Suppressor Cell Activity in Melanoma-draining Lymph Nodes

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ABSTRACT

We previously demonstrated that the cells of lymph nodes near to a melanoma respond less well to stimulation by mitogens, alloantigens, and interleukin 2 than do nodes further away. In this study we examined suppressor T-cell activity in nodes at different distances from primary melanoma, using a concanavalin A (Con A) suppression cell assay. Tumor-free regional nodes were classified as proximal, intermediate, and distal relative to primary melanoma. Lymph node lymphocytes (LNL) were stimulated with 50 μg/ml Con A for 48-72 h, inactivated, and then mixed with autologous peripheral blood lymphocytes. The peripheral blood lymphocytes-LNL mixtures were stimulated with phytohemagglutinin for 3 days. Proliferation was measured by [3H]thymidine uptake during the final 18 h of culture. In 13 patients, Con A-treated LNL from nodes near to tumor were more suppressive of the peripheral blood lymphocyte response to phytohemagglutinin than those from nodes located further from tumor. T-lymphocyte subset assessment before and after Con A treatment of LNL showed no significant changes in T4:T8 ratios. Con A-induced suppressor cells could be maintained in culture in the presence of recombinant interleukin 2 and retained their suppressive activity. LNL not exposed to Con A and maintained in culture with interleukin 2 did not show suppressor cell activity. Suppressor cell activity thus contributes to the weak immune reactivity of lymph nodes near to melanoma.

INTRODUCTION

Despite evidence that it is immunogenic (1-4), cutaneous malignant melanoma frequently metastasizes (5). The regional lymph nodes are most often the initial site of metastasis, with tumor colonization usually first affecting the on-line nodes nearest to the primary tumor. Little is known of the mechanisms whereby lymph nodes limit the survival and proliferation of tumor cells. Using histology and immunohistology, we have shown significantly reduced paracortical (T-zone) activity in nodes located near to primary or metastatic melanoma (6, 7). This finding of reduced immune reactivity in tumor-proximate nodes has been confirmed by studies of the response of individual tumor-orientated nodes to mitogens, alloantigens, and interleukin 2.

In this study we examined suppressor cell activity in melanoma-draining lymph nodes located at different distances from tumor. Suppressor cells are important modulators of the immune reactions of many diseases including cancer (8-12). Suppressor cells have been demonstrated in melanoma patients (13) and are considered to be prominent in melanoma-draining lymph nodes (14, 15). We used a highly reliable Con A suppressor induction cell assay that has been previously used to analyze suppressor cell activity in many diseases, including cancer (8, 9, 14, 16, 17).

MATERIALS AND METHODS

Lymph Nodes. Tumor-draining lymph nodes were obtained from axillary and groin dissections performed at UCLA during 1984-1985 for high-risk (Clark level, >III, Breslow thickness, >0.65 mm) Stage I primary melanoma. No patient received chemotherapy, immunotherapy, or radiotherapy prior to testing, nor was there evidence of local or systemic infection. Tissues were dissected aseptically within 15 min of removal. The specimen was divided in thirds: areas proximal to tumor (A-nodes), distal from tumor (C-nodes), and an intermediate zone (B-nodes). This approach accurately identified "on-line" lymph nodes where the relative relation of primary and nodes was simple (i.e., calf primary to groin nodes, or arm primary to axilla). This has been supported by observing patients in whom vital dye was injected into the primary tumor area at operation. 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. The distance between nodes in the different zones varied from 20 to 50 mm.

Lymph nodes were divided into portions for histology, to confirm the absence of melanoma, and for functional studies, by bisecting them from hilum to longest edge. The portions of each node used to prepare cell suspensions were therefore from histologically comparable portions of the different nodes. In this study the nodes examined were all identified histologically to be tumor free.

Tissues for the suppressor cell assay were placed in culture tubes containing cold sterile RPMI 1640 (Flow Laboratories, Inglewood, CA) containing antibiotics. Tissues for histopathology were fixed in neutral buffered formalin, processed conventionally, and assessed by the surgical pathology staff at UCLA.

Lymph nodes were processed into single cell suspensions by passage through a 60-mesh wire screen. The cell suspension was washed in RPMI 1640, and lymphocytes were isolated by Ficoll-Hypaque density gradient centrifugation. Lymphocytes from the interface were washed and resuspended in RPMI 1640 containing 20% (v/v) heat-inactivated human AB serum (Irvine Scientific, Santa Ana, CA) plus antibiotics (referred to as culture medium). The viability of the lymphocytes was determined by trypan blue exclusion and was usually >98%. The lymphocyte yield from individual nodes varied from 1-4 x 10⁶ cells. Only freshly isolated LNL were used in the Con A assay.

Reagents. PHA (Wellcome Diagnostics, Greenville, NC) was used at an optimal dose of 0.5 μg/ml. Recombinant IL-2 (Amergen, Thousand Oaks, CA) was used at a concentration of 20 units/10⁶ cells in culture medium. Con A (Calbiochem, San Diego, CA) was used at 50 μg/ml culture medium. Mitomycin C and α-d-mannose were purchased from Sigma Chemical (St. Louis, MO).

Con A-induced Suppressor Cell Assay. Freshly isolated LNL were incubated with or without Con A (50 μg/ml) for 48 h at 2 x 10⁶/ml culture medium. Incubation of lymphocytes with Con A (50 μg/ml) for 48-72 h was found to give maximum suppressor cell activity (17). After 48 h both Con A-incubated and control cells were washed 3 times with α-d-mannose (5 mg/ml) in medium to competitively remove the Con A from the cell surface membrane (17), resuspended in culture medium, and counted. Viability was >90% for Con A-incubated and control lymphocytes.

Autologous PBL were obtained 24 h before surgery, separated on a Ficoll-Hypaque gradient, washed, counted, and cryopreserved in liquid N₂. For cryopreservation, lymphocytes were resuspended in RPMI 1640, 10% dimethyl sulfoxide (Sigma), plus 40% heat-inactivated
agamma human AB serum (Biocell, Carson, CA). In the Con A assay PBL were thawed rapidly, washed twice in culture medium, and counted. Viability was >95%. The PBL were then resuspended at 10^6/ml in culture medium containing 0.5 μg/ml PHA. One hundred μl of PBL were added to each well of a 96-well microtiter plate (Fisher, CA). To each well 100 μl of mitomycin C-inactivated Con A-treated LNL (10^6/ml) or control LNL (10^6/ml) were added. After incubation with mitomycin C (50 μg/ml for 30 min at 37°C) cells were washed three times in culture medium. The cells were incubated at 37°C in a humidified, 7% CO₂ atmosphere incubator for 84 h. In the last 18 h of incubation cells were pulsed with 1 μCi ^3H]thymidine. Cells were harvested with a PHD harvester (Cambridge, MA) and counted in scintillation fluid.

Suppressor cell activity was measured as percentage of suppression of the PBL response to PHA. Data were expressed in cpm before being expressed as percentage of suppression.

\[
\text{% of suppression} = \left(1 - \frac{\text{cpm of Con A lymphocytes + PBL + PHA}}{\text{cpm of control lymphocytes + PBL + PHA}}\right) \times 100
\]

Cell Phenotype Analysis. To identify T-cell subsets we used a colored bead-rosetting assay (Bio-Rad, Richmond, CA) in which monoclonal antibodies specific for T-cell subsets (T4, helper-inducer subset; and T8, cytotoxic-suppressor subset) were coupled to beads.

RESULTS

We first examined Con A-induced suppressor cell activity in pooled TDLN (Fig. 1). Con A-induced suppressor cell activity in the pooled LNL from all patients was examined. The extent of suppressor activity varied from individual to individual (mean, 73.5%, range, 41.4 to 81.9%).

We next examined Con A-inducible suppressor cell activity on a node by node basis. Our previous studies showed that individual melanoma-draining lymph nodes vary in their immune reactivity, the strength of their reactivity varying with the proximity of each node to tumor. Significant node to node variation in Con A-inducible suppressor cell activity was observed (Table 1), although the absolute levels of activity varied from patient to patient. The pattern of reaction of tumor-orientated nodes, however, was constant. In most patients the nodes proximal to tumor (A-nodes) had more suppressor cell activity than those distal to tumor (C-nodes) (Table 2). Immediately located B-nodes were usually suppressive at a level in between the A- and C-nodes. The A-nodes were more suppressive than the B-nodes in 9 of 11 patients and in 10 of 11

![Fig. 1. Con A-induced suppressor cell activity of pooled lymph nodes from individual patients.](image)

**Table 1** Comparing Con A-induced suppressor cell activity of lymphocytes from tumor-free nodes at different distances from melanoma

<table>
<thead>
<tr>
<th>Patient</th>
<th>A-nodes &gt; C-nodes</th>
<th>B-nodes &gt; C-nodes</th>
<th>A-nodes &gt; B-nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9/11</td>
<td>9/11</td>
<td>10/11</td>
</tr>
<tr>
<td>2</td>
<td>9/11</td>
<td>9/11</td>
<td>10/11</td>
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<td>3</td>
<td>9/11</td>
<td>9/11</td>
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<tr>
<td>8</td>
<td>9/11</td>
<td>9/11</td>
<td>10/11</td>
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</table>

**Table 2** Con A-induced suppressor cell activity of melanoma-draining lymph nodes

Representative examples of Con A induced suppressor cell activity of individual TDLNs. In a TDLN group A-nodes are proximal, B-nodes are intermediate, and C-nodes are distal to tumor. Percentage of suppression is determined as described in “Materials and Methods.” The SE for each value is less than 5%. Summation and statistical analysis of data are given in Table 1.

<table>
<thead>
<tr>
<th>Patient</th>
<th>A-nodes &gt; C-nodes</th>
<th>B-nodes &gt; C-nodes</th>
<th>A-nodes &gt; B-nodes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9/11</td>
<td>9/11</td>
<td>10/11</td>
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<td>9/11</td>
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<tr>
<td>8</td>
<td>9/11</td>
<td>9/11</td>
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</tbody>
</table>

**Table 3** Effect of mitomycin C on activity of Con A-induced suppressor cells

Con A-induced suppressor cells were examined for their suppressor activity when inactivated with mitomycin C and not inactivated. Suppressor cells were added to PBL at a 1:1 ratio and assessed for their activity in the presence of PHA.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Nodes</th>
<th>Not inactivated</th>
<th>Mitomycin C inactivated</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>A</td>
<td>16.3</td>
<td>1.3</td>
</tr>
<tr>
<td>B</td>
<td>10.0</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>C</td>
<td>10.2</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>A</td>
<td>10.2</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>B</td>
<td>10.0</td>
<td>0</td>
<td>1.0</td>
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<tr>
<td>C</td>
<td>10.2</td>
<td>0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Patients were more suppressive than the C-nodes (Table 1). The B-node was more suppressive than the C-nodes in 9 of 11 patients. Nodal lymphocytes not exposed to Con A showed weak suppressor cell activity which also varied in strength from node to node.

To assess whether Con A-induced suppressor cell activity required active proliferating cells, Con A-exposed LNL were treated with mitomycin C before being added to PBL. In Table 3 we compare the suppressive activity of Con A-exposed LNL before and after mitomycin C treatment. Mitomycin C treatment reduced the extent to which Con A-exposed LNL from most nodes suppressed the PBL response to PHA. Despite the reduction of activity LNL from tumor-proximate A-nodes re-
Table 4 Comparison of the T-cell subset composition of TDLN before and after Con A treatment

<table>
<thead>
<tr>
<th>T-cell subset</th>
<th>Before Con A incubation</th>
<th>After Con A incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suppressor-cytotoxic T8+</td>
<td>13.8</td>
<td>15.0</td>
</tr>
<tr>
<td>Helper-inducer T4+</td>
<td>33.0</td>
<td>35.1</td>
</tr>
</tbody>
</table>

Fig. 2. Dose response of mitomycin C-treated Con A suppressor cells. Con A-induced suppressor cells were added to autologous PBL at different ratios and stimulated with PHA. Con A-induced suppressor cells were cultured with PBL at 1:1, 1:0.5, and 1:0.25 ratios. Results were expressed as percentage of suppression; SE for each bar is less than 5%. This is representative of five experiments.

DISCUSSION

Individual melanoma-draining lymph nodes show varying immunocompetence, some being immune stimulated, others being immune suppressed. In previous studies of such nodes assessing histology, immunohistology, proliferation responses to mitogens, alloantigens, and IL-2 we demonstrated that differences in activity relate to the position of each node relative to tumor*. In this study we examined suppressor cell activity in groups of nodes and in individual melanoma-orientated nodes.

Analysis of suppressor cell activity in humans is difficult due to variability among individuals, problems of assay reproducibility, lack of autologous antigen-specific effector cells on which suppressor cells can be tested, and the limited number of lymphocytes available from each patient. We used the Con A suppressor cell assay because it is reliable and reproducible (9, 17), requires small numbers of cells, and correlates with in vivo immune suppression (9, 14, 16). Additionally, it is well suited to comparing reactive lymphoid populations derived from different nodes of a single individual.

High levels of Con A-induced suppressor cell activity in pooled melanoma-draining LNL supported our previous observations that immune suppression in such nodes does exist. The overall average Con A suppressor cell activity among the patients was 73%. Examination of LNL from single tumor-oriented nodes indicated substantial node to node variation in suppressor cell activity. Nodes closest to tumor were significantly more suppressive than those located further away. There is thus zoning of the frequency of (Con A-inducible) suppressor and suppressor cells in a pattern that conforms to the zoning of immune competence that we observed previously. That node to node variations in Con A-inducible suppressor cell activity are likely to be biologically relevant is strongly suggested by the direct relationship between suppressor cell numbers and extent of reduction of the PBL responses to PHA.

Suppressor cell activity was found to be strongest with metabolically active LNL and reduced, although not abolished in mitomycin C-treated LNL. Although mitomycin C reduced absolute suppressor cell activity, nodes nearest tumor still displayed the greatest suppressive activity.

Others have suggested that Con A-induced human suppressor cells act via a soluble suppressor factor; however, no such factor has yet been purified from human cells. A soluble immune suppressor-cytotoxic T-lymphocytes (T8+) in all patients' LNL suspensions examined. The T4:T8 ratio after 48 h of exposure to Con A did not significantly differ from that seen prior to exposure (Table 4).

To assess the long term activity of induced suppressor cell activity we cultured Con A-exposed suppressive LNL in the presence of 20 units recombinant IL-2 for 6 days. The cells retained their capacity to suppress the response of PBL to PHA, but usually at a lower level than cells from the same source before culture (Fig. 3). It was difficult to maintain Con A-induced suppressor cells in culture beyond 6 days, but in one patient we were successful in culturing them for 16 days. Control cells for these experiments were LNL stimulated with IL-2 alone. Suppressor cell activity was maintained throughout the culture period. The maintenance of human suppressor cell cultures is technically difficult. In many patients the cells failed to proliferate and after 6 days the suppressor cells were too few to be assayed. Control lymphocytes, not preexposed to Con A, survive poorly beyond 6 days despite being cultured with recombinant IL-2.
suppressor factor has been isolated from Con A-activated murine T-cells (18). The synthesis of a suppressor factor would certainly require metabolically active cells. Inactivated suppressor cells, however, exert an effect by direct cell surface interaction with target cells. This type of contact would suppress the proliferative response of the target cell. Suppressor cell function may not require full metabolic activity (14, 19), and our data support this possibility. Con A-induced suppressor cells may possibly act by adsorbing lymphokines or other factors from the medium during incubation with target cells (14). In our studies this was not a major mechanism, since metabolically inactivated cells were less effective, despite still being capable of passive absorption (14).

We looked for changes in T-cell subsets in LNL exposed to Con A that might correlate with the development of suppressor cells. Significant changes in subset composition were not observed. This confirms the data of Damle and Gupta (19) and indicates that there are suppressor cells and their precursors in both the T4+ and T8+ subsets. Although most suppressor cells seem to express T8, it is known that some are T4 positive (20, 21). Dual marker studies may yet identify lymphocyte subsets that correlate better with suppressor cell activity. Identification of new T-cell subsets in the future may reveal suppressor T-cells that are neither T4+ nor T8+ positive. The level of suppressor activity in individual nodes does not correlate with the absolute number of T-cells, B-cells, or macrophages. In previous studies (7) we found similar numbers of T-cells, B-cells, and macrophages in the majority of the individual TDLN.

Suppressor cells express the TAC receptor and respond to IL-2 (14, 22), and we utilized these findings to maintain them in vitro for several passages. The ability to maintain human T-suppressor cells in culture will greatly assist their detailed analysis. The longest we have been able to maintain such cells in culture is 16 days and we have not yet established long term lines. We are investigating factor(s) or stimuli other than IL-2 that are necessary to maintain suppressor cells in vitro.

The Con A-induced suppressor cells may include nonspecific and antigen-specific suppressor T-cells. This assay may be used to amplify existing tumor-associated antigen-specific suppressor cells in TDLN. There is debate as to whether antigen-specific suppressor cells or antigen-nonspecific suppressor cells are more important in the immunosuppression seen in various diseases. We believe that complete assessment of immunosuppression in disease should include analysis of both types of suppressor cells.

The cause of increased suppressor cell activity in proximal TDLN remains to be determined. Possible factors include melanoma-derived products passing from tumor cells to the proximal nodes, and migration of suppressor cells from the tumor area (peritumoral lymphocytes) to the regional nodes, with possible additional recruitment and/or activation of presuppressor cells in the node and melanoma cells seeding in the nodes and inducing suppression. Suppressor cell activity may also reflect normal immunoregulatory activity toward high T-cell activity induced by antigenic stimulation. The first two possibilities are most likely the major inducers of suppression in nodes near to tumor. Certainly melanoma is a highly metastatic tumor that most often first spreads to the regional lymph nodes (23, 24). We hypothesize that lymph nodes draining the area of a primary melanoma may be made functionally incompetent by tumor products or tumor cell activation of the immunoregulatory network, and because of this are less able to kill tumor cells that enter them. The nodes nearest to a melanoma on the lymphatic drainage pathway are usually the first to be colonized by tumor (25), and are likely to be exposed to the highest concentration of tumor products [e.g., prostaglandins (26) or gangliosides (27)]. Although the proximal nodes are those through which tumor cells first filter, we postulate that nodal immunocompetence determines whether or not tumor cells will survive in them.

The Con A suppressor cell assay approach permitted us to assess suppressor cell activity in TDLN, providing excellent opportunities for phenotypic and functional analysis. This study has confirmed our immunohistological studies (6, 7), that lymph nodes proximal to melanoma have strong suppressor cell activity. Human melanoma can induce specific cytotoxic T-cells (15) and antibodies to tumor-associated antigen (28), which have been shown to have a role in controlling the disease. The presence of suppressor cell activity will reduce antitumor effector mechanisms and would therefore be detrimental to patient survival. We will now investigate the capacity of biological modifiers to reverse this zoned immunosuppression, in the belief that reduced suppressor activity will make it more difficult for melanoma cells to colonize TDLN and establish as metastases in them.

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