Lymphoid Subpopulation Changes in Regional Lymph Nodes in Squamous Head and Neck Cancer

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SUMMARY

Lymph nodes from 10 normal patients and regional lymph nodes (RLN) from 19 patients with squamous cancer of the head and neck were evaluated as to their lymphoid subpopulations. In comparison to normal lymph nodes, RLN from cancer patients demonstrated a marked increase in the proportion of cells with membrane immunoglobulin, the receptor for the third component of complement, and the receptor for the Fc portion of immunoglobulin G. The increased Fc receptor cells were not Fc-bearing thymus-derived lymphocytes, inasmuch as they separated with the non-sheep erythrocyte-lymphocyte rosette-forming population. The overall thymus-derived lymphocyte percentage in RLN was proportionally decreased. A transition from the normal lymph node composition to the altered lymphocyte profile seen in RLN was demonstrated on moving from distal lymph nodes to RLN within the lymphatic drainage of a tumor. Lymph nodes involved with tumor also showed the pattern of bursa equivalent cell population increases.

INTRODUCTION

RLN² constitute an early line defense against infection (3, 21), transplanted tissues (7, 22), and cancers (30). The effect of the RLN on tumor growth is controversial. Fisher and Fisher (10) and Alexander and Hall (1) have presented evidence that these local LN are important in initiation and maintenance of antitumor activity, while other investigators (5, 11, 12, 14) have been unable to demonstrate either in vitro antitumor activity by RLN cells or their importance in in vivo tumor control.

Many advances in relating pathological tissue changes to the nature of disease have followed the introduction of new methodologies for characterizing cellular changes, e.g., histochemical stains and electron microscopy. Histological changes in RLN have been studied for some time (6, 8, 25). Recent developments in immunology permit the recognition of various normal lymphoid subpopulations by surface receptor characteristics. Evaluation of RLN composition by these immunological techniques is indicated to explore the alterations on the lymphoid tissue closest to the neoplastic growth. This paper analyzes the changes in lymphoid subpopulation in LN under the stimulus of proximity to active neoplastic tissue. In comparison to NLN, RLN from cancer patients demonstrated a profound increase in the proportion of cells bearing Mlg, the receptor for the 3rd component of complement (C³), and the receptor for the Fc portion of IgG (Fc). Concomitantly, there was a decrease in the percentage of T-cells in RLN.

MATERIALS AND METHODS

Patients. LN were obtained from 18 patients with squamous cancer of the head and neck who were undergoing LN dissection as part of their surgical treatment. Patients had received no prior radiation, chemotherapy, or immunotherapy except where specifically noted. No patient had evidence of distant metastases. Two patients with squamous cancer of the head and neck who received radiation therapy consisting of 4500 rads over 5 weeks preoperatively supplied irradiated LN. NLN were obtained from patients undergoing cosmetic surgery. The mean age for head and neck patients was 60 ± 9 years; for normals it was 42 ± 12 years.

Lymphocytes. All LN studied were from the cervical or axillary regions. Only LN greater than 0.5 cm in diameter and immediately draining the tumor were used as RLN. RLN that were grossly or microscopically involved with tumor were excluded, except where otherwise noted. DLN were taken from the distant margins of the same surgical specimens. The LN were obtained fresh, placed in a 100- x 20-mm Falcon petri dish with MEM HEPES, and then teased apart using two 18-gauge needles. To remove debris, the resultant suspension was passed through a 12-mL plastic syringe with a 1-mL nylon wool plug. The cells were then washed 3 times in MEM HEPES and brought to a final concentration of 10⁵/ml. PBL were obtained by Ficoll-Hypaque separation as previously reported (29). PBL from 7 patients were obtained at least 1 month after surgery.

Rosette Procedures. T-cells were enumerated by E-rosette formation (18). Sheep RBC (Mission Labs, Rosemead, Calif.) were washed 4 times in 0.15 M PBS, and resuspended as a 1% suspension in fetal calf serum (Reheis Chemical Co., Chicago, Ill.) that had been heat inactivated at 56° for 30 min and absorbed with 10% sheep RBC at both 4° and 37° for 1 hr. Equal volumes of this 1% suspension and lympho-
cytes at 0.5 × 10^7/ml were mixed with 10- x 75-mm Pyrex tube (Scientific Products, Irvine, Calif.) and incubated at 4° for at least 1 hr. The pellet was then resuspended by gently rotating the tube on its long axis, 0.05 ml of crystal violet stain was added, and an aliquot was counted at the hemocytometer chamber. Lymphocytes with 3 or more sheep RBC were counted as rosettes.

Cells with a receptor for the Fc portion of IgG (Fc cells) were enumerated by rosette formation between lymphocytes and trypsinized sheep RBC coated with hyperimmune rabbit anti-sheep RBC antibody (32). Trypsinization prevents the formation of lymphocyte E-rosettes. Twenty ml of 5% suspension of sheep RBC in MEM HEPES were incubated with 100 mg of lyophilized trypsin (Sigma Chemical Co., St. Louis, Mo.) for 30 min at 37° followed by 3 more washes. The antibody-coated trypsinized sheep RBC were then suspended in MEM HEPES to a final concentration of 10 × 10^7/ml. The rosetting and counting procedure was the same as for E-rosettes.

Complement receptor-bearing cells (C′ cells) were enumerated by lymphocyte rosette formation with Zymosan (17). Zymosan particles (Sigma) were swollen in boiling PBS for 30 min, washed twice with PBS, and adjusted to 1 mg zymosan per ml. Fresh human sera, 0.5 ml/mg zymosan, were added, and the mixture was incubated with agitation at 37° for 30 min. The zymosan-complement complex was washed 3 times in PBS, adjusted to a final concentration of 1 mg Zyme-C per ml, aliquoted, and stored at −70°. Upon thawing, it was stored at 4° and used for 3 days. The lymphocyte zymosan rosettes were formed and counted by a procedure identical to that for E-rosettes except that the mixture was not incubated at 37° and was shaken prior to counting.

Immunofluorescence. Lymphocytes with MIg were labeled by direct immunofluorescence (27). After incubation to remove cytotoxic IgG (19), between 0.5 and 0.1 × 10^7 lymphocytes were incubated at 23° with a 1:10 dilution of polyvalent goat anti-human immunoglobulin (Meloy Laboratories, Springfield, Va.) in MEM HEPES with 0.01% azide. The cells were then washed 3 times with cold MEM HEPES plus 0.01% azide, mounted on a glass slide, and examined with epifluorescence on a Leitz Orthoplan microscope. The percentage of fluorescent cells was determined, and the presence or absence of capping was noted. For removal of any aggregated immunoglobulin that would bind to Fc receptors, the antisera were originally ultracentrifuged at 45,000 × g for 90 min, stored at 4°, and resup at 10,000 × g for 30 min just prior to use.

LN T- and B-Cell Separation. A fractionation of LN T- and B-cells was affected by E-rosette separation on Ficoll-Hypaque (13) except that the sheep RBC were pretreated with 2-aminoethylsulfoxonium bromide hydrobromide. This modified technique yields highly purified T- and B-lymphocyte fractions (29).

Miscellaneous. Viability was tested by trypan blue dye exclusion (0.40%). Esterase-positive cells (monocytes) were counted by the α-naphthyl acetate method (31), while contamination with granulocytes was enumerated by Wright's stain of cytocentrifuge-prepared slides. Phagocytic cells were determined by ingestion of 1-μm latex beads (32) (Dow Chemical Corp., Midland, Mich.). Latex beads were incubated with (0.1 to 0.2 × 10^7) cells in MEM HEPES plus 25% fetal calf serum for 60 min at 37° and washed 3 times with PBS; the percentage of cells with intracellular beads was determined after Wright's stain of these cells.

RESULTS

RLN. The majority of cells from LN immediately draining a primary cancer were T-cells (54.3%). However, this percentage was significantly less than seen in NLN, or the patients' or normals' PBL (p < 0.001) (Table 1). Along with this decrease in percentage of T-cells was an increase in cells carrying the C′ receptor, MIg, and Fc receptor (p < 0.01), the latter population having the largest proportional increase. There was no significant difference between the percentage of these 3 populations (Fc, C′, MIg; p > 0.05). These changes were not due to systemic changes in circulating lymphocytes as the patients' PBL subpopulations were unchanged from normal PBL. Since Fc receptors can be expressed on B- or T-cells (2), T-cells were purified and retested for the presence of Fc-positive cells. NLN T-cell fractions showed 5.66 ± 2.65% (S.D.) Fc-positive cells while 5.39 ± 4.85% of RLN T-cells expressed an Fc receptor. Conclusive evidence that the T-cell population with an Fc receptor for IgG was not expanded will rest on simultaneous double marker studies. The MIg-positive cells showed capping of the fluorescent stain at a level equal to that seen in PBL or LN L cells. There was no difference.

<table>
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<tr>
<th>Table 1</th>
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<tr>
<td>Percentage of cells bearing sheep RBC receptor, Fc receptor, C′, and MIg from normal blood, normal lymph nodes, patients' blood, RLN's, and DLN's</td>
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<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Sheep RBC receptor cell</th>
<th>Fc</th>
<th>C′</th>
<th>MIg</th>
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<tbody>
<tr>
<td><strong>Lymphoid subpopulations in LN</strong></td>
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<tr>
<td>RLN</td>
<td>23</td>
<td>54 ± 3.7a</td>
<td>25 ± 6.6</td>
<td>20 ± 4.0</td>
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<tr>
<td>NLN</td>
<td>10</td>
<td>74 ± 5.6</td>
<td>7 ± 3.8</td>
<td>9 ± 3.5</td>
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<tr>
<td>DLN</td>
<td>6</td>
<td>68 ± 8.3</td>
<td>12 ± 7.2</td>
<td>12 ± 5.1</td>
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<td><strong>Lymphoid subpopulations in blood and NLN</strong></td>
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</tr>
<tr>
<td>Normal blood</td>
<td>10</td>
<td>65 ± 6.1</td>
<td>17 ± 5.3</td>
<td>12 ± 4.4</td>
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<tr>
<td>Patient blood</td>
<td>7</td>
<td>64 ± 5.2</td>
<td>19 ± 6.0</td>
<td>11 ± 4.9</td>
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</table>

* Mean ± S.D.
findings cannot be accounted for by erosion of the primary tumor to a body surface with secondary bacterial invasion, since this did not occur in 7 cases. Granulocytes, esterase-positive cells, and phagocytic cells comprised less than 1% of each sample from RLN as they did for NLN. The lack of phagocytic and esterase-positive cells was not due to loss of these populations during LN disruption or filtration, inasmuch as their number was not increased in the prefiltration lymphoid suspension even when all cells were mechanically freed from the Petri dish. Viability was 84.7 ± 3.4%.

To determine whether these changes in LN composition were related to proximity to the primary tumor or could be found diffusely in the LN, LN from different anatomical locations were studied from 2 patients undergoing radical neck dissection for squamous cancer of the head and neck. The cellular composition was determined for an immediately draining node greater than 0.5 cm in diameter (RLN), several small nodes (<0.3 cm) from the same anatomical area, and a distant lymph node (Table 2). In one case, the location of the tumor (laryngeal) allowed for evaluation of a nonregional LN (submandibular) ≥0.5 cm in diameter. In the other case, several nodes with gross metastases were found. The lymphocyte composition of one of these involved nodes was determined. The gross tumor was dissected away, and the node was processed as described under “Materials and Methods.” No tumor cells could be identified in the final lymphoid cell suspension.

A progression from NLN composition in the distant LN to abnormal composition in the RLN was apparent. The small LN from the same area as the RLN exhibited a cellular profile intermediate between the 2 extremes. The large nonregional LN did not differ from normal, whereas the LN grossly involved with tumor showed the lowest percentage of T-cells and highest percentage of Mlg-positive cells (B-cells). DLN from 5 other patients also demonstrated lymphocyte subpopulations close to that in RLN (Table 1).

Radiated RLN contained a more normal proportion of cells with the various markers than did nonradiated RLN. The radiated RLN from 2 patients had an average composition of 61% T, 17% Fc, 15% C', and 12% Mlg cells; while radiated DLN consisted of an average of 63% T, 13% Fc, 15% C', and 10% Mlg cells.

**DISCUSSION**

The RLN has long been known to consist of both T- and B-cell areas (24). The relative proportion of these cells in LN can be estimated only crudely by histological or rosette overlay techniques. Application of cell surface characterization techniques allowed us to define a consistent pattern of alterations in lymphocyte subpopulations in RLN in patients with squamous cancer of the head and neck.

LN immediately draining a localized cancer (RLN) showed a clear distinction in lymphoid subpopulations as compared to NLN. T-cell percentage was decreased, while B-cell percentage (C' and Mlg positive) was increased. This should not be taken to mean that there was a decrease in absolute T-cell number but rather that there was greater proliferation or accumulation of these other cell types (Fc, C', Mlg).

In animals with early implanted tumors, uniform increase in RLN weight has been shown to occur (9). The increase in Fc receptor cells (3.5-fold) in RLN could have been due to an increase in T- or B-cell expression of this marker. When T-cells were purified, however, the number of Fc-positive cells in this population was not increased. Surprisingly, all RLN showed these changes, although differences in reaction patterns as defined histologically are well described in squamous cancer of the head and neck (14) as well as other cancers (12, 13).

The differences between RLN and NLN cells should not be taken as absolute differences, for this study was de-

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**Table 2**

| Lymphoid subpopulations in LN at various distances from a primary cancer: percentage of T-cells, Fc receptor cells, C' receptor cells, and Mlg-bearing cells in various LN |
|---|---|---|---|---|
| **Patient with squamous cancer of the larynx** | **T-cells** | **Fc** | **C'** | **Mlg** |
| RLN (deep cervical) | 62 | 20 | 19 | 25 |
| Small (deep cervical) | 68 | 16 | 13 | 17 |
| Distant (posterior cervical) | 73 | 7 | 10 | 12 |
| Nonregional (submandibular) | 70 | 9 | 11 | 14 |
| **Patient with retromolar squamous cancer** | **T-cells** | **Fc** | **C'** | **Mlg** |
| RLN (submandibular) | 55 | 23 | 24 | 25 |
| Small (submandibular) | 66 | 13 | 15 | 19 |
| Distant (posterior cervical) | 76 | 10 | 12 | 12 |
| Tumor involved (submandibular) | 46 | 21 | 18 | 33 |
signed to define NLN lymphoid subpopulations and to look for evidence for changes from normal in RLN. Therefore, the LN immediately adjacent to tumors were contrasted to NLN. A continuum of LN composition exists as evidenced by our studies on RLN and DLN from these same patients.

RLN will be exposed to tumor-related products prior to vascularization of minute tumors. Thus RLN may initiate and modulate the initial host tumor relationship. The regional areas of expanded B-lymphocyte activity may play a crucial role as localized sites of antibody synthesis. While antibody may arm certain Fc receptor cells to kill tumor cells (20, 26), both NLN and RLN human lymphocytes, in contrast to blood and spleen lymphocytes, are incapable of mediating lysis of antibody-coated target cells (23, 28). On the other hand, antibody complexed with tumor antigen has been shown to be capable of blocking lysis of tumor cells by cytolytically active lymphocytes (4, 15). In RLN high concentrations of antibody and tumor antigen may well be focused upon the RLN lymphocytes responsible for the development of anti-tumor cell-mediated immunity leading to inhibition of the early and effective expression of this activity.

Our data that normal cervical axillary LN were mainly comprised of T-cells confirm the report of Holowiecki et al. (16), who found 70 ± 10% T-cells in normal abdominal LN. The number of Mlg-positive cells in NLN was less than in peripheral blood or in the LN studied by Holowiecki et al. (22 ± 16; p < 0.05). Abdominal LN may be exposed to greater stimulation than can cervical or axillary LN due to antigens entering through the gastrointestinal tract. Also, their material came from patients with peptic ulcer disease or cholelithiasis, and some of these nodes may have been reactive, which would account for the wide range in their sample. Variations in immunofluorescent technique may also be responsible for the difference. Recently, Samarut et al. (28) reported on the composition of 6 normal abdominal lymph nodes. They also noted a T-cell predominance (62 to 75%). B-cell (C' receptor lymphocytes and cells reactive with an anti-B-cell antisemur) percentages were similar to normal peripheral blood. Fc receptor lymphocytes were markedly decreased in the NLN examined (0.6 to 4.3%) in agreement with our findings.

Both NLN and RLN cell suspensions showed a consistent and virtual absence of macrophages as defined by both esterase stain and phagocytic function. Indeed, Zucher with an anti-B-cell antiserum) percentages were similar to 75%). B-cell (C' receptor lymphocytes and cells reactive to mouse lymphocytes. J. Exp. Med., 139:1175–1188, 1974.


28. Samarut, C., Brochier, J., and Revillard, J. P. Distribution of Cells

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