We investigated the correlation between the detection of clonal rearrangement of the T-cell antigen receptor gene (TCRR) in lymph node tissue with histopathologic lymph node classification in 33 patients with mycosis fungoides (LN1-2). Dermatopathic nodes with clusters of atypical cells (LN3), and nodes effaced with lymphoma (LN4) and 10 of 11 LN4 nodes. Further, the detection of TCRR extracted from lymph nodes that were histologically unininvolved (LN1-2), dermatopathic nodes with clusters of atypical cells (LN3), and nodes effaced with lymphoma (LN4) and found TCRR in none of five LN1-2 nodes, 8 of 17 LN3 nodes, and 10 of 11 LN4 nodes. Further, the detection of TCRR correlated with presence of palpable adenopathy ($P^2 < .0001$) and was associated with a worse survival ($P^2 = .0024$). Within the subgroup of patients with LN3 nodes, there was a trend ($P^2 = .14$) toward inferior survival if nodes were involved by TCRR, irrespective of extent of skin disease. We conclude that detection of TCRR in nodes from mycosis fungoides patients is an objective and reliable means of assessing tumor infiltration of lymph node and is associated with an inferior survival. This is a US government work. There are no restrictions on its use.

MYCOSIS FUNGOIDES (MF) is an uncommon neoplasm of mature helper T lymphocytes that is initially manifest in skin in the form of plaques, tumors, or erythroderma. While the disease has a high frequency of extracutaneous spread, involvement of lymph nodes, blood, and viscera may not be clinically apparent in early stages of the disease. A previously published multivariate analysis of clinical parameters and histopathologic staging studies and their prognostic implications in MF has shown that histopathologic staging of lymph node and viscera provides prognostic data, complementing the clinical assessment of skin disease and adenopathy. The National Cancer Institute (NCI) staging system for MF takes into account both clinical parameters, such as type and extent of cutaneous disease, and histopathologic involvement of lymph node and viscera.

The NCI lymph node classification system, which categorizes nodes as LN0 to LN4 based on histopathologic assessment of nodal architecture and presence of atypical convoluted cells, has been used to stratify patients into good, intermediate, and poor prognosis groups. Lymph nodes showing preserved nodal architecture and scattered atypical cells (LN1) or small clusters of atypical lymphoid cells with convoluted nuclei (LN2) carry the most favorable prognosis. Nodes containing large clusters of atypical cells with preserved architecture (LN3) confer a less favorable prognosis compared with nodes classified as LN1 or LN2. LN4 nodes, in which nodal architecture is effaced by tumor cells, are associated with the least favorable clinical outcome.

While assessment of clinical parameters such as the type and extent of cutaneous involvement and presence of palpable adenopathy has proven useful in assigning patients to a prognostic category, histopathologic staging of lymph node and viscera identifies those patients with otherwise favorable clinical characteristics who have an inferior survival. There is a consistent association between limited skin involvement, absence of blood or visceral disease, and LN1-2 node histology, as compared with more advanced skin stage and the presence of blood or visceral disease in patients with effaced (LN4) nodes. Patients with LN3 node histology represent a more heterogeneous clinical group with respect to skin disease, adenopathy, and presence of circulating atypical cells.

Because of the heterogeneity of accompanying prognostic features in patients with LN3 nodes by light microscopic histopathology, and in view of the difficulty in consistent light microscopic interpretation among different pathologists, we sought another means of classifying the degree of nodal involvement and assessing prognosis of patients with MF.

Previous studies have shown that clonal populations of malignant lymphocytes may be detected in skin, blood, and lymph node of patients with MF using hybridization with nucleic acid probes for the T-cell antigen receptor genes. Rearrangements of the T-cell receptor (TCRR) or Ig genes have served as molecular markers for the diagnosis of lymphomas and for the presence of malignant cells after therapy in malignancies of T- or B-cell origin.

We sought to obtain an objective assessment of the extent of lymph node involvement by retrospectively analyzing lymph nodes in each histologically defined LN category for rearrangements of the T-cell antigen receptor and correlating these findings with the light microscopic findings. Our results suggest that the presence of detectable TCRR in lymph nodes of patients with mycosis fungoides complements the light microscopic lymph node classification and

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correlates with presence of palpable adenopathy and advanced skin stage. Further, detection of TCRR in lymph nodes is associated with an inferior survival regardless of histopathologic LN class, suggesting that TCRR may be of prognostic as well as diagnostic value in MF.

MATERIALS AND METHODS

 Patients. Lymph node biopsy specimens were obtained from patients with mycosis fungoides evaluated at the NCI-VA and the NCI-Navy Medical Oncology Branches between 1975 and 1990. During this period, 154 patients with mycosis fungoides were accrued, and 143 underwent lymph node biopsy. All biopsies were obtained as part of pretreatment determination of extent of tumor spread specified by clinical protocols approved by the appropriate Institutional Review Boards. Tissue from lymph nodes was obtained at the time of surgical biopsy of palpable nodes or, if no node was palpable, a blind lymph node biopsy from either the inguinal or scalene region. The region for blind biopsy was selected on the basis of drainage from the area of worst skin involvement. Lymph node tissue was frozen for research purposes in 64 patients. Of these 64, 15 were histopathologically classified as LN2, 35 as LN3, and 14 as LN4. The selection of patients for molecular analysis in this study was derived from this group and was based solely on availability of adequate quantities of frozen tissue from which DNA could be extracted. Of the 33 specimens studied, five were classified as LN2, 17 as LN3, and 11 as LN4.

To insure the accuracy and reproducibility of the LN stage used in this study, histopathologic classification of all lymph nodes represented the consensus opinion of three pathologists (A.G., I.L., and J.C.). To avoid sampling error as a factor in the determination of histopathologic staging of the nodes, multiple sections through each node were examined.

Preparation of DNA and Southern blot analysis. Lymph nodes were either processed via teasing and centrifugation on a Ficoll-Hypaque gradient with cryopreservation of the mononuclear cell layer, or frozen at −70°C directly on arrival from the operating suite. High molecular weight DNA was prepared from the above specimens either by ultracentrifugation on a cesium chloride gradient or by the proteinase K/RNase A method as described elsewhere. After determining the concentration of the DNA by its ultraviolet absorbance at 260 nm wavelength, restriction endonuclease digestion was performed using 10 μg of DNA for each of three reactions with the enzymes EcoRI, BamHI, and HindIII. The fragments from each reaction were then separated by electrophoresis in a 0.8% agarose gel and after denaturation and neutralization transferred to a nitrocellulose filter using standard techniques. Hybridizations were performed in 10% dextran sulfate, 40% formamide, 4X SSC, 1X Denhardt’s solution, 20 mmol/L Tris, and 20 μg/mL salmon sperm DNA at 42°C, and filters were washed between 60 and 65°C in 0.1X SSC and 0.1% sodium dodecyl sulfate. Autoradiography was performed using Kodak XAR film (Eastman Kodak, Rochester, NY) for 24 and 168 hours.

Four probes (templates for which were kindly provided by Drs C. Felix and I. Kirsch, NCI, Bethesda, MD) were labeled to greater than 10^6 cpm/μg with dCTP p32 by the Random Primer Method from the following fragments: (1) 400-bp Bgl II/Bgl II fragment from the constant region of the TCRβ2 locus; (2) 2.3-kb HindIII/EcoRI fragment from the joining region of TCR β locus; (3) 220-bp BamHI/BamHI fragment from the constant region of the TCRγ locus; and (4) 2.0-kb RsaI/HindIII fragment from the constant region of the Ig heavy chain locus.

A TCRR was defined in standard fashion as detection of a nongermline band in two digests, or a nongermline band in one digest that was clearly not a polymorphism, a partial digestion, or a plasmid contaminant.

Statistical analysis. Survival duration was calculated from the on-study date until the date of death or last follow-up. The Kaplan-Meier method was used to calculate the probability of survival as a function of time. The Mantel-Haenszel technique was used to identify the significance of the difference between curves, with the reported P values being two-sided.

RESULTS

Correlation of TCRR with lymph node histology. Table 1 shows the staging system for mycosis fungoides currently used at the NCI. According to this system, lymph node classification is based on histopathologic features, including preservation of node architecture, dermatopathic changes in the T-cell zones, and presence of small or large clusters of atypical lymphocytes with convoluted nuclei. Patients with LN1 or LN2 node histology and without visceral involvement are stage I, II, or III, depending on skin stage. Patients with LN3 or LN4 node histology are stage IVA; those with visceral disease are stage IVB.

The results of Southern blot analysis on patient lymph node tissue according to histopathologic classification are presented in Table 2. Clonal TCRRs were detected using a TCR constant region probe on Southern blots containing three restriction digests of genomic DNA for each patient tissue studied. As shown in Table 2, TCRR in early and advanced lymph nodes mirrored the findings on light microscopic histopathology, with none of the five clearly uninvolved (LN1 or LN2) nodes and 10 of 11 (91%) clearly effaced (LN4) nodes showing a TCRR. The LN3 nodes represented a more heterogeneous group, however, with 8 of 17 (47%) showing a TCRR.

Table 1. Staging System for MF

<table>
<thead>
<tr>
<th>Stage</th>
<th>Toney</th>
<th>T2: Generalized plaque, 10% or greater of body surface area</th>
<th>T3: Cutaneous tumor (one or more)</th>
<th>T4: Erythroderma (generalized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad+:</td>
<td>Palpable adenopathy</td>
<td>Ad-: No palpable adenopathy</td>
<td>LN1: Reactive node</td>
<td>LN2: Dermatopathic node, small clusters of convoluted cells</td>
</tr>
<tr>
<td>Ad-:</td>
<td>LN3: Dermatopathic node, large clusters of convoluted cells</td>
<td>LN4: Lymph node effacement</td>
<td>V+: Positive visceral biopsy</td>
<td></td>
</tr>
<tr>
<td>V-:</td>
<td>Negative visceral biopsy</td>
<td>Stages</td>
<td>IA: T1; Ad--; LN1; LN2; V--</td>
<td>IB: T2; Ad--; LN1; LN2; V--</td>
</tr>
<tr>
<td>IIA:</td>
<td>LN1; LN2; V-</td>
<td>IIB: T3; Ad++;LN1; LN2; V--</td>
<td>III: T4; Ad++; LN1; LN2; V-</td>
<td></td>
</tr>
<tr>
<td>IVA:</td>
<td>T1-T4; Ad++; LN3; LN4; V-</td>
<td>IVB: T1-T4; Ad++; LN1-LN4; V+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Clinical Features According to Lymph Node Histopathologic Class

<table>
<thead>
<tr>
<th>LN Class</th>
<th>TCRR</th>
<th>Skin Stage</th>
<th>Blood</th>
<th>Viscera</th>
<th>Adenopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
<td>T4</td>
</tr>
<tr>
<td>LN1/LN2</td>
<td></td>
<td>5</td>
<td>1</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>(N=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN3</td>
<td></td>
<td>8</td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>(N=17)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LN4</td>
<td></td>
<td>10</td>
<td>0</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>(N=11)</td>
<td></td>
<td></td>
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</table>

Fig 1. LN3 node without detectable TCRR. The patient was a 45-year-old man with stage IVA disease (T3, AD–). (A) Low-power photomicrograph showing dermatopathic adenopathy with widened T-cell areas, but with preservation of architecture and normal lymphoid follicles (hematoxylin-eosin stain; original magnification ×23). (B) A cluster of atypical lymphoid cells (open arrow; original magnification ×450) with hyperchromatic and cerebriform nuclei (insert, solid arrow; original magnification ×1,400) characteristic of mycosis fungoides (hematoxylin-eosin stain). (C) Southern blots of BamHI and EcoRI restriction digests of patient (P) and control (C) DNA hybridized to TCRβ constant region probe as outlined.

corresponding histologic sections from representative TCRR(+) and TCRR(–) LN3 patients. As shown, the TCRR(–) and TCRR(+) LN3 nodes were indistinguishable based on histopathologic appearance. Lymph node DNA was also probed for rearrangements at other TCR antigen loci, including the TCRγ and TCRδ loci, as well as the Ig heavy chain locus. No LN with an undetectable TCRβ rearrangement had a detectable δ, γ, or Ig heavy chain gene rearrangement (data not shown).

Clinical features and survival. Table 2 outlines the clinical features of the patients whose nodes were studied for TCRR, stratified by LN stage and by presence of TCRR. The five LN1/LN2 patients all lacked detectable TCRR
and had early stage, plaque-only skin disease without evidence of blood or visceral involvement or palpable adenopathy. The patients with LN4 or effaced nodes had histopathologic evidence of involvement in extracutaneous sites and more extensive disease as assessed by clinical parameters. Eight of 11 had erythroderma, 10 had circulating Sézary cells, six had positive visceral biopsies, and 10 had palpable adenopathy.

Because the TCRR data identified two subgroups within the LN3 patients studied, the clinical features of each subgroup were compared to determine which parameters correlated with detectable TCRR. As outlined in Table 2, none of the 17 LN3 patients studied had evidence of visceral disease as determined by biopsy of the liver or bone marrow. Of eight LN3 TCRR(+) patients, six (75%) had advanced skin disease (T3 or T4), and five had circulating Sézary cells, suggesting an overall greater total body tumor burden in these patients. Of nine LN3 TCRR(−) patients, only three (33%) had extensive skin disease and one had circulating Sézary cells. All of these associations were statistically significant ($P^2 < .05$) by Fisher’s exact test.

Clinical assessment of the entire group of studied patients as stratified by TCRR is presented in Table 3. The detection of TCRR correlates with presence of advanced disease irrespective of the LN subgroup. Of 15 TCRR(−) patients, 11 had plaque stage skin disease (T1 or T2), three...
had skin tumors (T3), and only one had erythroderma (T4). In TCRR(+) patients, 12 of 18 had erythroderma, four had tumor stage disease, and only two had plaques. All patients with positive visceral biopsies were TCRR(-), as were 14 of 16 with circulating Sézary cells.

Of 20 patients with palpable nodes, 17 (85%) had TCRR ($P^2 < .0001$). Only 1 of 13 (9%) of nonpalpable nodes obtained by blind node biopsy showed a TCRR. Among the LN3 patients (Table 2), 7 of 10 with palpable nodes had TCRR ($P^2 = .073$).

Figure 3A shows Kaplan-Meier survival curves for all patients on whom TCRR studies were performed, stratified by histopathologic LN class. As shown, median survival time for patients with LN2 nodes has not been reached. Patients with LN4 nodes had a median survival of 11 months. Patients with LN3 nodes without detectable TCRR showed a median survival of 3 years, probably comparable with that which the LN2 patients might experience, whereas the TCRR(+) LN-3 patients' survival (median, 21 months) was similar to that of the LN4 patients, with no patient surviving beyond 3 years. The observed survival differences between the LN3 patients that are TCRR(+) and those that are TCRR(−) are not statistically significant ($P^2 = .14$), possibly because of the small sample sizes.

Because of the observed similarities in survival between the LN2 and LN3 TCRR(−) patients and the LN4 and LN3 TCRR(+) patients, survival curves were constructed retrospectively comparing patients based on the presence or absence of TCRR (Fig 3B). When patients are considered without regard to histopathologic type, but only with regard to the presence or absence of TCRR, a statistically significant ($P^2 = .0024$) survival difference is shown. Although the numbers are small, these results suggest that the presence of TCRR in MF lymph node tissue may be associated with an inferior survival.

### DISCUSSION

Our results show that detection of TCRR in lymph nodes of patients with MF correlates with histopathologic assessment of lymph node infiltration with atypical lymphocytes in both early and late stage nodes. In patients whose lymph nodes show clusters of atypical cells without effacement by light microscopy, the presence of TCRR provides an objective measure of extent of node involvement. Retrospective analysis of patient outcome based on the occurrence of TCRR shows that TCRR is an important prognostic determinant, especially in patients with noneffaced nodes.

Rearrangements of the genes encoding the TCR have been shown to be markers of clonal T-cell proliferation in T-cell neoplasms and in nonmalignant cutaneous T-cell disorders such as lymphomatoid papulosis. Detectable TCRRs have been reported in dermatopathic lymph nodes and in plaque stage skin lesions in mycosis fungoides patients, as well as in the peripheral blood of Sézary patients and in histologically effaced lymph nodes. Weiss et al examined nine dermatopathic nodes and detected TCRR in six, but clinical outcome was not examined. Ralfkiaer et al reported that TCRRs were detected in nine nodes from advanced stage patients with Sézary Syndrome or tumor stage disease, while no rearrangements were found in nodes from 12 patients with plaque stage disease.

In the current study, we have extended these observations by correlating the histopathologic appearance of nodes, the presence of TCRR, and prognosis. We show an association between histopathologic classification of lymph node tissue and TCRR.
nodes and the ability to detect clonal markers of malignancy using standard Southern blotting techniques. We have shown that a TCRR is not detectable at the level of sensitivity of Southern blotting techniques in a limited sampling of histopathologically uninvolved nodes classified as LN1 or LN2, is almost uniformly found in clearly effaced (LN4) nodes, and is variably present in nodes showing minimal infiltration and dermatopathic features (LN3). While application of the newer and more sensitive technology of polymerase chain amplification may yield detectable clonal TCRR even in histopathologically uninvolved tissues, Southern blotting has been shown to be a reproducible technique capable of detecting a clonal subpopulation comprising as little as 10% of the total cell population studied.

Our results validate the concept that detection of a clonal TCRR among nodes with varying degrees of histopathologic involvement is a measure of degree of tumor infiltration with MF. Most of the patients with limited skin involvement (11 of 13 T1 or T2 patients) had undetectable TCRR, consistent with the findings of Ralfkiaer et al. Likewise, 16 of 20 patients with advanced skin disease (T3 or T4), 14 of 16 with blood involvement, 17 of 20 with palpable nodes, and all six with visceral involvement had TCRR.

Our molecular analysis of LN3 nodes identified two groups of patients with histopathologically identical disease but with clear differences in detectable clonal TCRR. The LN3 histologic grouping represents nodes that are not effaced and, therefore, by conventional histologic criteria would not be considered to be involved by lymphoma. However, these nodes have imprecisely defined “large clusters of cytologically atypical cells” usually occurring in a background of dermatopathic changes. The LN3 classification has been associated with an inferior prognosis compared with the LN1-2. Given the difficult task of morphologically distinguishing between clusters of activated lymphocytes and malignant cells in noneffaced, dermatopathic nodes, TCRR data may prove to be a more objective means by which to predict clinical outcome.

The survival differences observed between TCRR(−) and TCRR(+) patients suggest that the presence of a TCRR, along with or perhaps irrespective of histopathologic lymph node classification, may be a predictor of adverse outcome. Survival curves for the LN2, LN3, and LN4 groups studied here parallel those reported in a larger series. The survival for the LN3 TCRR(+) patients paralleled that of the LN4 patients, whereas that of the LN3 TCRR(−) patients was similar to that of the LN2 patients, and overall the TCRR(−) patients clearly had a more favorable outcome than their TCRR(+) counterparts.

Because of the strong correlation between TCRR and palpable adenopathy in this study, TCRR is not an independent prognostic indicator. Sausville et al reported in a multivariate analysis of prognostic features in MF that adenopathy was of marginal significance when skin stage and visceral involvement were taken into account. Further, 25% of good prognosis patients with LN2 nodes in that study had adenopathy, suggesting that adenopathy was not a good predictor of extent of histopathologic lymph node involvement. In our study, only 10 of the 17 LN3 patients had adenopathy, one patient with adenopathy had TCRR, and three with adenopathy had no TCRR.

These results suggest that TCRR may be of use in an investigative setting as an adjunct to conventional histopathologic and clinical staging criteria in identifying MF patients with early skin stage and noneffaced (LN2 or LN3) nodes who may benefit from aggressive, potentially curative therapies such as total skin electron beam irradiation.

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Prognostic implications of evaluation for lymph node involvement by T-cell antigen receptor gene rearrangement in mycosis fungoides

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