Tumor Immunity in Rat Lymph Nodes Following Transplantation

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Inguinal lymph nodes in the Buffalo rat were autotransplanted to the popliteal fossa by an intact vascular pedicle or by microvascular anastomosis. These revascularized nodes had normal histology and made spontaneous afferent and efferent lymphatic reconnection with surrounding lymphatic vessels, as documented by ink and silicone rubber injection studies. Lymphoscintigraphy with ^{99m}Tc antimony sulfide colloid correctly predicted the 44 of 120 node transplants that had made afferent reconnection. To demonstrate immunologic activity of lymph nodes following transplantation, a cellular adherence assay was employed to detect cell-mediated cytotoxicity of lymph node cells isolated from rats sensitized to an allogeneic gliosarcoma. Cytotoxicity was detected in nontransplanted regional nodes sensitized to tumor ($p < 0.01$) and in nodes transplanted by vascular pedicle and then sensitized to tumor ($p < 0.001$). This study demonstrates that lymph nodes can be transplanted with restoration of functional lymphatic anatomy, and that following transplantation, lymph nodes retain the ability to mount an immune response against tumor.

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REGIONAL LYMPH NODES play a major role in the initiation and maintenance of immunity in some animals, but in man their function has not been defined conclusively. Controversy over prophylactic lymphadenectomy for the extirpation of malignancy centers around the possible effect this might have on host defense. The replacement of lymph nodes in a clinical setting has not been attempted, but the capability of lymph node transplantation now exists and would appear to be a solution to this dilemma. The potential to amplify the immune state by a surgical approach may someday expand the surgeon's role in the treatment of malignancy, but this must first rest on the demonstration of direct patient benefit. One step towards achieving this is to demonstrate immunologic activity of nodes following transplantation in the laboratory animal.

A lymph node grafted from one site to another without its direct vascular blood supply undergoes destruction by progressive fibrosis.[15,25] However, lymph nodes remain viable when transposed on a vascular pedicle or when revascularized by microsurgical anastomosis.[10,11,15,23,25]

In earlier studies by Futrell et al.,[10] revascularized lymph nodes underwent cellular depletion and repopulation during the first month following transplantation. Cellular depletion may have been related to a prolonged ischemia time. Shesol et al.[23] reported normal histology in transplanted nodes. They also documented uptake of ^{198}Au colloid following autotransplantation and showed that the greatest likelihood of isotope uptake will occur in nodes transplanted immediately to a lymphadenectomized site. Presumably this is because afferent lymphatics in the recipient site remain open for a short time following disruption by lymphadenectomy and can make reconnection with the severed afferent lymphatics of the transplanted nodes. Groth et al.[11] corrected factor VIII deficiency in dogs by revascularized node homografts and by spleen transplantation. Sustained levels of factor VIII were detected prior to rejection, suggesting that lymphorecticuloendothelial tissue produces this factor.

The purpose of this study has been twofold: (1) to determine whether a lymph node transplanted from one site to another with its vascular supply intact can still

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develop immunity against tumor, and (2) to document the restoration of functional lymphatic anatomy by showing afferent and efferent lymphatic reconnections with transplanted nodes.

Materials and Methods

Rats

Two- to 4-month-old male inbred Buffalo rats (obtained through the National Cancer Institute from Charles River Breeding Laboratories) were used to detect cell-mediated cytotoxicity of lymphocytes from native and transplanted lymph nodes.

Tumor and Cell Culture

An allogeneic, non-metastasizing gliosarcoma (GS-9L), originally induced by intravenous (IV) injection of N-nitrosomethylurea in the F344 rat,[3] was maintained in the laboratory by continuous monolayer cell cultures in growth medium, consisting of medium M199, 17% NCS, 40 mM HEPES, 80 μ/ml penicillin, 80 μ/ml streptomycin and 0.20 mcg/ml amphotericin B (Grand Island Biological Co., Grand Island, NY).

Transplantation of Lymph Nodes

Following popliteal lymphadenectomy, inguinal lymph nodes in the rat can be transposed to the vacant popliteal fossa by the vascular pedicle supplying the nodal tissue, the superficial epigastric artery and vein, or transplanted from the contralateral side and revascularized by microsurgical anastomosis. For the latter procedure, it was preferred to use the superficial epigastric vessels supplying the lymph nodes as a sidebranch from the common femoral vessels and perform end-to-side anastomoses of the donor common femoral vessels to the common femoral vessels of the recipient side (Fig. 1). The anastomoses were performed under 40× magnification using the Zeiss OpMi-7P/H operation microscope and 11-0 nylon suture.

Inguinal nodes in 36 rats were transplanted for histologic studies: 12 with lymph nodes transplanted by vascular pedicle, 12 with lymph nodes revascularized by microsurgical anastomosis, and 12 with lymph nodes transplanted as nonvascularized grafts. Three of each group were excised at weekly intervals for four weeks and prepared for histologic examination with hematoxylin-eosin (H & E).

In a second study, inguinal nodes were transplanted to the popliteal fossa by vascular pedicle in 120 rats to determine if any specific method of dissection resulted in greater likelihood of afferent lymphatic reconnection.

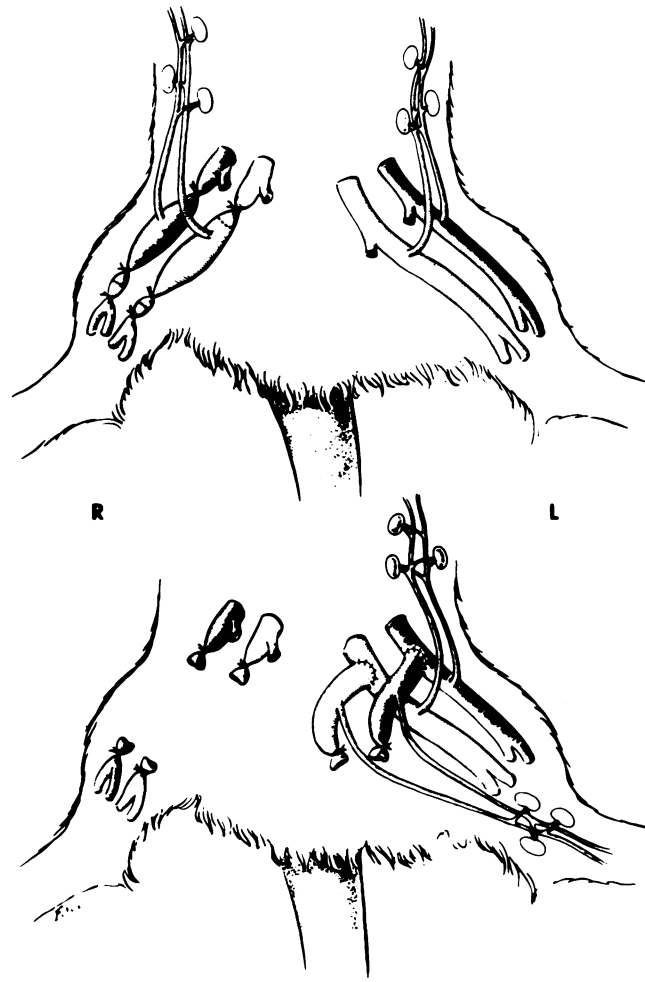

FIG. 1. Right inguinal fat pad and lymph nodes supplied by the superficial epigastric artery and vein shown excised and revascularized by end-to-side anastomoses of the common femoral vessels. Nodes are placed in the left popliteal fossa following popliteal lymphadenectomy.

These rats were divided into six surgical groups representing different methods of dissection in preparing the donor nodes and in performing the lymphadenectomy at the recipient site (Table 1). Following surgery, animals in each group were imaged by ^{99m}Tc antimony sulfide colloid (ASC) weekly for five weeks and then dissected 24 hours following intradermal pedal injections of india ink. Normal popliteal nodes consistently stain intensely after such injections, and this technique was employed to assess success of afferent lymphatic reconnection with transplanted nodes. Lymphoscintigraphy was evaluated as a method to noninvasively select functioning transplants.

Efferent lymphatic reconnection was also studied in several revascularized nodes by ink or Microfil silicone rubber (Canton Bio-Medical Products, Boulder, CO) injected directly into the node with a 32-gauge needle. This technique required killing the animal.

TABLE 1. *Incidence of Afferent Lymphatic Reconnection to Transplanted Nodes with Different Methods of Surgical Dissection*

Surgical Technique	Number	Per cent Reconnected	
Excision of popliteal node alone			
Inguinal node pad incorporating subdermal plexus	14	29%	N.S.*
Inguinal node pad cut as a 2 × 2 cm block of tissue	25	32%	
Inguinal node pad skeletonized to expose nodes	27	37%	
Excision of popliteal node and all surrounding fat			
Inguinal node pad incorporating subdermal plexus	6	17%	
Inguinal node pad cut as a 2 × 2 cm block of tissue	20	25%	
Inguinal node pad skeletonized to expose nodes	28	57% ($p < 0.05$)	

* All combinations of these groups are not significantly different by chi-square test.

Cytotoxicity Test—Native Popliteal Nodes

Normal intact rats were used to identify the time of maximal cytotoxic response against the immunizing tumor. Fifty male Buffalo rats were each immunized with 10^6 allogeneic GS-9L tumor cells in 0.1cc of M199 by intradermal injection of the dorsolateral left foot. Twenty control rats received injections of medium without tumor cells. Native popliteal nodes from sensitized and control rats were aseptically excised at different times after inoculation and assayed for cytotoxic activity.

Each node was aseptically teased apart by needle dissection, freeing the lymphocytes in medium M199. Three times these cells were washed with 20 ml of medium and centrifuged at 300 g for eight minutes at 4 C. Between each wash, vortex agitation and low speed centrifugation was used aggregate and sediment dead cells according to the technique of Parish et al.[16] After the third wash, the cells were resuspended in 2 ml of growth medium and counted in a hemacytometer. Viability was assessed by Trypan blue exclusion and was usually greater than 85%. Cell concentrations were adjusted to 10^5 cell/ml.

Lymphocytes were then plated on GS-9L tumor cells using a modification of the cellular adherence assay system reported by Takasugi and Klein[24] (Fig. 2). A suspension of GS-9L tumor cells in growth medium was randomly pipetted in 200 μl volumes to deliver 100 tumor target cells into each well of a Micro Test II plate (Falcon Plastics Inc., Oxnard, CA). The plates were then incubated for eight hours at 37 C in moist

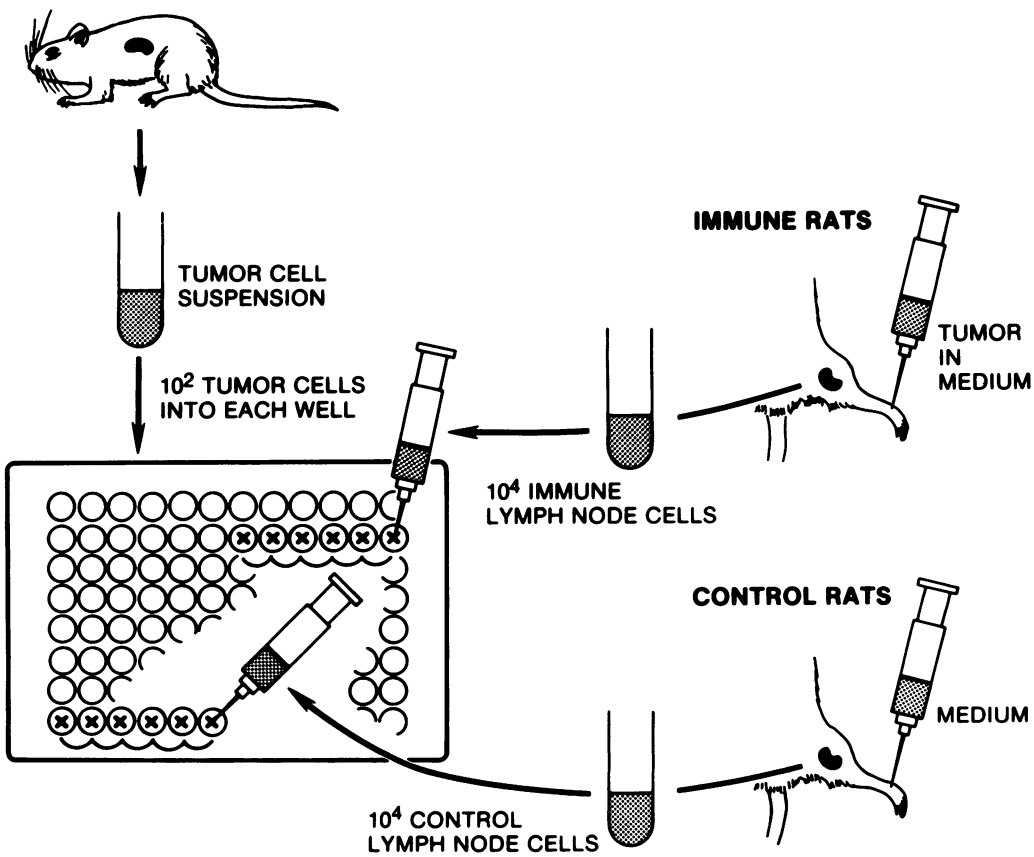

FIG. 2. Schematic diagram of the cell-mediated cytotoxicity assay. Tumor cells are first plated in each well, allowed to adhere, and then immune and control lymph node cells are added. The difference in the number of tumor cells remaining after 40 hours incubation provides an index of cytotoxicity.

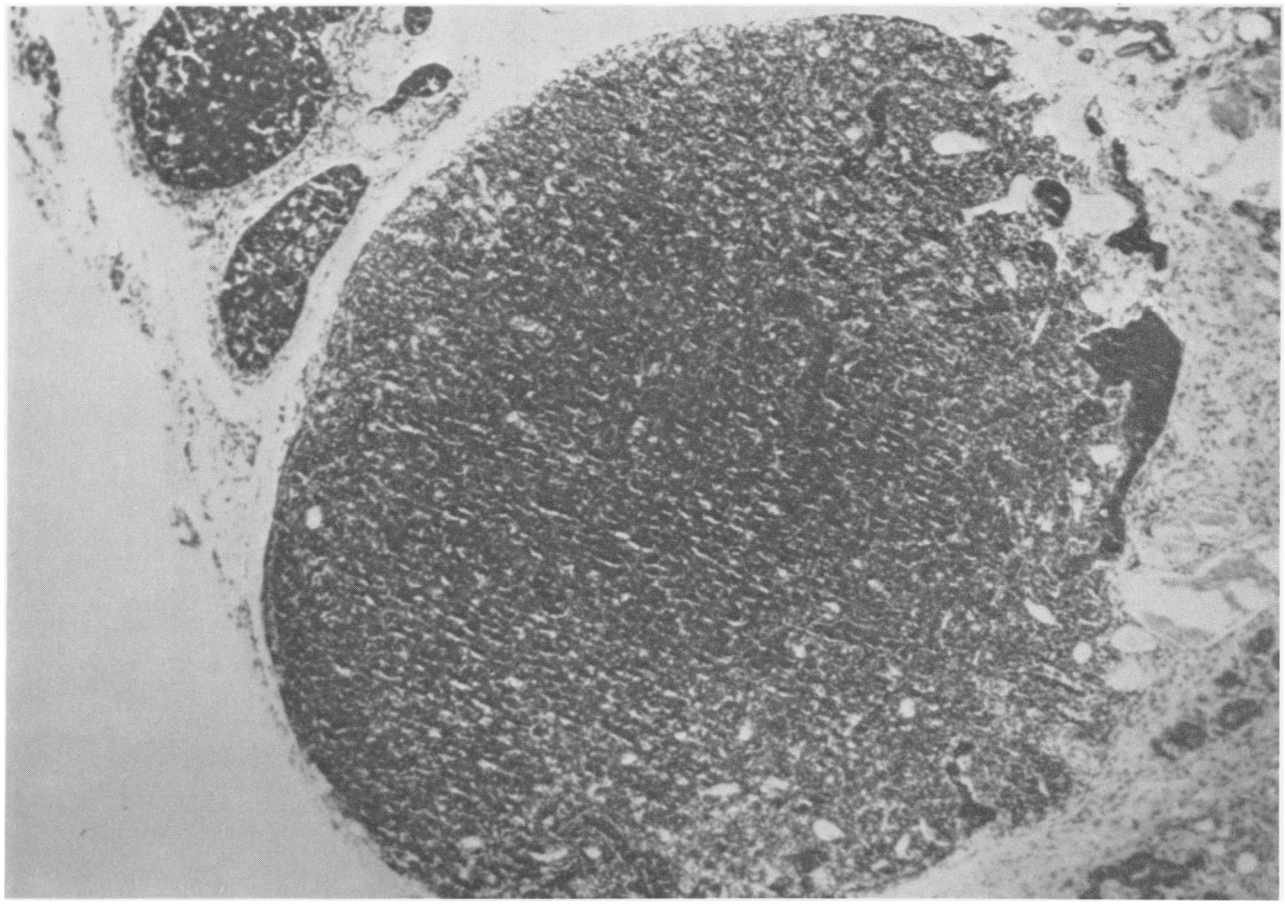

FIG. 3. Two weeks following transplantation and revascularization, showing normal nodal histology (hematoxylin-eosin, ×32).

air to allow adherence of tumor cells to the bottom surfaces of the wells. Lymph node cell suspensions prepared as above were coded and randomly pipetted in 100 μl volumes to deliver 10^4 lymphocytes into each well for a lymphocyte to tumor target cell ratio of 100:1. Four to six replicate tests were performed for each lymph node cell suspension. The Micro Test plates were then returned to the incubator for 40 hours after which they were gently washed three times with M199 to remove lymphocytes and detached non-viable tumor cells. The adherent cells were fixed with ethanol, stained with giemsa, and visually counted.

Percentage cytotoxicity was calculated as follows:

$$\frac{nC - nT}{nC} \times 100\%$$

where nC is the mean number of tumor cells remaining in control wells and nT in the test wells. An index of cytotoxicity was calculated for each immune rat from the mean of its four to six replicate tests and the mean from the two control animals on each day. Significant cytotoxicity was determined by the Sign Test and represents greater killing or growth inhibition of tumor cells by immune lymph node cells compared to controls.

Cytotoxicity Test—Transplanted Nodes

In a second study, inguinal nodes were transplanted to the vacant popliteal fossa by vascular pedicle in 60 male Buffalo rats. Donor nodes were skeletonized, and all fat was excised from the recipient site during popliteal lymphadenectomy. Four and five weeks following transplantation lymphoscintigraphy with ^{99m}Tc ASC was performed: 31 were found to have excellent uptake of the isotope by the transplanted nodes and these rats were randomly divided into immune and control groups. The immune group (22 rats) received 10^6 GS-9L tumor cells in 0.1 cc M199 by intradermal injection of the dorsolateral foot. The control group (nine rats) received injections of medium without tumor. The transplanted nodes from sensitized or control rats were aseptically excised at days 3, 7, and 8 following inoculation and assayed for cytotoxic activity in the same manner as described for the intact rats.

Results

Twelve nodes transplanted by vascular pedicle and 11 nodes transplanted by microsurgical anastomosis showed normal histology with some hemosiderin pig-

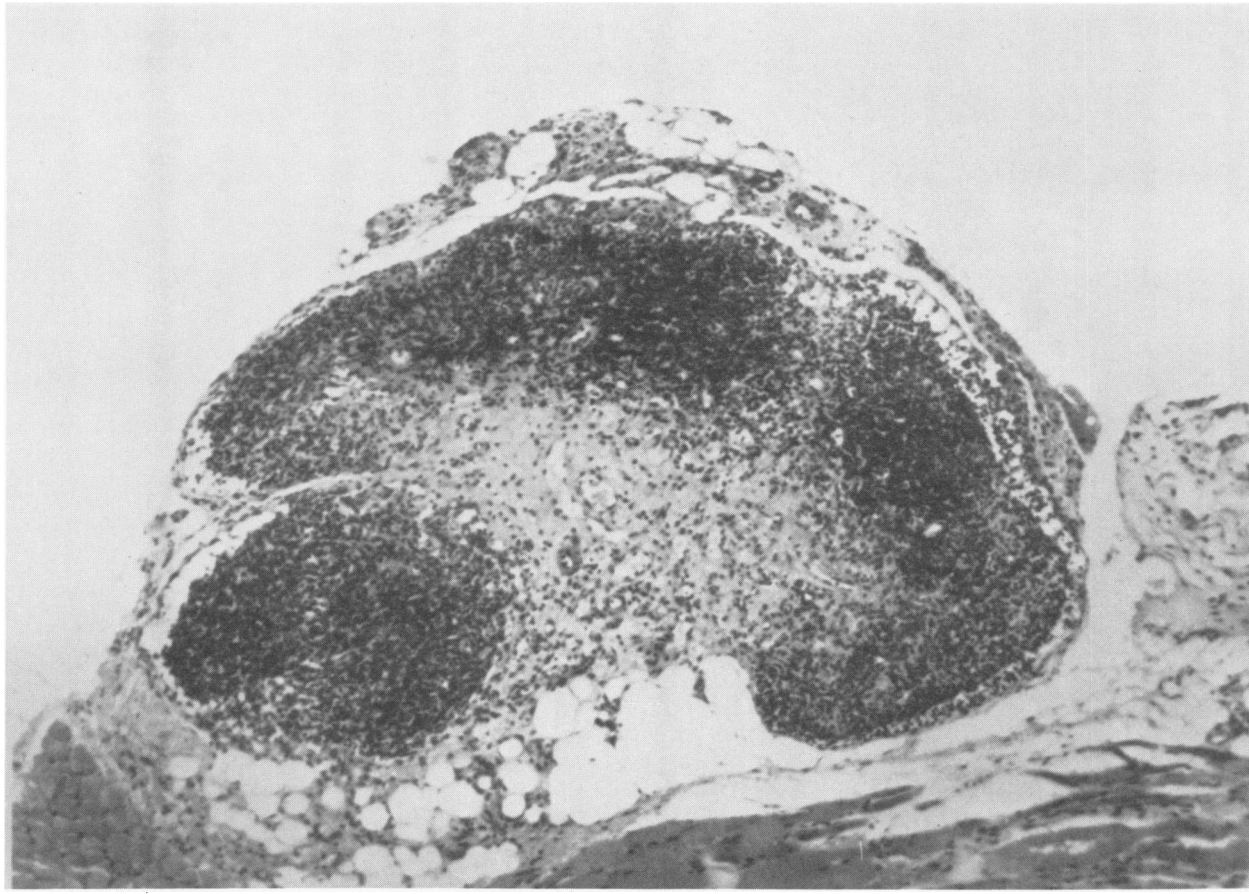

FIG. 4. Two weeks following grafting without a blood supply, showing loss of nodal architecture and fibrosis (hematoxylin-eosin, ×32).

ment (Fig. 3). There was one necrotic node in the anastomotic group. In contrast, nodes transplanted without a blood supply uniformly underwent progressive fibrosis (Fig. 4).

The data in Table 1 summarize the study of 120 rats undergoing different methods of dissection and indicate that for afferent lymphatic reconnection the ideal method of dissection is to skeletonize and expose the donor nodes and excise the native popliteal node and all surrounding fat from the recipient site. This partic-

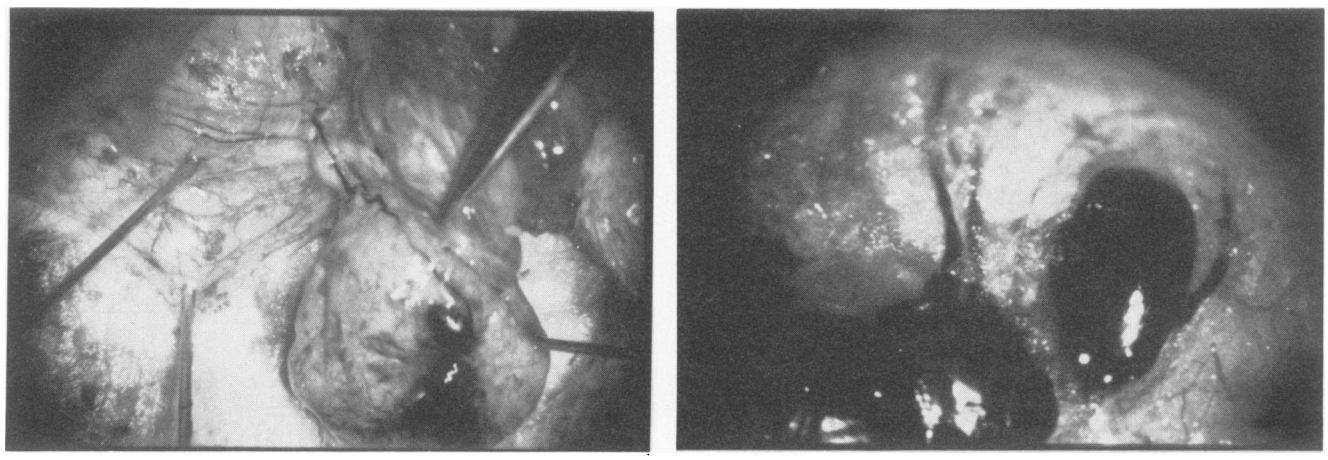

FIG. 5. *Left*, afferent lymphatics draining india ink from the foot making reconnection with transplanted nodes. *Right*, restoration of functional lymphatic anatomy to a group of three revascularized lymph nodes in a site previously occupied by one popliteal node.

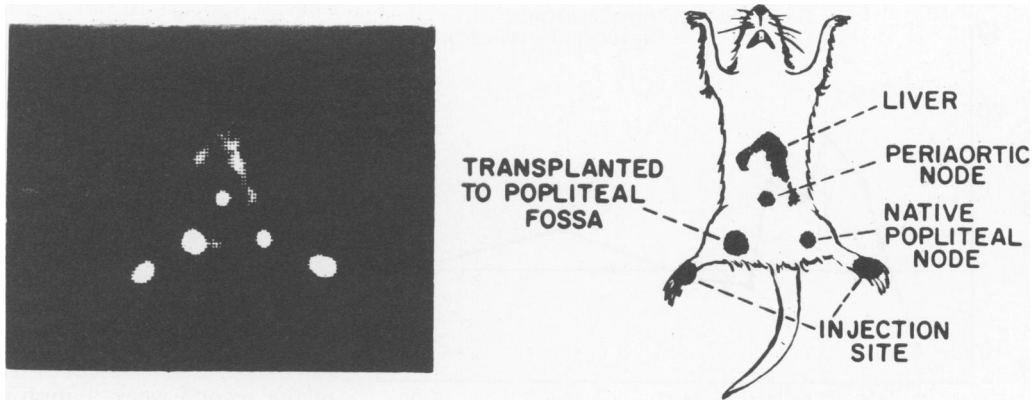

FIG. 6. Lymphoscintography with ^{99m}Tc ASC, 200 μCi injected intradermally and imaged two to three hours later. After three weeks following surgery, presence or absence of uptake at popliteal site correlates exactly with demonstration of afferent lymphatic reconnection at dissection.

ular method afforded significantly greater incidence of afferent reconnection than five other methods (57% *vs.* 17–37%; $p < 0.05$) and was the surgical technique chosen to study immunologic function of transplanted nodes. Figure 5 shows afferent reconnection with transplanted vascularized nodes, visualized by ink injection at the time of dissection. Dissection of unstained transplanted nodes demonstrated lymphatic reconnection within the fat bypassing the nodes completely.

Uptake by lymphoscintigraphy after the third week correlated exactly with demonstrable afferent lymphatic reconnections seen in 44 of the 120 rats. Lymphoscintigraphy proved to be a reproducible means of predicting success of afferent reconnection, and it was employed to noninvasively select functioning transplants for the immunologic study (Fig. 6).

In nodes transplanted by microsurgical anastomosis, the efferent lymphatics in the vascular pedicle were disrupted at the time of vascular division. They were seen to make reconnection with disrupted lymphatics at the site of the anastomosis in the recipient groin (Fig. 7). The technique of intranodal injection for efferent lymphatic studies required sacrifice of the animal in contrast to lymphoscintigraphy (used to identify afferent reconnection), which was noninvasive and reproducible.

Native popliteal nodes from 50 normal intact rats exposed to tumor inoculation demonstrated peak cytotoxicity against the tumor on day 7, with a mean of 17% (Fig. 8). A 95% confidence limit for the mean of relative differences shows exclusion of zero, which reflects significant positive cytotoxicity for that day. From day 7 through day 11, 12 of 14 rats had positive cytotoxic indices, which reflects cytotoxicity over that time interval (Sign Test; $p < 0.01$). Tumors became palpable at one to two weeks and then regressed.

From this it was known to focus on cytotoxicity at the end of the first week following inoculation, when cytotoxicity appeared to peak at the popliteal site. Figure 9 shows that 95% confidence limit for the transplanted nodes excludes zero on days 7 and 8 demonstrating significant positive cytotoxicity on each day.

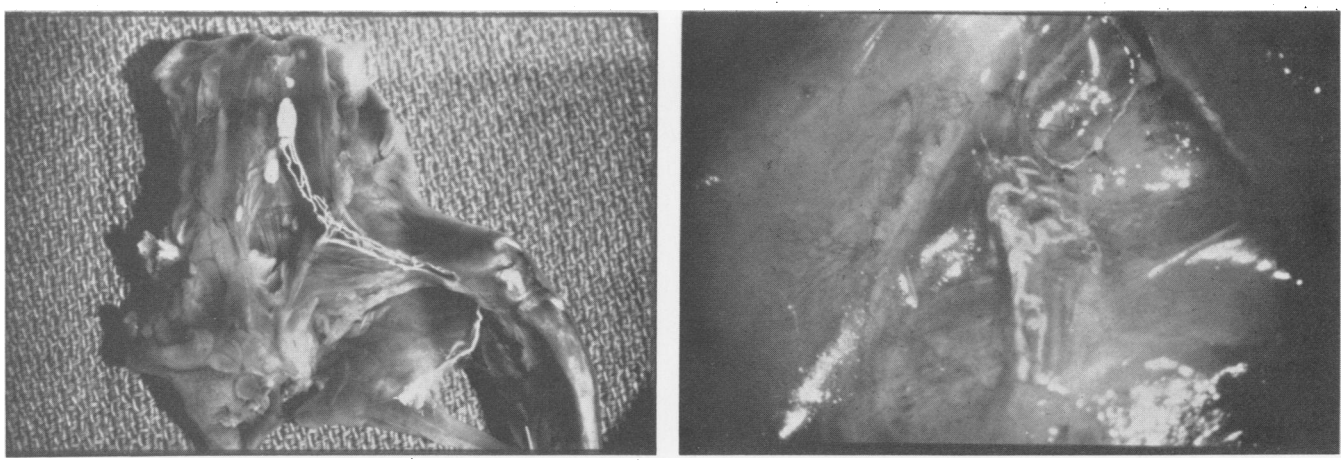

FIG. 7. *Left*, microfil injected directly into transplanted node at popliteal site and showing efferent lymphatics draining up the thigh and abdomen to the periaortic nodes. Specimen is cleared with glycerin. *Right*, efferent lymphatics in the vascular pedicle making reconnection with disrupted lymphatics at the site of the microvascular anastomoses.

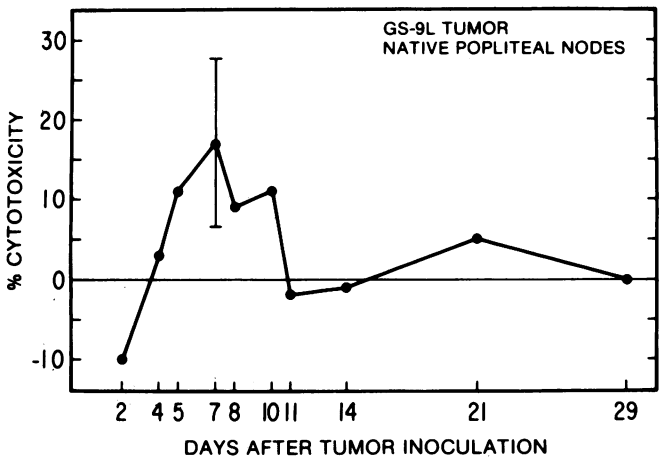

FIG. 8. The cytotoxic response of cells from draining popliteal nodes in intact rats following inoculation of tumor. This study was used to identify the time of maximal cytotoxic response at the popliteal site.

On these days, 14 of 15 rats had positive indices (Sign Test; $p < 0.001$).

Discussion

These experimental results demonstrate that rat inguinal lymph nodes, which are transplanted by vascular pedicle, retain the ability to mount a cytotoxic response against an immunizing tumor as determined by an *in vitro* cellular adherence assay. In this allogeneic system, cytotoxicity is probably elicited by both tumor-specific and differing histocompatability antigens.[1] Lymph node

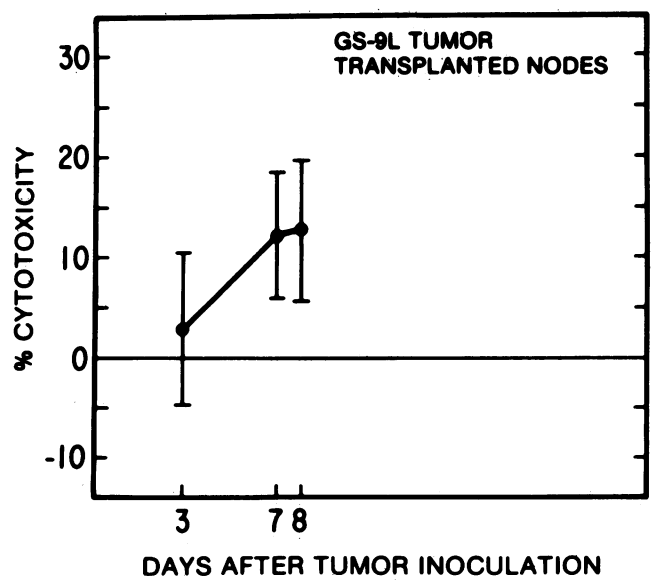

FIG. 9. The cytotoxic response of cells from inguinal nodes transplanted by vascular pedicle to the popliteal site and then sensitized by tumor inoculation.

anergy appears to develop by two weeks, before complete regression of the palpable tumors. Such a decline in cytotoxicity is commonly observed[19] and is often associated with a decline in paracortical T-cell response and a rise in B-cell activity.[14] Although not proven in this system, the cytotoxic cells are assumed to be T-cells, based on the work of other investigators.[4] Similarly, Irvin and Eustace[13] showed a decline in anti-tumor response of lymph node cells studied by adoptive transfer. When harvested within the first week following sensitization lymph node cells caused accelerated rejection of tumor allografts. However, when harvested in the second week, lymph node cells conferred tumor growth enhancement, and this was associated with higher antibody titers.

Cell-mediated cytotoxicity represents an immune response within the lymph node. Therefore, this study provides evidence for the recreation of an intact afferent arc of immune function following lymph node transplantation, similar or identical to normal nodes. In the experimental design, however, the controls are not interchangeable, and one cannot statistically compare the levels of immune response in native nodes with those in transplanted nodes. This study also does not assess the efferent arc of immune function, *i.e.*, the systemic response against growing tumor *in vivo*.

Regional lymph nodes may provide a mechanism for the early sensitization of the host against foreign antigen.[9] Some investigators have suggested that lymphadenectomy may result in greater tumor growth, increased metastases, and less resistance to a subsequent tumor challenge,[5,8,17,18,22] while other workers have found no evidence for this.[2,12,26] Thus it is a controversial issue and reflects a system where multiple interactions are involved at the cellular and humoral level.[21]

Lymph node cytotoxicity against naturally occurring tumors in man is a well-recognized phenomenon,[20] but its clinical relevance is as yet undefined. The prospect of clinically applying lymph node transplantation techniques raises several important questions: Is lymphatic reconnection with transplanted nodes a fortuitous phenomenon or are there trophic factors involved, the study of which may be helpful in the surgical treatment of lymphedema? Should surgeons who perform lymphadenectomy consider replacing the nodes with healthy nodes from another site to afford optimal regional antigen contact and potentially increased regional protection against infection, further tumor progression or additional primaries? Can a patient's immune potential be amplified above normal by surgical manipulation? This rather uncomplicated and reproducible model of lymph node transplantation can be employed for numerous further investigations.

Summary

This study demonstrates that vascularized rat inguinal nodes make spontaneous lymphatic reconnection following transplantation and that these nodes, identified as functional by lymphoscintigraphy are still capable of mounting a cytotoxic response against an immunizing tumor. This indicates the successful recreation of an intact afferent arc of immune function following lymph node transplantation.

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