Variations in Functional Immunocompetence of Individual Tumor-draining Lymph Nodes in Humans

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ABSTRACT

In studying the histology and immunohistology of tumor-draining lymph nodes, we observed that nodes close to tumor showed reduced paracortical activity relative to those further away. To assess whether this reduction was paralleled by alterations in the functional activity of nodes, we examined the immunocompetence of individually oriented tumor-draining lymph nodes. We assessed the unstimulated activity of lymph node lymphocytes and their responses to phytohemagglutinin, interleukin 2, and one-way alloantigenic stimulation (mixed lymphocyte reaction) in vitro. Regional nodes from patients with malignant melanoma or breast cancer were classified as proximal, intermediate, or distal relative to tumor. Node groups with and without tumor metastases in them were studied. Significant variations in \( [\text{H}] \text{thymidine} \) uptake were noted with the unstimulated and stimulated lymphocytes of different lymph nodes from the same individual. Nodes near to tumor were less responsive than those located farther away; some of the latter showed relative immunostimulation. Tumor-draining node groups thus demonstrated a random variation in the strength of reaction of individual nodes. There are zones of low and high lymph node reactivity, related to the position of each node relative to tumor. Tumor-derived immunosuppressive products and/or immunoregulation down-regulates lymph node functional activity, creating conditions that may permit the survival of tumor cells and the establishment of metastases. It is suggested that immunosuppression of nodes nearest to tumor may facilitate the early establishment of metastases.

INTRODUCTION

In most patients with melanoma or breast cancer, RLN\(^3\) are the initial site of metastases, with those nodes closest to the primary tumor usually the first colonized by tumor cells. Establishment of metastases depends upon interactions of host factors, including immunological factors, and tumor cell characteristics. RLN help generate tumor immunity and may represent a barrier to secondary tumor growth (1–5). Despite this, the significance and function of RLN in human malignant disease remain largely unexamined. To study RLN more fully, we considered that each node should be oriented accurately (proximal, intermediate, or distal) to tumor and assessed separately (6).

We previously examined nodal reactivity by histology and immunohistology in individually oriented RLN and found that nodes partly replaced by or near to melanoma showed reduced paracortical activity relative to nodes further from tumor.\(^4\) Our studies indicated that individual RLN were heterogeneous in the extent of observed reaction, and that reactivity varied with the position of the node relative to tumor. This strongly suggested that, in any assessment of their immunocompetence, lymphoid cells from RLN should be examined on a node-by-node basis. Pooling of lymphocytes from different nodes seemed likely to lead to an uninterpretable mixture of stimulated and suppressed lymphoid cells.

In this study, we examined the functional immunocompetence of tumor-involved and tumor-free RLN groups. We assessed whether lymph node lymphocytes from the individual nodes of a patient's RLN would react similarly on \textit{in vitro} testing or whether there would be variations in functional activity between nodes, corresponding with the observed histological differences. We further questioned whether such inter-nodal differences, if they existed, would be random or would have an identifiable pattern and, given a pattern of reaction, whether this would relate to the position of the nodes relative to tumor.

MATERIALS AND METHODS

Lymph Nodes. We studied specimens from 84 patients who underwent lymphadnectomy at UCLA in 1984. Tumor-draining nodes were obtained from 66 axillary and 18 groin dissections performed for clinical Stage II melanoma (12 patients), high-risk (thick, deep) Stage I primary melanoma (37 patients), Stage I breast cancer (20 patients), or Stage II breast cancer (15 patients).

When data from nodes of patients with either breast cancer or melanoma were similar, we stated results for these two tumors together. None of the patients studied received chemotherapy, immunotherapy, or radiotherapy prior to testing. Evidence of local or systemic infection was not present in any patient at the time of surgery.

Tissues were dissected fresh and individual nodes were oriented relative to tumor, or the edge of the specimen nearest to tumor, and to each other node. The specimen was divided into thirds: areas nearest tumor (A nodes), furthest from tumor (C nodes), and a middle zone (B nodes). This approach accurately identified "on-line" lymph nodes in most individuals where the anatomic relation of primary and nodes was simple (e.g., calf primary to groin nodes, or arm primary to axilla). This has been supported by observing patients in whom vital dye was injected in the area of the primary tumor at the time of operation.\(^5\)

A sketch was made of each dissection, and the distance of each node from tumor or the tumor proximate edge of the dissection specimen was noted. When the node group was identified as containing tumor, by histopathology the individual nodes were recategorized as tumor replaced, very near to tumor (0–19 mm from tumorous node), near to tumor (20–50 mm from tumorous node), and remote from tumor (>50 mm from tumorous node).

Lymph nodes were divided into portions for diagnostic histology and for functional studies by bisecting them from hilum to the longest edge. The portions of each node used to prepare cell suspensions were therefore from anatomically comparable portions of the different nodes.

Tissue for functional immunology was placed in tubes with cold sterile RPMI 1640 (Flow Laboratories, Inglewood, CA) containing penicillin, streptomycin, and fungizone (GIBCO, Grand Island, NY). Tissue for histopathology was fixed in neutral buffered formalin, processed conventionally, assessed by the staff of the Division of Surgical Pathology at UCLA, and reviewed by A. J. C.

Cell Separation. Fat was trimmed off the nodes, and single-cell...
cytes were isolated by standard Ficoll-Hypaque density gradient cen-
then in RPMI 1640 containing 20% (v/v) heat-inactivated human AB 
suspensions were made by mechanical passage through a 60 mesh wire 
medium). Lymphocytes were counted, and viability (usually >98%) was 
recess lymphocytes. Excess lymphocytes were resus-

Additional nodes varied from 1 to 4 x 10^7 cells. All assays were carried out 
with freshly isolated lymphocytes. Excess lymphocytes were resus-

Experiments were performed on lymphocytes from the gibbon 
Reagents. PHA (Wellcome Diagnostics, Greenville, NC) was used as a 
non-specific mitogen to stimulate lymphocytes. IL-2 was obtained 
from supernatants of the gibbon lymphosarcoma cell line, MLA 144 
(8), seeded at 2 x 10^6 cells/ml in RPMI 1640 plus 5% human AB heat-
inactivated serum, and harvested after 48-72 h. The MLA-144 cell line is a high producer of IL-2 without exogenous stimulation (8). Super-

When sufficient supernatant had been accumulated, it was thawed, 
cultured with 25 units of IL-2. There were significant differences 
1. Significant differences in the response of different nodes 
from the same individual were seen in 57% of Stage I patients and 
were used for analysis of the mitogen stimulation studies (7). Lymphocytes cultured in medium alone did not proliferate as well as those cultured in the presence of mitogens or IL-2. We therefore did not subtract the counts obtained with medium-cultural lymphocytes from the substantially higher levels ob-
ained from lymphocytes cultured with mitogens or IL-2. We chose not to express the results as ratios, i.e., counts of IL-2-
or mitogen-stimulated lymphocytes over counts of nonstimu-
lated lymphocytes, because of the variability of proliferation of 
unstimulated lymphocytes from one node to another in a node group (7).

Stimulation of lymphocytes by antigens and mitogens has 
been associated with production of IL-2, and the ability of 
lymphocytes to respond to this lymphokine is integral to the 
developing immune reaction (11). We therefore investigated 
the ability of lymphoid cells from individual oriented nodes to 
respond to IL-2. Dose-response studies of lymphocyte re-
sponses to IL-2, at concentrations ranging from 10 to 40 units 
IL-2, were initially carried out. Optimum responses were ob-
tained with 25 units of IL-2. There were significant differences 

Cloning of [3H]dThd was measured by liquid scintillation counting. Data were 
analyzed on the basis of cpm and were converted to logarithms for 
analysis. The coefficient of variation of the assays over the four quad-
ruplicate microwells was less than 15%.

Allogeneic Mixed Lymphocyte Reaction. Lymphocytes were stimu-
lated at various ratios with RAlJ lymphoblastoid cells that had been 
inactivated with mitomycin C (50 µg/ml) for 30 min in a 37°C water 
bath. They were then washed three times in culture medium before 
being seeded in quadruplicate microwells and incubated at 37°C for 
126 h. In the last 18 h, cells were pulsed with [3H]dThd. Harvesting 
and counting were performed as described above.

Data Analysis. Since data from many immunological assays are often 
skewed, with frequent "outliers," nonparametric techniques, not 
requiring a normal distribution of data, were used whenever possible.

When we studied the heterogeneity of reaction between nodes, quad-
ruplicate determinations from each node allowed us to compare be-
tween-node variations with variations within single node replicates from 
each patient. If between-node variation was significantly greater than 
the variation within replicate observations of cells from a single node 
(Kruskal-Wallis test, P < 0.05), the patient's nodes were considered to show 
significant node-to-node heterogeneity for that particular reac-
tion. The Kruskal-Wallis test is a nonparametric alternative to the 
analysis of variance (10).

When we sought "zoned" patterns of reactivity, we obtained an 
average value for each parameter for the A node, the B node, and the 
C node in each patient by taking the geometric mean of four replicate 
values for each node. Statistical significance was tested using a Wil-
coxon signed-rank test on the logarithms of the individual patient ratios 
(10). The 95% confidence intervals determined were based on the 
Wilcoxon signed-rank test. These statistical techniques are nonpara-

| Stimulation | Total Patients | Patients showing significant inter-
node variation | % of patients showing sig-
nificant inter-
odinal variation |
<table>
<thead>
<tr>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Tumor-free (Stage I)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated</td>
<td>47</td>
<td>20</td>
<td>43</td>
</tr>
<tr>
<td>PHA</td>
<td>49</td>
<td>28</td>
<td>57</td>
</tr>
<tr>
<td>IL-2</td>
<td>39</td>
<td>26</td>
<td>67</td>
</tr>
<tr>
<td>MLR</td>
<td>24</td>
<td>13</td>
<td>54</td>
</tr>
<tr>
<td>Tumor present (Stage II')</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unstimulated</td>
<td>25</td>
<td>14</td>
<td>56</td>
</tr>
<tr>
<td>PHA</td>
<td>25</td>
<td>18</td>
<td>72</td>
</tr>
<tr>
<td>IL-2</td>
<td>16</td>
<td>15</td>
<td>79</td>
</tr>
<tr>
<td>MLR</td>
<td>9</td>
<td>6</td>
<td>67</td>
</tr>
</tbody>
</table>

* Lymphocyte stimulation measured by [3H]dThd uptake. "Unstimulated lymph-
cytes" refers to incubation in culture medium alone. Mitogen stimulation was 
with PHA, 0.1 µg/ml. Lymphokine stimulation with IL-2 was at 25 units. The 
MLR stimulation was one way, with mitomycin C-inactivated RAlJ cells as a 
respondersimulator ratio of 1:1.

f The criteria for assessing the significance of internodal variation are described 
in "Materials and Methods." P < 0.05.

f Patients with at least one tumor-involved node on histological examination.

result
in the response of individual nodes to IL-2 in 67% of Stage I patients and 79% of Stage II patients (Table 1).

We used lymphoblastoid RAJI cells in one way allogeneic mixed lymphocyte reactions because of their reputation as strong stimulators in allogeneic interactions. On the basis of our initial studies of RAJI-induced MLR, we used an inactivated RAJI cells: lymphocyte ratio of 1:1. Significant node-to-node variations in response to RAJI cells were observed in 54% of Stage I patients and 67% of Stage II patients. Responses to alloantigen exposure varied considerably, perhaps reflecting the extent of histocompatibility differences between the patient's lymphocytes and the RAJI cells.

Do Variations in Node Reaction Relate to Tumor Proximity? We investigated whether the reactivity of a node varied according to its proximity to tumor (zoned pattern). Nodes were classified into A (proximal), B (middle), and C (distal) groups relative to tumor. Table 2 shows examples of data obtained in experiments using lymphocytes from zoned lymph nodes, and Table 3 summarizes the series of experiments undertaken to study zonal variations in lymphocyte responsiveness.

When nodal lymphocytes from patients with Stage I disease (tumor-negative node groups) were cultured in medium alone, the A nodes proliferated significantly less than the B nodes ($P < 0.005$). The response of the A nodes to stimulation by mitogens, alloantigens, or IL-2 was less than that of the B nodes in most patients (56–71%). The difference between these responses was either significant (IL-2, $P < 0.05$; PHA, $P < 0.05$) or very close to formal significance (MLR, $P < 0.06$). The most striking difference was in the unstimulated nodes.

We also compared the reactivity of the B nodes and C nodes.

**Table 2** Representative experiments showing internodal variations of lymph node lymphocyte reactivity.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tumor</th>
<th>Lymph nodes</th>
<th>Unstimulated (cpm)</th>
<th>PHA (0.1 g/ml)</th>
<th>IL-2 (25 units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>Melanoma</td>
<td>A1</td>
<td>379 ± 64</td>
<td>12,690 ± 1,638</td>
<td>5,299 ± 228</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B1</td>
<td>4,024 ± 615</td>
<td>14,948 ± 662</td>
<td>42,041 ± 2,553</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C1</td>
<td>1,206 ± 262</td>
<td>8,556 ± 407</td>
<td>3,921 ± 563</td>
</tr>
<tr>
<td>42</td>
<td>Melanoma</td>
<td>A1</td>
<td>793 ± 351</td>
<td>11,128 ± 315</td>
<td>2,742 ± 386</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B1</td>
<td>1,941 ± 350</td>
<td>14,578 ± 125</td>
<td>3,095 ± 550</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C1</td>
<td>3,934 ± 86</td>
<td>23,473 ± 734</td>
<td>25,496 ± 2,101</td>
</tr>
<tr>
<td>54</td>
<td>Breast cancer</td>
<td>A1</td>
<td>1,008 ± 357</td>
<td>14,428 ± 1,237</td>
<td>5,881 ± 1,014</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B1</td>
<td>1,070 ± 203</td>
<td>23,345 ± 380</td>
<td>8,969 ± 85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C1</td>
<td>1,117 ± 260</td>
<td>16,123 ± 3,494</td>
<td>1,171 ± 198</td>
</tr>
</tbody>
</table>

* Mean ± SD of mean cpm due to uptake of [3H]Thd. Means derived from four replicate cultures.

**Table 3** Reactivity of lymph nodes at different distances from tumor (zoned responses): Stage I patients without tumor in lymph node group.

| Stimulation | Patients A | A < B Total (%) | P  
|-------------|------------|-----------------|---
| Unstimulated| 25/36      | 69              | 0.005
| PHA         | 24/36      | 67              | 0.05
| IL-2        | 22/31      | 71              | 0.05
| MLR         | 10/18      | 56              | 0.06

* Assay of [3H]Thd uptake. Stimulants were used at the following concentrations: PHA, 0.01 μg/ml; IL-2, 25 units; and MLR, responderstimulator ratio, 1:1. Not all tests were performed on each patient.

**DISCUSSION**

In previous histological studies (6), we demonstrated that reaction strength varied from node to node at different levels of the lymph node group, and that these variations related to tumor proximity. Now we have examined the functional immunocompetence of lymphoid cells from individual nodes using various stimulators of lymphocyte proliferation.

In our initial analysis of internodal heterogeneity, we did not consider the distance of the individual lymph nodes from the primary tumor. Between 43 and 79% of patients (the actual proportion depending upon the stimulant used) showed significant variations in reaction strength from node to node, regardless of whether the node group was invaded by tumor. The reactivity of nodal lymphocytes cultured in medium alone varied from node to node, but this variation was not as great as when the lymphocytes were stimulated. Unless previously stimulated by an immunogen, lymphocytes cultured in medium alone do not normally replicate. The proliferation of cells in medium alone may thus indicate the extent of priming in vitro. For example, in other studies nodal lymphocytes from patients who received BCG preoperatively proliferated more rapidly on culture in medium alone than did those from individuals who had not received BCG. Nodal lymphocytes responded differently to the various types of stimulation, indicating differences in the characteristics of both the mitogenic stimuli used and the lymph node cells that respond to them. In response to PHA, previously activated and immunocompetent lymphocytes are stimulated nonspecifically to proliferate. In the mixed lymphocyte reaction with RAJI cells as stimulators, the cells stimulated to proliferate are those competent to respond to alloantigens, primarily to the HLA-Dr antigenic determinants (16, 17). The lymphocyte response to exogenous IL-2 is a specific reaction, mainly involving lymphocytes previously activated in vivo that express IL-2 receptors. Priming with antigen and IL-2 stimulates T-cell growth (11). We investigated the effect of IL-2 in the belief that T-cells that had been exposed to tumor antigen in vivo have a higher response than the B nodes but this frequency was not significantly above chance. In 67% of the patients, the C nodes responded more strongly than the B nodes to stimulation with PHA ($P < 0.05$). Comparable figures for responses to IL-2 and MLR were 50 and 40%, respectively, neither being significant.

Tissue was studied from 15 Stage II patients in whom tumor had spread to the regional lymph nodes (Table 4). Eight of these individuals had malignant melanoma, and seven had breast cancer. The patterns of reaction of the nodal lymphocytes were similar, and data from the two groups are combined. The nodes lying within 20 mm of tumor were less reactive in most patients (unstimulated, 10 of 12; PHA, 9 of 12; and IL-2, 9 of 11) than nodes further from tumor (20–50 mm). These differences achieved formal statistical significance ($P < 0.05$) in five, seven, and six individuals respectively. The middle-distance nodes (20–50 mm) were in turn more reactive than the nodes located at more than 50 mm from tumor (unstimulated, 6 of 7; PHA, 5 of 7; and IL-2, 3 of 5). These differences were statistically significant in four, five, and three individuals, respectively. The relationship between nodes at less than 20 mm and those at more than 50 mm was more complex, with no pattern clearly predominating, tumor proximate nodes being less reactive than remote nodes in some individuals and more reactive in others.

* D. S. B. Hoon, unpublished data.
would have become responsive to IL-2, although such responsivity could have been reduced by inhibitory factors such as tumor antigens or prostaglandins (18, 19). Our data strongly suggest that lymphocytes from some of the nodes tested had been stimulated in vivo. We are currently investigating the effects of melanoma-associated antigens and prostaglandins on T-cell responses to IL-2.

The results of our in vitro assessments of functional immunocompetence confirm the existence of node-to-node response heterogeneity and of the “zoning pattern” observed in our previous histological and immunohistological studies. In tumor-free node groups, A nodes were generally less responsive than B nodes; this difference was statistically significant or close to significant. The C nodes were more responsive than B nodes; this difference was statistically significant or close to significant. The PHA-stimulated cell proliferation and maximal reactivity of midzone nodes were again observed patterns this group. Each patient essentially must be considered as an individual, with varying absolute amounts of tumor present and different degrees of penetration of the node group. When multiple nodes are involved, their accurate zoning is difficult because of the many interconnections among these nodes. It is also difficult to be certain that nodes from a tumor-positive node group are devoid of occult tumor cells and that there are no tumor cells in the afferent lymphatics. The tumor-positive patients represent an interesting group, and we continue to study them.

Accurate orientation of nodes proved quite difficult, particularly when there were several tumor-containing nodes present, or being impossible to be certain that every node examined received a lymphatic directly from a tumorous node. Orientation is also difficult in node groups from which tumor is absent, as the point at which tumor-draining lymphatics enter the skin, enter the lymphatic tract, and terminate in the lymph node is often difficult to determine. However, we selected nodes for study with careful regard to their anatomical relationship to the primary tumor and its lymphatic drainage. Studies in progress using vital dyes to identify drainage pathways suggest that our approach is valid at least for individuals where the anatomic relationship of primary tumor and nodes is straightforward, e.g., arm/axilla. The frequency with which we observed patterns of a zoned reaction would certainly have been higher if we could have overcome some of these difficulties.

The differential reactivity between nodes within each patient was also observed when cryopreserved lymphocytes from indi-
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