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INTRODUCTION

Use of the intralymphatic route for administration of tumor cell vaccines in active specific immunotherapy has been found to be safe and potentially effective in previous investigations (5, 6, 7, 8). Following active specific immunotherapy, evidence of tumor associated immune responses has been observed in human patients (7) and animals with tumor xenografts (2, 8). In most instances, both humoral and cellular immune responses have been demonstrated following intralymphatic immunization with xenografts (2). In general, the purpose of previous studies has been to evaluate the safety and immunotherapeutic potential of the intralymphatic route and emphasis has been placed on detection of possible deleterious effects, assessment of consequent immune responses and clinical changes. To better understand the biological basis for effects observed following active specific immunotherapy, it is important to investigate events which take place between the time of intralymphatic infusion and the observation of apparent consequential immune or clinical effects.

The present investigation was undertaken to determine the fate and immediate consequences of intralymphatic injections of cellular materials under the conditions used for intralymphatic immunotherapy. A canine animal model was used to permit the infusion of high specific activity radiolabeled cells for scanning studies.

Although the distribution of intralymphatically injected contrast material is widely known in diagnostic radiology, the distribution of intact immunogenic cells similarly injected was not known. To approach this problem, serial total body gamma scanning was used to determine the distribution of infused radiolabeled cells and to observe how this distribution changed with time after injection. The use of a readily releasable and rapidly cleared submembrane label allowed some assessment of infused cell cytolysis after intralymphatic injection.

Another area of particular interest was the extent of spread of regional lymph nodes draining the sites of infusion. Histopathologic studies were used to detect alterations in lymph node architecture and immediate cellular reactions to the infused viable tumor cells. In vitro cytotoxicity testing was then used to detect the development of effector functions associated with immune reactivity.

MATERIAL AND METHODS

Animals: Three categories of dogs were used in these studies.
- Acute dogs: Randomly bred dogs of either sex and undetermined ages were obtained from local agencies through the UCLA Animal Facility. Although the past medical history of these dogs was unknown, there was no apparent signs of disease at the time of use.
- Tumor-bearing dogs: Privately owned dogs of various breeds were referred for intralymphatic immunotherapy at the Leo G. Rigler Center at UCLA by veterinarians in the community. Use of these dogs for this study was obtained by informed consent of their owners.
- Inbred dogs: Inbred 1-2 year old female beagles were obtained from Marshall Research Animals, Inc., North Rose, New York. All dogs had been vaccinated for canine distemper, hepatitis, parvovirus and were clinically free of any disease at the time of the study. All susceptible dogs had also been previously vaccinated for oral papillomas.

Cells: The murine tumor line, EL4, was maintained in the ascitic form by serial transplantation in C57Bl/6 mice. Cells used for infusion were obtained ten days after transplantation. EL4 was obtained from Dr. Benjamin Bonavida, UCLA School of Medicine.

Autochthonous and allogeneic cells were obtained and prepared as previously described (8) and stored in liquid nitrogen until use. Prior to infusion the autochthonous cells were grown to a density of 10,000 rads in a Gamacell 220 over approximately 2 minutes.

Cells were radiolabeled with $^{51}$Cr-sodium chromate (ICN, Irvine, California) by incubation of $1 \times 10^9$ viable cells in 200 microcuries (specific activity: approximately 900 Ci/gm) for 90 minutes at 37°C. The cells were then washed four times, counted and resuspended in sterile Hank’s Balanced Salt Solution (HBSS) (Grand Island Biological Co.) for injection.

Intralymphatic infusions of either labeled or unlabeled cells were done under general anesthesia in a distal lymphatics of one limb at a rate of 1 ml per minute as previously described (6, 8).

Gamma scans were performed using a Picker Dynacamera standardized against 0.3 Mv energy photons: 5000 counts were recorded over 5 to 10 minutes.

Histology: Tissues removed for histopathologic examination were immediately placed in 10% buffered formaldehyde fixative. Paraffin sections and hematoxylin and eosin stains were routinely prepared for microscopic examination. Special stains, e.g. acid fast on PAS stains were also used in some instances to confirm or exclude nonexperimental causative agents.

In vitro cytotoxicity tests: In vitro cytotoxicity against EL4 was determined by the $^{51}$Cr-release technique of Brunner et al. (3) and modified for use of separated canine peripheral blood lymphocytes and canine lymph node cells as effector cells. Cell viability was calculated on the basis of the method of Ting and Morris (9). Single cell suspensions of canine lymph node cells were obtained by mincing excised lymph nodes and suspensions found to be greater than 90% viable were used for testing.

FATE OF INFUSED CELLS

To determine the fate of intact tumor cells injected via the intralymphatic route, $10^9$ $^{51}$Cr-labeled EL4 cells in 1 ml of HBSS were infused at a rate of 1 ml per minute into the distal peripheral lymphatic vessels of the hind limbs of two dogs. Whole body gamma scans were used to monitor the course of the injected cells. By 30 minutes after completion of the infusion, the radiolabel was found to be concentrated in the first echelon regional lymph nodes draining the site of infusion and was no longer demonstrable at the site of infusion. Detectable radiolabel remained in the regional nodes of injected animals for 4 weeks after injection. No label was detected in distant nodes, lungs, liver or spleens of the animals at any time (fig. 1).

To compare the distribution of cells injected via various routes (intra-venous, subcutaneous, intradermal and intralymphatic), $10^9$ radiolabeled EL4 cells in 1 ml of HBSS were infused as described above in four pairs of dogs using a different route of administration for each pair. Whole body gamma scans were performed at thirty minutes and then serially up to 28 days when indicated. In intravenously injected animals, radiolabel was found concentrated in the liver and spleen as indicated in Table 1. Label

<table>
<thead>
<tr>
<th>ROUTE INJECTION</th>
<th>SITE OF INJECTION</th>
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<th>LUNGS</th>
<th>LIVER</th>
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0 indicates no detectable radioactivity over organ; + indicates levels of radioactivity detectable for a few days; ++ indicates high levels of radioactivity detectable for a few days; +++ indicates very high levels of radioactivity persisting for several weeks.
was also detectable in the lungs and marginally at the site of injection. No label could be found in regional or distant lymph nodes. Five days after intravenous injection, label was no longer detectable at any site.

Animals receiving subcutaneous injections of the same number of radiolabeled cells in the same volume retained label at the site of injection for 9 days. At 24 hours after injections, label was detectable in small amounts in regional nodes draining the site of injection, but was no longer detectable by day 5. No label was found in the lungs, liver or spleen at any time. Intravenous injection

Animals receiving intradermal injections of the same number of radiolabeled cells showed a pattern of distribution similar to those receiving subcutaneous injections, however, label remained concentrated at the site of injection for 4 weeks. By 24 hours label was detectable in regional nodes draining the site of injection and was retained in small amounts in the nodes for 9 days. No label was observed in the lungs, liver or spleens of these animals at any time following injection. (fig. 2).

Animals receiving intralymphatic injections of equal numbers of labeled cells showed the pattern of distribution described above. Label was retained in first echelon regional nodes draining the site of infusion for 4 weeks and no label was observed at the site of injection, distant nodes, liver or spleen at any time.

These observations indicate that the use of the intralymphatic route of injection results in selective retention of the injected cells in regional lymph nodes for prolonged periods of time. It is to be noted that °Cr as sodium chromate does not result in long term retention of label by infused lymph nodes. Unbound label is rapidly cleared by the kidneys and is found primarily in the urinary bladder within several hours after infusion. The long term retention of °Cr label in lymph nodes following intralymphatic infusion of labeled cells strongly suggests that the labeled cells are trapped in the lymph nodes as intact cells without major membrane damage or cytolysis. One mechanism to account for this observation could be phagocytosis by lymph node histiocytes or other node cells without prior cytolysis of the infused cells.

Effect of Concentration, Viability and Origin of Fate of Infused Cells

To determine the effect of injected cell concentration on the distribution and fate of the injected cells, pairs of dogs were given intralymphatic infusions as previously described, but with either a higher concentration of EL4 cells (10⁷ cells in 1 ml) or lower concentration of cells (10⁶ cells in 5 ml). As summarized in Table 1, neither an increase nor a decrease in concentration within the range tested (2 x 10⁷ cells per ml to 10⁸ cells per ml) showed any significant effect on the distribution of label following intralymphatic injection.

To determine if the presence of non-viable tumor cells in the injection suspension would prevent retention of the viable tumor cells by regional nodes, pairs of dogs were infused with a suspension of 10⁷ chromated EL4 cells mixed with 1.9 x 10⁶ unlabeled, dead EL4 cells. No alteration was observed in the distribution and concentration of the radiolabeled viable cells in the draining lymph node. The distribution of the non-viable cells could not be determined at this time, since non-viable cells do not retain sodium chromate, however, the presence of a large number of dead cells did not appear to prevent trapping of viable cells by the lymph nodes.

Since only malignant xenogeneic cells had been used for these experiments, it was of interest to determine if normal, autologous cells would be distributed in the same way following intralymphatic infusion. To investigate this question, pairs of dogs were injected with either 10⁷ °Cr-labeled autogenous peripheral blood lymphocytes in 1 ml of HBSS or 10⁷ °Cr-labeled autogenous peripheral blood lymphocytes in 1 ml which had also received 10,000 rads in a cobalt blood irradiator (Gammacell 220) immediately before infusion. Serial whole body gamma scans following infusion showed that normal lymphocytes whether irradiated or not showed the same pattern of distribution and clearance as EL4 cells.

Effect of Local Node Irradiation Prior to Intralymphatic Infusion

To determine the effect of regional node irradiation on the fate of intralymphatically infused cells, three dogs were given 1000 rads in one fraction to both popliteal node regions. Thirty minutes after irradiation, one dog was infused with 10⁷ EL4 cells in 1 ml of HBSS in one limb only. The second dog was similarly infused 5 days after irradiation and the third dog, two weeks after irradiation. All dogs were serially scanned following the infusion of EL4. The dogs which had received infusion 30 minutes and 5 days following irradiation of the popliteal regions demonstrated no detectable differences from normal dogs in the distribution and retention of the labeled cells. The dog which had been infused two weeks after irradiation, however, showed a normal distribution of label.
immediately following infusion, but appeared to clear the label from the irradiated nodes more rapidly than the other animals. Retained radiolabel was negligible in infused nodes 15 days after infusion as compared to 4 weeks in the other dogs. The inability to retain label for the expected period of time may reflect chronic changes in lymph node cellularity following irradiation.

**Effect of Tumor Burden on the Fate of Infused Cells**

Since the intralymphatic route is being used for the introduction of immunotherapeutic agents in the treatment of malignant diseases, it was of considerable interest to determine if the presence of an existing tumor affected the distribution of infused cells and the ability of the lymph nodes to retain the infused materials.

To begin investigation of this question, two dogs with mammary carcinomas were selected for study. Both dogs received 10^7 irradiated Cr-labeled autologous tumor cells in 1 ml of HBSS infused intralymphatically to apparently normal lymph node areas. Subsequent serial scans showed non-involved nodes in tumor-bearing animals show no detectable isotope in trapping materials injected via the intralymphatic route.

To determine the effect of nodal involvement on the fate of intralymphatically infused material, four dogs with disseminated lymphoma were studied. All dogs had involved nodes draining the site of the intralymphatic injection. One dog received 10^7 labeled EL4 cells (xenogeneic), a second dog received 10^7 similarly labeled allogeneic lymphoma cells and the remaining two dogs each received 10^7 similarly labeled autologous tumor cells. Subsequent serial scans of the four dogs showed that when the lymph nodes draining the site of infusion are themselves involved by malignant proliferation, the infused material is not effectively trapped in the first echelon nodes, indicating that non-involved nodes in tumor-bearing animals show no detectable isotope in trapping materials injected via the intralymphatic route.

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In order to reduce reactions to previous or concomitant diseases and to identify more precisely histological reactions consequent to intralymphatic infusion, 11 carefully screened, inbred beagles, received intralymphatically 10^7 EL4 cells in one posterior limb. The popliteal nodes were examined on day 3, 5, 7, 10, 15 and 21, and on days 2, 3, 5, 8 (2 dogs), 15 (2 dogs), 22 29, 36 after infusion (Table II). One half of each lymph node was prepared for histological examination. The other half was processed for lymphocyte storage. For comparison, one additional inbred beagle was injected intralymphatically with sterile saline and lymph nodes were removed for examination.

Serial histopathologic studies of the popliteal nodes in inbred beagles showed that cortical and paracortical reactions were clearly detectable table 48 hours after intralymphatic infusion of EL4 cells, became intense on day 3, peaked on day 8 when the volume of the lymph node was 3 or 4 times its normal size and subsided in the paracortical area on day 22. The lymph nodes appeared normal in structure and size on day 36 (Table II). Histiocytosis, hyperemia and edema of the infused lymph node appeared within 30 minutes and subsided within 8 hours. Transient histiocytosis was present again 4 weeks after infusion. No histologic changes were observed after infusion of saline alone (Table II).

**Effect of Cytopathic Drugs on the Histologic Reaction**

Two dogs were given daily intravenous infusions of 100 mg (5.5 mg/kg) cyclophosphamide for 15 days. On day 10, a 30 per cent and 40 per cent drop of the circulating lymphocytes were observed and 10^7 EL4 cells in 1 ml of saline were administered intralymphatically in one posterior limb. On day 15 the popliteal nodes were removed bilaterally. The lymphocytolytic response of the stimulated lymph nodes was not altered by the intravenous infusions of cyclophosphamide and an increase of 50 per cent and 100 per cent was observed in the circulating lymphocytes counts following intralymphatic infusion.

**Effect of Ionizing Radiations**

Histologic studies were performed on 3 dogs which had received 1,000 rads to the popliteal nodes bilaterally and had been intralymphatically infused on one side with radiolabeled tumor.
Normal (+) cortical and paracortical lymphopoiesis. No increased diffuse lymphocytosis (0/+) (120 ×).

Figure 3. Lymph node.

Increase in lymphopoiesis (++) and diffusion (++) between the follicles and in the medullary zone (120 ×).

Figure 4. Lymph node.

Marked increase (+++) of the lymphopoiesis to the extent of confluency (diffusion ++++) (120 ×).

Figure 5 and 5a.

5a: Magnification of interfollicular confluent lymphopoiesis (540 ×).
cells either 30 minutes, 5 days or 2 weeks after irradiation. Lymph nodes from dogs which had been infused 30 minutes and 5 days after irradiation showed persistent lymphoid proliferation (6 and 8 weeks) which was more discrete than when the infusion was given 2 weeks after irradiation. In all three dogs, the contralateral, irradiated but not infused lymph nodes showed only typical post-irradiation changes, i.e. lymphocytic depletion and presence of plasma cells, eosinophils and neutrophils.

**CELLULAR IMMUNE RESPONSES**

Peripheral blood lymphocytes were harvested from all beagles immediately prior to intralymphatic infusion of EL4 and again 15 and 30 days after infusion. For comparative purposes, direct cytotoxicity of all lymphocyte samples against EL4 was assayed simultaneously. Results of these studies showed that the longer the time interval prior to lymph node biopsy, the larger the number of lymphoid cells were left in place from day 1 until day 8, the more cytotoxicity was detected in the peripheral blood (fig. 6). These results were consistent with the fate of radiolabeled cells and the observed regional histologic reactions. When the lymph nodes were removed on day 20, baseline significant changes in peripheral blood lymphocytic cytotoxicity were noted on day 15 and 30 after infusion.

**LYMPH NODES FROM DOGS WHICH**

Lymph node cells from draining and contra­ lateral nodes were similar assayed for cytotoxicity to EL4 at various effector to target cell ratios. Subsequent levels of cytotoxicity was observed in stimulated node cells only on days 15, 22 and 29 after infusion.

**DISCUSSION**

These experiments document the differences in the fate and consequences of intact cellular immunogens administered by the intralymphatic route as compared to other parenteral routes of administration. The conditions of intralymphatic administration used in these studies, i.e. high concentrations of cells infused as a bolus over 1 to 5 minutes, which were those which have been employed in vivo (6), were used to establish the fate of radiolabeled intact cells and infused cells were observed in higher echelons of tumor nodes via lymphatic pathways in a disease state and the observations presented in this study should be interpreted only in the context of the condition under which they were observed.

In animals with normal lymph nodes, intralymphatic injection of radiolabeled intact cells resulted in detection of radiolabel exclusively in the first echelon of lymph nodes. Although silver gamma scanning may not be sufficiently sensitive to detect small numbers of injected cells which could traverse first echelon lymph nodes to higher levels, the additional absence of histopathologic changes in higher echelons of distant lymph nodes suggest that insufficient numbers of cells reached these sites to exceed concentration thresholds for stimulation of nodal lymphoid proliferation by immunogenic materials. Our observations, therefore, suggest that under the conditions employed, intact tumor cells injected via the intralymphatic route are trapped by lymphatic pathways for varying periods following although their microscopic identification in the lymph nodes would require more refined techniques than those presently available. Direct tumor cell proliferation, which would make them more readily detectable was not observed in any case. Fisher and Fisher (4) have reported only transient retention and no histopathologic changes following intralymphatic infusion of rabbit red cells into rabbits. The differences between the two observations may be attributable to differences in the conditions of administration. These experiments attempted to simulate physiological condition of lymphatic drainage, whereas in the present study, such conditions were intentionally avoided. Adjuvant effects of irradiation would also be due to intrinsic differences in the injected materials. Ada et al. (1) have reported differential trapping of different antigens in lymph nodes following footpad or intravenous injection and found only transient retention at canine red cells were used. However, they found no significant correlation between the extent of trapping and the immunogenicity of the material. In our studies, intralymphatic injection of separated autologous peripheral blood lymphocytes in two dogs resulted in detectable trapping of radiolabeled material, but histologic preparations of lymph nodes draining the site of injection showed no evidence of cortical or paracortical lymphocytic proliferation.

When the infused lymph nodes were involved by malignant processes, the first echelon of lymph nodes showed minimal detectable uptake and infused cells were observed in higher echelons of the involved lymph nodes indicating that while the trapping functions of the involved lymph nodes were severely diminished, they were not totally abolished. This may in part explain our earlier observations of responses to active specific intralymphatic immunotherapy in cases with nodal involvement and even lymphoma in dogs (6, 7). However, one could postulate that neither uptake nor response would be observed if the lymph nodes were totally replaced by malignant tissue and the prognosis should be extremely poor.

The cortical and paracortical lymphopoi­ esi observed in normal lymph node areas after intralymphatic injection of EL4 is consistent with the development of an anti-EL4 immune response. Intralymphatic infusion of inert particles was found to result in a simple trapping effect with transient histiocytosis, but no cortical or paracortical lymphocytic activity. In EL4 injected dogs lymphocytic activity was consistently associated with the development of in vitro peripheral blood lymphocytic cytotoxicity to EL4. This development of cellular cytotoxicity could be abolished by removal of the infused cells (fig. 6). After 8 days, cytotoxicity could be diminished but not abolished by removal of infused nodes (fig. 6). Dogs with intact lymph nodes develop varying levels of peripheral blood lymphocyte cytotoxicity following intralymphatic injection of the immunizing cells. The kinetics of peripheral blood cytotoxic activity roughly paralleled histologic and clinical findings. Lymph node lymphocytes, however, only showed minimally detectable cytotoxicity to EL4 at 22 days after irradiation. The absence of cytotoxic lymphocytes in infused lymph nodes during periods of peripheral blood lymphocyte cytotoxicity is observed in cases where irradiation cytotoxic effector cells may leave the lymph node and indicated functional differences between lymph node and peripheral blood lymphocyte cytotoxicity following infusion of weakly immunogenic materials.

Collection and in vitro testing of lymphocytes from effervescent lymph vessels of stimulated nodes would provide more accurate information on the development of cell mediated immunity following immunization via the intralymphatic route.

The duration of the histologic reaction (5 to 6 weeks) in intact dogs was somewhat longer than would be predicted by either clinical data (6, 8) or in vitro studies (2) and should be taken into account for optimizing treatment schedules in repeated intralymphatic immunotherapy.

The distribution of intralymphatically infused radiolabeled cells was not modified by irradiation of first echelon lymph nodes with 1 000 rads in one fraction, 30 minutes or 5 days prior to infusion. Although nodal lymphocytic depression was observed under these conditions, lymph nodes from dogs which had been infused within 5 days of irradiation, not only did not show the expected lymphocytic depression, but exhibited marked lymphopenia in cortical and paracortical areas. In one case, lymphoblastic activity was observed up to 8 weeks after infusion. When intralymphatic infusions were performed 2 weeks after irradiation, distribution and retention of infused materials remained the same as in unirradiated lymph nodes, but lymphocytic activity was greatly decreased.

The capacity of infused lymph nodes for marked lymphoid proliferation even after 1 000 rads or intravenous cyclophosphamide may be of great potential value to patients following radiation therapy or chemotherapy and deserves further investigation.

In conclusion, these studies demonstrate the biologic uniqueness of the intralymphatic route of immunization. Previous preclinical and clinical studies suggest that the full potential of intralymphatic infusion of immunostimulatory materials has not yet been realized. Future studies should be directed toward increased manipulation of resulting immune responses by selective stimulation of lymph nodes beyond the first echelon.
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REFERENCES

INTRODUCTION
Il est admis que les cellules de mammifères ont une durée de vie limitée en culture (3). Ceci est encore plus vrai lorsqu'il s'agit d'cellules différenciées (4, 15). Par ailleurs, la transformation spontanée de cellules de rongeurs en culture a souvent été observée (13, 14). Nous avons voulu savoir ce qu'il en était de ces phénomènes dans des cellules en voie de différenciation et, plus précisément, si, tout en conservant leur caractère différencié, des cellules pouvaient être maintenues in culture suffisamment longtemps pour permettre l'étude dans des cultures parallèles de transformations provoquées. Nous avons donc retenu dans ce but des cellules de différentes régions du cerveau d'embryons de souris que nous avons mises en culture. La nature gliale de la plupart de ces cultures a pu être établie sans équivoque en montrant la présence d'une protéine gliale spécifique (gliarial fibrillary acidic protein, ou GFAP). Parmi ces lignées, cinq sont restées non transplantables chez l'animal pendant la durée de l'expérience. La sixième, au contraire, a subi une transformation spontanée à la 11e génération cellulaire. Cette culture, après transformation, comme les tumeurs qu'elle provoque, a gardé son caractère différencié neurogial.


SUMMARY: Spontaneous transformation and long term culture of foetal mouse glial cells

Six cell lines originated from foetal mouse brain were maintained in vitro for more than two years. The morphology of most of these cultures suggests their glial character. This is corroborated, for some lines, by the presence of a specific glial protein (Glial fibrillary acidic protein, or GFAP). Five out of these lines remained not transplantable in animals during all the experiments. However one of them went through a spontaneous transformation at the eleventh population doubling. This culture, after transformation remained glial and when injected in animals, induced GFAP containing tumors.


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Transformation spontanée et maintien prolongé in vitro de cellules gliales d'embryons de souris

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