vaccine blank. No reactions to the vaccine blank were observed at any time, although conventional recall skin test antigens administered at the same time elicited strong delayed hypersensitivity responses.

Following each intralymphatic injection, the animals were closely observed for signs of systemic anaphylactic reactions. None were observed at any time.

Hematologic factors of intralymphatic injections

Hematologic studies were routinely performed before and after intralymphatic injections. The transient appearance of lymphoblastoid cells in the peripheral blood for approximately 48 hr post injection was occasionally observed. Whether these cells were normal lymphoblastoid cells or injected tumor cells was not determined. Additionally, 48–60 hr post injection, the animals showed a marked increase in peripheral blood monocytes which appeared to be related to changes in the size of diseased lymph nodes.

Effect of intralymphatic immunization on tumor growth and clinical status

Although the primary purpose of this pilot study was to determine whether intralymphatic injections were technically possible and could be tolerated by the animals with the intention of subsequently investigating its potential as a specific immunotherapeutic modality in a controlled manner if the method itself was not technically and clinically prohibitive, one of the most interesting apparent effects of intralymphatic injection of modified autochthonous tumor cells was the rapid decrease in the size of the affected lymph nodes demonstrated by treated animals. Figure 1 shows the total measurable lymphadenopathies of a dog over a three month course during which intralymphatic injections...
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Fig. 1. Total volume (cm³) of measurable peripheral nodes of dogs I-IV during course of intralymphatic injections of neuraminidase-treated, irradiated autochthonous tumor cells. Vertical arrows indicate days on which intralymphatic injections were performed.

were initially given followed by a rapid decrease in node volume. Intralymphatic injections were then discontinued, during which time node size began to increase. Intralymphatic injections with autochthonous vaccine were reinitiated and followed by a second decrease in node size. The decreases in node volume appeared to be associated with an increase in peripheral blood monocytes. Histologic examination of involved nodes after intralymphatic injections of treated autochthonous cells showed a marked decrease in malignant lymphoblastoid cells with residual fibroblastoid activity and large numbers of histocytes as compared with node biopsies taken before any injections (Figs. 2 and 3).

Prior to treatment, all animals demonstrated marked splenomegaly, which decreased markedly during the course of injections. One animal had preponderant hepatosplenic involvement and severely abnormal liver function values. Concomitant to a decrease in the size of the palpable nodes following intralymphatic injections was a marked decrease in the size of the liver and spleen and progressive improvement of liver function values.

Although supportive treatment in the form of antibiotics and intravenous administrations of Ringer's lactate were given as needed, no other form of antitumor treatment was undertaken. It is, perhaps, striking that all of the treated animals showed marked regressions of tumor growth and clinical improvement although the small number of animals utilized in this preliminary study preclude drawing conclusions as to the efficacy of this mode of antitumor stimulation at the present time.

DISCUSSION

The route of administration may play an important role in specific immunotherapy of malignant tumors. *In vitro* stimulation of lymphocytes by autochthonous or syngenic tumor and subsequent direct cytotoxicity to
Fig. 3. 100× cervical lymph node biopsy of dog 1 following two series of intralymphatic injections of treated autochthonous tumor cells. Slide shows marked decrease in lymphoblastoid cells with residual perivascular infiltration and large areas of collagen.

...the stimulating cells has been demonstrated in several animal tumor systems. The failure of many specific immunotherapy attempts may reflect the effects of a number of interrelated factors which may prevent the in vivo generation of adequate populations of specific effector cells by interaction at the afferent, central or efferent levels of immune responsiveness. One of these factors on the afferent level may be mechanisms which circumvent recognition of tumor cells by the appropriate populations of immunoreactive cells. The underlying rationale for investigation of an intralymphatic route of immunization has been based on the hypothesis that the lymph nodes may contain significant populations of unstimulated but potentially immunoreactive cells which may require certain threshold of antigen concentration to generate an effective response. Thus, by the introduction of fairly large amounts of material directly into the lymphatics, high intranodal concentrations may be achieved without disruption of normal node function and antigen processing. Hence, the aim of this initial study was to determine if large amounts of cellular material could be introduced directly into lymphatic vessels. The 42 injections performed clearly indicated that repeated intralymphatic injections could be performed easily and with minimal discomfort. This finding now permits investigation of cellular and humoral immune responses subsequent to intralymphatic administration of antigenic material and comparison with responses elicited by administration via more conventional routes.

Due to a lack of controls and systematic evaluation of antitumor immune parameters following treatment, we have no evidence at the present time that the observed regressions in treated animals have an immunologic basis, although immune mechanisms are theoretically possible. Tsoi et al. have found evidence for the existence of tumor-associated antigens in canine lymphosarcomas and have demonstrated in vitro proliferative responses to autochthonous tumor cells in approximately 60% of the tumor-bearing dogs studied. Presence of tumor-associated cell surface antigens which can elicit proliferative lymphoid responses under appropriate conditions of presentation might result in sufficient immunologic reactivity to account for the apparent decrease in tumor burden following intralymphatic immunization. Additionally, the tumor cells used for vaccination had been treated with Vibrio cholerae neuraminidase under conditions which remove significant quantities of cell-surface sialic acid. Such treatment has been shown to increase tumor cell immunogenicity possibly by uncov-
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...ering cell surface structure capable of eliciting immune reactions in vivo and may have been further contributory to the observed responses. Proposed involvement of these factors is, however, purely speculative since apparent clinical improvement could have been due to a number of inflammatory toxic and immunologic mechanisms, which remain to be identified.

Investigations are presently underway to evaluate cellular and humoral responses of normal dogs to known cellular immunogens following immunization via the intralymphatic route as compared to more conventional routes of immunization in an effect to determine the relative efficacy of this method. In addition, tumor-bearing animals are being treated in a controlled randomized study and evaluated for changes in humoral and cellular responses to their autochthonous tumor cells following treatment.

REFERENCES