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Lymphocyte Subset Alterations in Nodes Regional to Human Melanoma[1]

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ABSTRACT

The lymphocyte subpopulations in tumor-draining lymph nodes of melanoma patients were determined using two-color flow cytometry. Data were analyzed according to: (*a*) the staging of the melanoma; (*b*) whether or not the nodes contained tumor; and (*c*) their distance from the primary tumor.

Compared with Stage I patients (without metastasis), uninvolved nodes of stage II patients (with nodal metastases) had a significant decrease in helper/inducer ($CD4^+$) T-cells ($P < 0.001$), with a corresponding increase in cytotoxic/suppressor ($CD8^+$) cells ($P < 0.001$) and Leu 19^+ natural killer ($CD56^+$) cells ($P < 0.01$). In some patients the presence of tumor within a node was associated with a large decrease in $CD3^+$ total T-cells, whereas in others tumor involvement had little influence on lymphocyte phenotypes. When analyzed by distance from the primary tumor, nodes closest to tumor in Stage I patients contained a smaller percentage of $CD19^+$ B-cells. In Stage II, tumor-free nodes nearest to tumor showed an increase in $CD19^+$ cells, but statistical significance was not reached. $CD56^+$ natural killer cells increased progressively in nodes near tumor and were more numerous in Stage II uninvolved nodes compared with Stage I nodes.

Alterations in phenotypically defined lymph node lymphocytes occur in nodes regional to melanoma as the disease progresses, as growth of metastases occurs, and in tumor-free nodes nearest to tumor. These alterations may be essential to the establishment and progression of metastases.

INTRODUCTION

We have shown that the individual lymph nodes that comprise the nodal group regional to a human melanoma react to a degree that varies according to whether they are on the lymphatic pathway from tumor and their distance from tumor. Tumor-proximate nodes show reduced paracortical hyperplasia on histological assessment and relatively few paracortical interdigitating cells when examined by immunohistology (1, 2). Cells from nodes near tumor show a reduced response to mitogens and alloantigens (3) and contain more Con A[3]-inducible suppressor cells than nodes further from tumor (4). In most melanoma patients whose tumors metastasize the nodes nearest to the primary tumor are the first site of metastases (5, 6).

A variety of cells mediate immune responses to tumors. The lymphocyte subsets involved reflect the nature of the response and may determine the outcome of the tumor-host interaction. Different lymphocyte subsets can be recognized by distinctive surface-membrane markers, that can be accurately assessed by flow cytometry. Little quantification of LNL subpopulations by flow cytometry has been carried out in human subjects with solid tumors, although differences in nodal lymphocyte subsets have been reported between Stages I and II of breast carcinoma (7).

In this study, the frequencies of phenotypically defined lymph node lymphocytes in the regional nodes of melanoma patients were quantified by two-color flow cytometry. The nodes were analyzed according to the pathological staging of the melanoma, whether or not they contained metastatic tumor and according to their distance from the primary tumor.

MATERIALS AND METHODS

Patients and Lymph Nodes. Twenty-four patients with malignant melanoma whose ages ranged from 35 to 72 yr were studied. The axillary nodes were removed for clinical Stage I melanoma (14 patients, 21 nodes) or for clinical Stage II melanoma (10 patients, 19 normal and 8 metastatic nodes). Each lymph node was oriented according to its distance from tumor. The first tumor-draining node was identified in most cases by injection of an inert blue dye (Patent Blue or isosulfan blue, 1%) into the primary tumor area during operation.[4] This highlights the relevant lymphatic channels and at least the first draining node. The distance (in mm) of each individual node was measured from the blue node, from the highest tumor-containing node, or from the edge of the dissection specimen nearest to the primary, if a blue node was not seen. Half of each node was retained for histology, and the rest was placed in cold sterile medium (RPMI 1640) supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2.5 μg/ml of Fungizone, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (complete medium).

Isolation of Lymphoid Cells. Freshly excised nodes were trimmed of fat and connective tissue, minced, and gently teased through a 60-mesh stainless steel wire screen. Mononuclear cells were isolated by standard Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density gradient separation and washed twice with complete medium. The cells were resuspended in complete medium containing 5% heat-inactivated human AB serum (Flow Laboratories, McLean, VA) at 10^6 cells per ml. In all instances viability exceeded 90% by trypan blue exclusion. Lymphoid cell yields varied from 10^6 to 10^8.

Cell Surface Markers and Monoclonal Antibody Staining. FITC-conjugated monoclonal antibodies to CD2, CD4, CD8, and CD19 and PE-conjugated monoclonal antibodies to CD3, CD11b (CR3), HLA-DR, CD56 (Leu19), and CD25 (Tac) were purchased from Becton Dickinson (Mountain View, CA). The PE-conjugated antibody to CD45R (2H4) was purchased from Coulter Immunology (Coulter Corp., Hialeah, FL). Details of the panel of monoclonal antibodies used for two-color analysis are given in Table 1.

The staining technique was direct immunofluorescence (two-color), in which 10^5 to 10^6 cells in 50 μl of test solution (RPMI 1640 containing 0.5% bovine serum albumin and 0.1% sodium azide) were incubated with 20 μl of FITC-conjugated antibody, followed by 20 μl of PE-conjugated antibody. For anti-CD45R, 5 μl/100 μl of cell suspension were used, according to the manufacturer's instruction. The incubation was at 4°C for 1 h or overnight. Negative controls contained test solution only. The cells were then washed and resuspended in 1.0 ml of the same solution for analysis.

Flow-cytometric Analysis. The stained cells were examined by flow cytometry (Cytofluorograf, Ortho Instruments, Westwood, MA). Lymphocytes were gated upon, using forward and orthogonal light scatter, and the percentage reactive with each of the monoclonal antibodies was measured.

Data Analysis. The lymph nodes were divided: (*a*) according to whether they contained metastatic deposits or were tumor free (assessed by histology, hematoxylin:eosin, and S-100 protein staining): (*b*) by pathological stage of melanoma (I and II); and (*c*) according to their

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[3] The abbreviations used are: Con A, concanavalin A; LNL, lymph node lymphocyte; NK, natural killer; FITC, Fluorescein L-isothiocyanate; PE, phycoerythrin.

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Table 1 *Cell markers examined alone and in combination*

Official name	Familial name	Lymphocyte subset
CD2	Leu 5b	T- and NK cells
CD3	Leu 4	Total T-cells
CD4	Leu 3a	Helper/inducer T-cells
CD8	Leu 2a	Suppressor/cytotoxic T-cells
CD19	Leu 12	B-cells
CD11b	Leu 15	NK and some Leu 2$^+$ cells
CD25	anti-TAC	Interleukin 2 receptor-bearing cells
CD56	Leu 19	NK cells
	HLA-DR	B-cell and activated lymphocytes
CD45R	2H4, Leu 18	T-subsets, NK, and B-cells
CD4 CD45R		Suppressor/inducer T-cell subset
CD4 DR		Activated T-helper/inducer cells
CD4 CD25		Activated T-helper/inducer cells
CD8 CD56		Cytotoxic T-cells subset

Table 2 *Distribution of LNL phenotypes in patients with Stage I and Stage II melanoma*

The percentage of cells positive for each marker is shown. n = number of nodes examined. With the exception of the single observations, markers were assessed on all samples. Uninvolved Stage II nodes had a lower percentage of CD4$^+$ cells ($P < 0.001$) (a) and a higher percentage of CD8$^+$ cells ($P < 0.001$) (b) and CD56$^+$ (Leu 19$^+$) NK cells ($P < 0.01$) (c) compared with Stage I nodes. Tumor-involved Stage II nodes were very similar to uninvolved Stage II nodes, except that CD3$^+$ total T-cells were very variable in number.

Cell marker	Stage I (n = 21)	Stage II (tumor free) (n = 19)	Stage II (tumor positive) (n = 8)
CD2	77.0 ± 2.9[a]	68.6 ± 8.7	81.3[b]
CD3	74.5 ± 0.9	71.6 ± 3.9	47.6 ± 20
CD45R	65.9 ± 4.1	59.2 ± 9.1	45.4[b]
CD4	69.4 ± 1.3 (a)	50.9 ± 2.1 (a)	51.7 ± 7.9 (a)
HLA DR	21.2 ± 2.2	22.0 ± 5.7	27.4 ± 8.5
CD19	10.6 ± 3.8	17.9 ± 2.1	17.6 ± 1.9
CD8	9.8 ± 0.2 (b)	16.7 ± 0.7 (b)	13.9 ± 5
CD11b	5.9 ± 0.1	10.3 ± 3.6	5.1[b]
CD56	1.9 ± 0.4 (c)	7.1 ± 1.6 (c)	6.5 ± 1.7
CD25	1.5 ± 0.4	0.8 ± 0.2	0.3[b]
CD4 CD45R	38.1 ± 2.7	34.7 ± 10.8	14.0[b]
CD4 DR	3.7 ± 0.2	2.3 ± 0.7	2.3 ± 1.1
CD4/CD8	8.5 ± 0.5	3.8 ± 0.2	5.2 ± 1.9

[a] Mean ± SE.
[b] Single observation.

distance from tumor. In the latter situation they were grouped in each stage as being ≤50 mm, 50 to 90 mm, or >90 mm from tumor. The standard error of the mean was determined for each group of nodes. Significance was assessed using Student's t test.

RESULTS

Comparison of LNL Subsets from Patients with Stage I and Stage II Melanoma (Table 2). LNL subsets in 21 Stage I nodes were compared with 19 uninvolved Stage II nodes without regard to their position relative to tumor. Nodes from Stage II patients had a significantly lower percentage of CD4$^+$ cells ($P < 0.001$), but more CD8$^+$ cells ($P < 0.001$) and CD56$^+$ (Leu 19) NK cells ($P < 0.01$). The CD4/CD8 ratio in Stage II nodes (3.8 ± 0.2) was less than half that of Stage I nodes (8.5 ± 0.5).

Comparison of Tumor-involved with Uninvolved Nodes in Stage II Melanoma (Table 2). In Stage II patients, we compared LNL in 8 nodes partly replaced by tumor with 19 nodes free of tumor. The LNL subsets were almost equally present in the two types of nodes, except for CD3$^+$ total T-cells. There were fewer CD3$^+$ cells in tumor-containing nodes (47.6 ± 20%) than in tumor-free nodes from Stage II patients (71.6 ± 3.9%). In tumor-involved nodes, however, the frequency of CD3$^+$ cells was quite variable, some having near normal values, while others were greatly depressed. Preliminary data suggested a decrease in the CD4$^+$ CD45R$^+$ (suppressor/inducer) subset in patients with low CD3$^+$ values. There was no compensatory increase in LNL expressing other markers in this subgroup of patients.

Comparison of Nodes at Different Distances from Tumor in Stage I Melanoma (Table 3). LNLs in 21 nodes from 8 patients with Stage I melanoma were analyzed according to their position relative to tumor. In most cases (92%) the node nearest tumor was identified by the blue dye technique. CD19$^+$ B-cells were significantly decreased in nodes located close to tumor (≤50 mm) relative to both remote (>90 mm) nodes ($P < 0.02$) and intermediate (50 to 90 mm) nodes ($P = 0.05$). No significant differences were observed in the frequency of cells bearing the other subset markers.

Comparison of Nodes at Different Distances from Tumor in Stage II Melanoma (Table 4). In 19 tumor-oriented nodes from 7 Stage II patients, there was a gradual increase in CD56$^+$ NK cells in nodes closer to tumor and a decrease in CD19$^+$ B-cells. These cells were more frequent in Stage II nodes relative to Stage I nodes. Preliminary data on the CD4 CD45R subset suggested that it too is decreased in nodes close to tumor in Stage II melanoma. Significant decreases in CD4$^+$ cells and in the CD4/CD8 ratio, compared with Stage I, were seen in Stage II nodes at all distances from the tumor (see also Table 3).

DISCUSSION

Phenotypic analysis of LNL from melanoma patients by two-color flow cytometry revealed significant alterations in nodal

Table 3 *Phenotypes of LNL from nodes at different distances from tumor in Stage I melanoma*

The percentage of cells positive for each marker is shown. n = number of nodes examined. With the exception of the single observations, markers were assessed on all samples. Significant decreases in the percentage of CD19$^+$ B-cell numbers in nodes ≤50 mm *versus* >90 mm ($P < 0.02$) and in nodes at ≤50 mm *versus* ≤50 to 90 mm from the tumor ($P = 0.05$) are identified (a).

	Distance from tumor		
Cell marker	≤50 mm (n = 7)	50–90 mm (n = 6)	>90 mm (n = 8)
CD3	76.3 ± 8.7[a]	73.9[b]	73.3 ± 7.0
CD45R	63.6 ± 1.5	73.8[b]	60.2 ± 7.0
CD4	68.8 ± 2.2	67.5 ± 5.4	72.0 ± 2.9
HLA DR	18.7 ± 2.0	19.2 ± 3.2	25.6 ± 4.0
CD19	3.9 ± 1.4 (a)	10.9 ± 3.3 (a)	17.1 ± 4.6 (a)
CD8	9.5 ± 1.4	10.3 ± 1.4	9.7 ± 1.4
CD11b	5.8 ± 1.5	6.0 ± 2.7	6.0 ± 2.5
CD56	1.8 ± 0.3	1.3 ± 1.4	2.6 ± 1.1
CD25	2.2 ± 0.8	1.5 ± 0.7	0.9 ± 0.5
CD4 CD45R	33.9 ± 1.8	43.0[b]	37.3 ± 3.4
CD4 DR	4.1 ± 1.1	3.6 ± 1.2	3.5 ± 0.9
CD4/CD8	9.0 ± 2.0	7.5 ± 1.6	9.1 ± 1.8

[a] Mean ± SE.
[b] Single observation.

Table 4 *Phenotypes of LNL from nodes at different distances from tumor in Stage II melanoma*

The percentage of cells positive for each marker is shown. n = number of nodes examined. With the exception of the single observations, markers were assessed on all samples. There is a gradual increase in (CD56) Leu 19$^+$ representation in nodes closer to tumor, although not statistically significant. The level of these cells in all Stage II nodes is higher than in all Stage I nodes, while CD4$^+$ (T-helper/inducer) cells and CD4/CD8 ratio are lower (see Table 3).

	Distance from tumor		
Cell marker	≤50 mm (n = 7)	50–90 mm (n = 6)	>90 mm (n = 8)
CD3	65.6 ± 4.5[a]	79.0[b]	70.2 ± 7.7
CD45R	45.7 ± 6.4	76.6[b]	55.4 ± 9.9
CD4	49.6 ± 4.7	55.0 ± 6.5	48.1 ± 8.7
HLA DR	27.7 ± 5.1	10.5 ± 3.1	27.7 ± 5.6
CD19	22.1 ± 2.4	15.7 ± 3.0	15.9 ± 3.7
CD8	17.5 ± 3.9	15.4 ± 2.5	17.3 ± 4.8
CD11b	17.2 ± 7.6	8.7 ± 4.0	5.1 ± 1.4
CD56	9.8 ± 3.6	7.2 ± 0.2	4.3 ± 1.0
CD25	1.1 ± 0.4	0.7 ± 0.2	0.5 ± 0.03
CD4 CD45R	13.3 ± 2.7	48.2[b]	42.5[b]
CD4 DR	3.6 ± 0.9	1.2 ± 1.6	2.0 ± 0.4
CD4/CD8	3.5 ± 1.0	3.7 ± 0.7	4.3 ± 1.8

[a] Mean ± SE.
[b] Single observation.

lymphocyte subpopulations associated with stage, distance from tumor, and presence of tumor in the nodes.

Previous investigators have revealed lymphocyte subset alterations in the peripheral blood of patients with a wide variety of diseases including rheumatoid arthritis, infectious mononucleosis, myasthenia gravis, mycosis fungoides, graft *versus* host disease, and leprosy (8). Little quantification of LNL subpopulations has been carried out in patients with solid tumors. Using flow cytometry, variations in lymphocyte subsets in regional lymph nodes have been reported at different stages of breast carcinoma (7). Other investigators have utilized the semiquantitative techniques of immunocytochemistry and fluorescence microscopy to identify LNL subsets in patients with cancer of the bladder and larynx (9), head and neck (10), prostate (11), and breast carcinoma (12). From these studies the effect of tumor burden on lymph node lymphocyte subpopulations remains unclear. This is probably because the studies are few, utilize different techniques, and deal with cancers of different types and stages.

A major problem in this type of study is the provision of baseline values. Ethical and practical considerations limit the availability of truly normal nodes. In theory, nodes could be obtainable from tissue removed during reconstructive surgery, but in practice such operations seldom yield nodal tissues. Nodes might also be removed specifically for research purposes during elective procedures, but the ethical considerations of this have always concerned us, and it is unlikely that nodes from the axilla, groin, or cervical area could be obtained in this way. On the basis of our previous studies of carefully oriented tumor-draining lymph nodes we consider that nodes located farthest from the primary tumor in Stage I melanoma, while not absolutely normal, probably represent the nearest to normality that can be attained in a study of this type. Furthermore, the lack of a true "normal" control does not detract from the "comparisons" that are made between nodes at different distances from tumor and between Stage I and Stage II patients.

The absence of phenotyping studies on normal nodal lymphocytes in the literature and the relatively few studies that have been done on cancer patients make comparisons of our results with those of others rather difficult. However, the lack of NK cells, the T-cell preponderance, and the high CD4/CD8 ratio in our "near normal" nodes are predictably different from peripheral blood lymphocytes values (10 to 25% NK cells; 28% $CD8^+$; 45% $CD4^+$) (Becton Dickinson). It is of some interest that studies on metastatic melanoma have shown that the majority of tumor-infiltrating lymphocytes are $CD8^+$ (13) unlike the situation in the nodes.

There were substantial differences between LNL populations from nodes of patients with Stage I and Stage II melanoma. There was a significant increase in $CD8^+$ cytotoxic/suppressor cells and $CD56^+$ (Leu 19^+) cells associated with Stage II, at the expense of $CD4^+$ helper/inducer T-cells. The CD4/CD8 ratio in Stage II was less than half that of Stage I. Reductions in $CD4^+$ cells and elevations of $CD8^+$ cells in Stage II patients are very similar to the findings of Morton *et al.* (7) from a study of early breast cancer and may reflect increased immunosuppression. The findings of fewer $CD4^+$ T-cells and more $CD8^+$ cells in association with a higher stage of disease were reproducible and significant. This is well illustrated by a representative case where these changes were seen in a single individual during disease progression (Fig. 1). This patient had bilateral lymphadenectomies separated by 5 mo. The first operation, a right axillary dissection, was undertaken for a high-risk (Clark level IV, invading the reticular dermis; and Breslow or tumor thickness of 2.15 mm) primary melanoma of the back. A second operation, 5 mo later, removed the nodes of the left axilla, which were clinically suspicious on palpation. Pathological examination showed no tumor in the right axillary nodes (first operation), but melanoma was found in the left axillary nodes (second operation). Comparison of the nodes excised on the second occasion (pathological Stage II disease) with those from the first operation (Stage I) showed increased $CD8^+$ cells and Leu 19^+ NK cells, but decreased $CD4^+$, $CD4^+$ DR^+, and HLA-DR cells. The CD4/CD8 ratio was reduced by half.

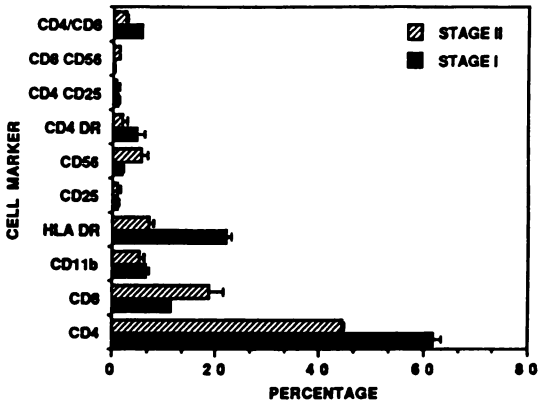

Fig. 1. Phenotypic differences in LNL from a single melanoma patient subjected to two lymphadenectomies on two separate occasions. The first was for Stage I melanoma and the second, for stage II.

The presence of tumor within a node was associated with extreme variability in the number of $CD3^+$ T-cells. Nodes from some patients had essentially a normal number of T-cells, whereas others had very low values. In nodes where T-cells were reduced, most other markers gave values similar to uninvolved Stage II nodes; however, preliminary data suggest that $CD4^+$ $CD45R^+$ (suppressor-inducer) subset of T-cells may be depressed. Loss of this marker has been associated with lymphocyte activation (14–16). Reduced detection of CD3 may represent a marker loss rather than a loss of T-cells as the number of $CD2^+$ cells was unchanged, and there appeared to be no compensatory rise in other populations. Further studies using CD45R and UCHL1 (helper-inducer), which are acquired concomitantly (16), may help define the events occurring in the nodes from this subgroup of patients.

When analyzed by their distance from nearest tumor, alterations in Stage I were quite minor. A decrease in $CD19^+$ B-cells was observed in nodes that lay nearest to tumor, but was not statistically significant. In Stage II, uninvolved nodes closest to tumor had more $CD19^+$ B-cells, although this was not statistically significant. Most significant was the linear increase in $CD56^+$ (Leu 19) NK cells in nodes close to tumor. In such nodes NK cells were more frequent than in Stage I nodes.

The extent to which alterations in lymphocyte phenotype can reflect functional changes is problematical. The profound differences in CD4/CD8 ratios between Stage I and II nodal lymphocytes are likely to mirror decreases in functional competence associated with disease progression. On the other hand, we have previously described defective reactivity in Stage I nodes close to tumor which are not obviously reflected by phenotypic changes. These observations were based on histology and immunohistology (1, 2), on poor responses to mitogens and alloantigens (3, 17), and on the presence of increased numbers of Con A-inducible suppressor cells in Stage I nodes (4). More recently we have demonstrated a lack of antitumor cytostatic activity in tumor-proximal nodes from Stage I and II

patients.[5] The mechanism underlying the lack of reactivity in these proximal nodes is currently unknown, but may involve tumor-derived immune-modulatory molecules such as gangliosides (18, 19). From the results of the present study, it seems that immunosuppression in tumor-oriented nodes may not always be associated with gross changes in lymphocyte populations, although we did find a tendency for increases in NK and B-cells in tumor-proximal nodes. Further investigations using more subtle markers of activation in conjunction with functional studies may be necessary to characterize the cell populations involved.

Our data reveal substantial differences in expression of LNL phenotypes at different stages of melanoma, between metastatic and tumor-free nodes, and some differences between nodes in relation to their distance from tumor. This study suggests that changes in the subsets of immunocompetent cells in regional nodes of melanoma patients may be essential elements in the metastatic process and in the progression of the cancer. Quantitation of lymphocyte subpopulations by sensitive techniques such as flow cytometry may help to identify those immunocompromised cancer patients who are at greatest risk of recurrence, who would be most likely to benefit from adjuvant therapy and for whom treatment-associated morbidity would be most tolerable.

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