Granzyme B-Expressing Peripheral T-Cell Lymphomas: Neoplastic Equivalents of Activated Cytotoxic T Cells With Preference for Mucosa-Associated Lymphoid Tissue Localization

By Peter C. de Bruin, J. Alain Kummer, Paul van der Valk, Peter van Heerde, Philip M. Kluin, Rein Willeminze, Gert J. Ossenkoppele, Thaddäus Radaszkiewicz, and Chris J.L.M. Meijer

T-cell non-Hodgkin’s lymphomas can be considered the neoplastic equivalents of immunologically functional, site-restricted T lymphocytes. Little is known about the occurrence and clinical behavior of T-cell lymphomas that are the neoplastic equivalents of different functional T-cell subsets. Here, we investigated the prevalence, preferential site, immunophenotype, and clinical behavior of the neoplastic equivalents of activated cytotoxic T cells (CTLs) in a group of 140 nodal and extranodal T-cell lymphomas. Activated CTLs were shown immunohistochemically with a monoclonal antibody against granzyme B, a major constituent of the cytotoxic granules of activated T cells. Granzyme B-positive T-cell lymphomas were mainly found in mucosa-associated lymphoid tissue (MALT; nose, 63% of the cases; gastrointestinal tract, 46%; and lung, 33%). Granzyme B-positive cases with primary localization in MALT were more often associated with angiogenesis (P = .005), necrosis (P = .002), and histologic characteristics of celiac disease in adjacent mucosa not involved with lymphoma. eosinophilia was more often observed in granzyme B-negative cases (P = .03). Most cases belonged to the pleomorphic medium- and large-cell group of the Kiel classification. CD30 expression was more often found in granzyme B-positive lymphomas of MALT (P = .04), whereas CD56 expression was exclusively found in nasal granzyme B-positive lymphomas. Immunophenotypically, most of the cases should be considered as neoplastic equivalents of activated CTLs based on the presence of T-cell markers on tumor cells. In two cases of nasal lymphoma, tumor cells probably were the neoplastic counterparts of natural killer cells. The prognosis of the granzyme B-positive gastrointestinal T-cell lymphomas was poor but did not differ from granzyme B-negative gastrointestinal T-cell lymphomas. This indicates that, in peripheral T-cell lymphomas, site of origin is more important as a prognostic parameter than derivation of activated CTLs. © 1994 by The American Society of Hematology.

MATERIALS AND METHODS

Patient selection. Paraffin wax-embedded specimens and, when available, frozen tissue specimens of T-cell lymphoma cases were selected from the files of the Comprehensive Cancer Center Amster-

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Granzyme B expression and pathologic findings. Granzyme B-positive neoplastic cells showed a diffuse granular reaction with a monomer of 33-kD protein. One of these MoAbs against granzyme B (GB9) gives a strong staining result on formalin-fixed, paraffin wax-embedded tissue and was subsequently used in this study. All nonlymphoid cells were completely negative for GB9 on tissue sections with the exception of polymorphonuclear (PMN) leukocytes. However, on Western blot, granzyme proteins were not detected in cell lysates of PMN leukocytes, indicating that staining of PMN leukocytes in tissue sections with GB9 is caused by cross-reactivity with PMN leukocyte-associated serine proteases. Immunostaining with GB9 was performed after antigen retrieval of tissue sections in microwave oven (2 × 5 minutes at 100°C; maximum power, 700 W) with citrate buffer (0.1 mol/L, pH 6.0), for 1 hour at room temperature. After blocking endogenous peroxidases, immunoperoxidase staining was performed using a biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA) and the streptavidin-biotin horseradish peroxidase complex (ABC; Dakopatts, Glostrup, Denmark) as second and third step. The peroxidase reaction was visualized using 3,3′ diaminobenzidine-tetrahydrochloride/H2O2 (Sigma, St Louis, MO).

According to the number of tumor cells positive for granzyme B, cases were divided into four categories: (1) 0% to 10%; (2) 11% to 20%; (3) 21% to 50%; and (4) more than 50% staining of the tumor cell population by granzyme B. Only the latter were considered to be granzyme B-positive lymphomas to avoid any possible misinterpretation of admixed reactive cells as tumor cells. As a control, sections were incubated with an irrelevant primary antibody (ATIII21) of the appropriate subclass (IgG1). Immunohistologic analysis for further characterization of granzyme B-positive cases and granzyme B-negative cases involved the use of a panel of MoAbs and polyclonal antibodies on frozen (n = 12) and paraffin-embedded tissue sections. They included on frozen sections leu-41 (CD16, and CDS6) followed by the alkaline phosphatase anti alkaline phosphatase (APAAP) method (APAAP, Dakopatts). Subsequently, these slides were incubated with biotinylated mouse antibody (Vector Laboratories, Burlingame, CA) and the avidin-biotin peroxidase complex (ABC, Dako, Glostrup, Denmark). The immunohistochemical stainings were performed with standard techniques using an avidin-biotin horseradish peroxidase complex method, with or without modifications as described for GB9, or using an indirect immunoperoxidase method. The enzyme histochemical staining for naphthol AS-D-chloroacetate esterase (Leder) was performed according to standard laboratory techniques.

To identify the phenotype of granzyme B-expressing cells in non-neoplastic lymphoid tissue, sequential frozen tissue sections were used for double stainings. Briefly, slides were incubated with primary MoAbs directed against different CD markers (CD3, CD4, CD8, CD16, and CD56) followed by the alkaline phosphatase anti alkaline phosphatase (APAAP) method (APAAP, Dakopatts). Bound alkaline phosphatase was then visualized by addition of naphthol AS-MX phosphate (Sigma) and Fast Blue BB in 0.2 mol/L Tris-HCL, pH 8.5. Endogenous alkaline phosphatase activity present in the tissue sections was blocked by addition of 1 mmol/L levamisole to the reaction mixture. Subsequently, these slides were incubated with biotinylated MoAbs against granzymes. Binding of these antibodies was detected using the streptavidin-biotin peroxidase method. Endogenous peroxidase activity was blocked by 0.1% sodium azide, 0.3% H2O2. Peroxidase activity was visualized using amino-ethyl-carbazole (Sigma).
or dot-like perinuclear staining. Overall, 29 of 140 (21%) T-cell lymphomas were found to be granzyme B-positive (ie, showed more than 50% staining of the tumor cell population; see Table 1). In an additional 2 primary gastrointestinal cases, 11% to 20% of the tumor cells were GB9-positive. No cases were found with 0% to 10% or 21% to 50% granzyme B-positive tumor cells. A total of 27 of 77 (38%) primary extranodal T-cell lymphomas and 2 of 63 (3%) primary nodal T-cell lymphomas were granzyme B-positive in more than 50% of the tumor cells. The extranodal granzyme B-positive cases were predominantly found in the nose and nasal cavities (5 of 8; 63%), gastrointestinal tract (20 of 46; 43%; see Fig 1), and lung (1 of 3; 33%; see Fig 2). The remaining granzyme B-positive extranodal case was localized in the skin (1 of 20; 5%). Mitotic cells were often GB9-positive in granzyme B-positive cases (Fig 3). In 1 gastrointestinal lymphoma case, interpretation was difficult because CD8-positive tumor cells were surrounded by numerous granzyme B-positive small-to-intermediate sized, CD8-positive, possibly reactive, cells.

The relationship between lymphoma subtype and granzyme B expression is depicted in Table 1. Most cases belonged to the pleomorphic medium- and large-cell subtype category. Pleomorphic small-cell, immunoblastic and, less frequently, large-cell anaplastic granzyme B-positive T-cell lymphomas were also observed.

Necrosis and angioinvasion were more prominent in the group of granzyme B-positive T-cell lymphomas, whereas eosinophilia was slightly more prominent in granzyme B-negative cases. Epitheliolitroptism in the overlying mucosa of neoplastic T cells did not differ between the granzyme B-positive or granzyme B-negative groups (Table 2). According to the criteria described by Chott and associates,39 the primary gastrointestinal lymphomas were separated in enteropathy-associated T-cell lymphomas (EATCLs; n = 13), EATCL-like lymphoma without enteropathy (EATCL-LLWE; n = 7) and T-cell lymphoma without enteropathy and without features of EATCL (non-EATCL; n = 14). Granzyme B-positive neoplastic cells were mainly associated with EATCL. A total of 77% (n = 10) of the EATCL cases versus 14% (n = 2) of the non-EATCL cases were granzyme B-positive. In 57% (n = 4) of EATCL-LLWE cases, granzyme B expression was detected.

In the granzyme B-positive cases of which Giemsa-stained imprint preparations were available (n = 4), numerous azurophilic granules were found in the cytoplasm of the neoplastic

<table>
<thead>
<tr>
<th>Table 2. Histopathologic Findings in Gastrointestinal and Nasal T-Cell Lymphomas in Relation to Granzyme B Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granzyme B positive (%)</td>
</tr>
<tr>
<td>-------------------------</td>
</tr>
<tr>
<td>Necrosis</td>
</tr>
<tr>
<td>Epitheliolitroptism</td>
</tr>
<tr>
<td>Angioinvasion</td>
</tr>
<tr>
<td>Eosinophilia</td>
</tr>
</tbody>
</table>

Abbreviation: NS, not significant.

* x² test.
cells. In a primary nasal granzyme B-positive case, the characteristic secondary lysosomes could be found using electron microscopy (Fig 4).

**Immunophenotypic analysis.** The phenotypes of T-cell lymphomas originating in gastrointestinal tract, nose, and lung are listed in Table 3. CD30 expression was found significantly more often in granzyme B-positive cases than in granzyme B negative cases. Moreover, there was a trend for the CD4⁺/CD8⁻ phenotype to be present in granzyme B-positive cases. Other markers demonstrable on paraffin wax-embedded tissue were not associated with granzyme B expression. From the markers that can only be used on frozen material (ie, TCRβ1, HML-1, and CD56), an association was found between CD56 expression and granzyme B-positive cases. Other markers demonstrable on paraffin wax-embedded tissue were not associated with granzyme B expression.

**Clinical features.** Patients were treated in different institutes in different countries, and therapy was, therefore, heterogeneous. The male:female ratio of patients with granzyme B-positive tumors was 1.25. The patients’ ages ranged from 6 to 82 years (median, 54 years).

Patients with primary nasal T-cell lymphoma presented with localized disease (stage IE according to Ann Arbor) in all but 1 case (stage IV according to Ann Arbor with cervical lymph node and liver involvement). None were leukemic. Of 5 patients with granzyme B-positive nasal tumors, 4 died of disease between 1 and 27 months (median, 4 months) after initial presentation; 1 patient was alive with disease with ongoing chemotherapy. Of 3 patients with granzyme B-negative nasal T-cell lymphomas, 1 died of disease after 4 months (stage IV disease), 1 died of pneumonia without evidence of disease after 14 months, and 1 is still alive after 53 months with disease in CR.

Patients with primary gastrointestinal T-cell lymphomas most often presented with tumors of the small intestine. Most

**Table 3. Phenotypes of T-Cell Lymphomas of MALT**

<table>
<thead>
<tr>
<th>T-cell Lymphomas of MALT</th>
<th>GI Tract T-NHL</th>
<th>Nasal T-NHL</th>
<th>Lung T-NHL</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>GrB⁺</td>
<td>19/20</td>
<td>2/4</td>
<td>0/1</td>
<td>21/25</td>
</tr>
<tr>
<td>GrB⁻</td>
<td>22/26</td>
<td>2/3</td>
<td>0/2</td>
<td>25/31</td>
</tr>
<tr>
<td>GrB⁺</td>
<td>5/20</td>
<td>0/1</td>
<td>0/1</td>
<td>6/26</td>
</tr>
<tr>
<td>GrB⁻</td>
<td>12/26</td>
<td>2/3</td>
<td>0/2</td>
<td>14/31</td>
</tr>
<tr>
<td>GrB⁺</td>
<td>5/20</td>
<td>0/1</td>
<td>0/1</td>
<td>6/26</td>
</tr>
<tr>
<td>GrB⁻</td>
<td>8/26</td>
<td>2/3</td>
<td>0/2</td>
<td>8/31</td>
</tr>
<tr>
<td>CD3⁺/CD8⁻</td>
<td>10/20</td>
<td>3/5</td>
<td>1/1</td>
<td>14/26</td>
</tr>
<tr>
<td>CD3⁺/CD8⁻</td>
<td>10/20</td>
<td>0/4</td>
<td>1/1</td>
<td>14/26</td>
</tr>
<tr>
<td>CD3⁺</td>
<td>1/3</td>
<td>0/1</td>
<td>0/1</td>
<td>1/3</td>
</tr>
<tr>
<td>CD3⁻</td>
<td>15/20</td>
<td>4/5</td>
<td>1/2</td>
<td>19/26</td>
</tr>
<tr>
<td>CD3⁻</td>
<td>14/19</td>
<td>3/4</td>
<td>1/1</td>
<td>18/24</td>
</tr>
<tr>
<td>TCRβ1⁺</td>
<td>3/3</td>
<td>0/1</td>
<td>0/1</td>
<td>3/3</td>
</tr>
<tr>
<td>HML-1⁺</td>
<td>2/3</td>
<td>0/2</td>
<td>0/1</td>
<td>2/3</td>
</tr>
<tr>
<td>CD56⁻</td>
<td>0/2</td>
<td>0/2</td>
<td>0/1</td>
<td>0/2</td>
</tr>
</tbody>
</table>

**Abbreviations:** GI, gastrointestinal; NS, not significant; GrB, granzyme B; T-NHL, T-cell non-Hodgkin's lymphoma; ND, not done.

*χ² test.
patients presented with stage I or II disease (n = 31). When more generalized disease was present (n = 8), spread was often apart from lymph nodes to other mucosal sites (lung, stomach, and palate). No apparent differences were noted with regard to clinical presentation between granzyme B-positive or granzyme B-negative cases. Median survival was 5 and 3.5 months for granzyme B-positive and granzyme B-negative tumors, respectively, (P = .5). No significant differences in survival were noted between patients with CD4-positive and CD8-positive or CD4-negative and CD8-negative gastrointestinal T-cell lymphomas (P > .1). This also accounted for CD30-positive gastrointestinal T-cell lymphomas when compared with CD30-negative gastrointestinal T-cell lymphomas (P = .15).

Both patients with nodal granzyme B-positive lymphomas were young (6 and 17 years), with stage I and III disease and with disease in CR of long duration (25 and 70 months, respectively) after aggressive chemotherapy. The remaining granzyme B-positive primary cutaneous lymphomas had a favorable course (alive with disease in CR after 104 months).

**DISCUSSION**

We have shown that a substantial percentage (21%) of our T-cell non-Hodgkin’s lymphomas are granzyme B-positive, indicating that they probably are the neoplastic equivalents of activated CTLs. Primary extranodal peripheral T-cell lymphomas that made up a significant proportion of our study group almost exclusively accounted for this substantial percentage of granzyme B-positive T-cell lymphomas that included 10 of 13 EATCL cases. Of the 29 granzyme B-positive cases, 26 were localized in mucosal sites, ie, nose, gastrointestinal tract, and lung. Only few were localized in lymph nodes (n = 2) or skin (n = 1). Therefore, these lymphomas have a preference for localization in MALT.

Histologically granzyme B-positive cases showed significantly more angiogenesis and necrosis and less infiltration with eosinophils than granzyme B-negative lymphomas. Most granzyme B-positive cases belonged to the pleomorphic subtype categories of the updated Kiel classification. Moreover, granzyme B-positive lymphomas of the gut were associated with histologic characteristics of celiac disease, ie, villous atrophy and intraepithelial lymphocytosis in the adjacent normal mucosa. The association between clinical and/or histologic evidence of celiac disease and the occurrence of primary gastrointestinal T-cell lymphoma has been well-established.31–33 Interestingly, most cases of T-cell lymphoma with eosinophilia of the gastrointestinal tract have not been associated with celiac disease.34

Activation of resting CTLs induces morphologic changes with the occurrence of azurophilic granules.35 In granzyme B-positive cases of which Giemsa stained cytologic slides were available, azurophilic granules were found. Reports of peripheral T-cell lymphomas with azurophilic granules are scarce, and cases reported most often had extranodal disease and often mucosal involvement.36–41 In general, cytotoxic granules are enveloped by a lipid bilayer and contain small internal vesicles and an electron-dense core surrounded by a membrane.42–44 Granules with identical morphology were also found, by electron microscopy, in the cytoplasm of the granzyme B-positive tumor cells of the nasal T-cell lymphoma investigated.

Low levels of granzyme B are constitutively expressed by NK cells but not by unstimulated CD3+ CD8- or CD3+ CD8- unstimulated peripheral blood lymphocytes.43–44 This might be the reason for the low number of CD3-positive granzyme B-positive cells in control lymphoid tissue. NK cells are CD3-negative, CD56-positive, and do not rearrange TCR genes,50,51 but are otherwise remarkably similar to T cells with respect to the expression of other membrane antigens.52 The T-cell origin of our granzyme B-positive gastrointestinal and cutaneous and of part of the nasal cases was confirmed by the presence of CD3. Two nasal granzyme B-positive cases were CD56-positive and CD3-negative. These might represent lymphomas of NK cell origin. In accordance, non-major histocompatibility complex-restricted cytotoxicity by tumor cells has been described recently for CD3-negative, CD56-positive nasal T-cell lymphoma with TCR genes in germline configuration.53 Several studies have addressed the relation between CD56 expression and T-cell lymphomas.40,54,55 CD56-positive gastrointestinal T-cell lymphomas were reported to have a propensity for extranodal sites, in particular the upper aerodigestive tract,44 which is in agreement with our findings. Moreover, CD56-positive lymphomas were reported to share a very aggressive clinical behavior and resistance to conventional chemotherapy.40

Although our results do not provide conclusive evidence as to whether granzyme B-positive lymphomas are neoplastic counterparts from IELs or activated CTLs in the lamina propria of the small intestine,32 we slightly favor the first option. In mice, IELs have been shown to express granzymes57 and are predominantly γδ TCR-positive. In humans, IELs are mostly aβ TCR-positive58 and probably also express granzyme proteins after activation. IELs are increased in celiac disease, and granzyme B-positive IELs seemed to be more prominent in the adjacent nonlymphoma involved mucosa in cases classified as EATCL or EATCL-like lymphoma without enteropathy. In addition, normal human small intestine CD3+ CD8- CD4- T lymphocytes comprise a minor fraction of IELs (6%) that are increased in celiac disease.59 This phenotype proved to be the dominant phenotype in granzyme B-positive gastrointestinal T-cell lymphomas.

We can only speculate on the association between signs of celiac disease and the occurrence of tumors derived from activated CTLs. It has been assumed that the mucosal permeability for luminal antigens is increased in celiac disease because of local and systemic IgG responses to gluten and other dietary proteins.58 This then might exert a cytotoxic T-cell response. The continuous antigenic stimulation might contribute to malignant transformation as has been proposed for gastric B-cell lymphomas in association with the presence of Helicobacter pylori.59,60

In conclusion, granzyme B-positive T-cell lymphomas are predominantly localized in MALT and differ from granzyme B-negative T-cell lymphomas in increased frequency of angioinvasion and necrosis but less infiltration with eosinophils. Skin and lymph nodes are occasionally involved. Although in some nasal cases an NK cell nature can not be excluded, most cases should be considered as neoplastic.
equivalents of activated CTLs. The prognosis of the granzyme B-positive gastrointestinal T-cell lymphomas is as equally poor as the prognosis of granzyme B-negative gastrointestinal T-cell lymphomas, indicating that site of origin rather than derivation of activated CTLs predicts clinical outcome of peripheral T-cell lymphomas.

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