Activation of Infiltrating Cytotoxic T Lymphocytes and Lymphoma Cell Apoptotic Rates in Gastric MALT Lymphomas

Differences between High-Grade and Low-Grade Cases

Massimo Guidoboni,* Claudio Doglioni,[†]
Licia Laurino,[†] Mauro Boiocchi,* and
Riccardo Dolcetti*

From the Division of Experimental Oncology 1,* Centro di
Riferimento Oncologico, Aviano, Italy; and the Department of
Pathology,[†] Belluno City Hospital, Belluno, Italy

In this study, we have characterized infiltrating T lymphocytes from 13 low-grade and 17 high-grade primary gastric MALT lymphomas by immunohistochemistry, with particular regard to the presence, activation, and topographic distribution of cytotoxic effectors. Although the prevalence of CD4+ and CD8+ cells was similar in low- and high-grade lymphomas, higher numbers of TIA-1+ cytotoxic effectors were found in this latter group of cases (11.6 versus 7.8%; $P = 0.004$). Activation of CD8+ cytotoxic T lymphocytes (CTLs) was significantly more pronounced in high- than in low-grade lymphomas, as shown by immunostaining for perforin (8.7 versus 4.0%; $P = 0.001$) and granzyme-B (GrB) (8.7% versus 3.0%; $P < 0.0001$). Of note, CD20/GrB double labeling showed that high-grade lymphomas carried a markedly higher content (about ninefold) of activated CTLs relative to the number of CD20+ lymphoma B cells (0.081 ± 0.076 versus 0.009 ± 0.011 ; $P < 0.0001$). Moreover, high-grade lymphomas showed significantly increased apoptotic rates compared to low-grade cases (5.3 and 1.1% of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells, respectively; $P < 0.0001$). In the whole series, the percentage of GrB+ cells and the GrB+/CD20+ ratio showed a strong linear correlation with the number of TUNEL-labeled cells. These findings, together with the frequent colocalization of CTLs and TUNEL+ neoplastic cells, suggested that apoptotic death of lymphoma cells may be due at least in part to the killing by cytotoxic effectors. Our results are consistent with the occurrence of host antitumor cell-mediated immune responses in gastric MALT lymphomas. Moreover, the finding of stronger cytotoxic responses in high- than in low-grade cases is of

potential usefulness in the design of more effective therapeutic strategies for the management of these disorders. (Am J Pathol 1999, 155:823–829)

Gastric B-cell lymphomas are frequently associated with longstanding chronic infection by *Helicobacter pylori*, and several lines of evidence indicate that this infectious agent plays a critical role in the pathogenesis of these disorders.^{1,2} In fact, it has been shown that *H. pylori* infection is responsible for the acquisition of reactive mucosa-associated lymphoid tissue (MALT), physiologically not present in the gastric mucosa, thus providing the cellular milieu from which B-cell clonal expansions may arise.³ Moreover, T lymphocytes infiltrating low-grade MALT lymphomas stimulated with *H. pylori*-derived antigens were shown to strongly sustain the growth and differentiation of clonal B cells *in vitro*.^{4,5} More recently, it has been reported that these responses are largely dependent on CD40-mediated stimulation coupled with signaling induced by cytokines interleukin (IL)-4 and -10 produced by helper T cells of the Th2 type.^{4,6} Although accumulated evidence clearly supports the growth-promoting role of the CD4+ helper subset in the pathogenesis of gastric MALT lymphomas,⁷ only limited information is currently available on the presence of host antitumor immune responses in these tumors, particularly those mediated by cytotoxic effectors.

Cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells are the major effectors of cytolytic responses, and the molecular mechanisms by which they kill target cells have been recently clarified.^{8–10} Both classes of cytotoxic effectors induce apoptosis in target cells by a granule exocytosis pathway; these granules carry both a pore-forming protein, such as perforin,¹¹ and serine-esterases, such as granzymes.¹² Differences were found in the expression pattern of these proteins between NK and

Supported in part by Associazione Italiana per la Ricerca sul Cancro and Ricerca Sanitaria Finalizzata Regione Veneto.

Accepted for publication June 17, 1999.

Address reprint requests to Dr. Mauro Boiocchi, Division of Experimental Oncology 1, Centro di Riferimento Oncologico, via Pedemontana Occidentale, 12–33081 Aviano (PN), Italy.

CD8+ CTLs, in that the former constitutively express both perforin and granzyme B, whereas the latter produce perforin and granzyme-B (GrB) only on activation induced by antigen recognition.⁸ Other granule-associated proteins are expressed by CD8+ CTLs: among them, T-cell-restricted intracellular antigen 1 (TIA-1) is expressed by cytotoxic effectors independently of their activation status, thus being a reliable marker for CD8+ lymphocytes with cytotoxic potential.^{13,14} Antibodies recognizing these markers have recently become available, thus allowing the identification of activated cytotoxic effectors *in situ*.

The aim of the present study was to better define the nature of reactive T cells infiltrating primary gastric MALT lymphomas and, particularly, to shed light on the role of cytotoxic immune responses in these disorders. To this end, we analyzed the presence, activation status, and spatial distribution of cytotoxic effectors in 13 low- and 17 high-grade gastric MALT lymphomas. Moreover, possible relationships between the number of activated cytotoxic effectors and the extent of lymphoma cell apoptosis were also investigated by an *in situ* end-labeling method.

Materials and Methods

Tissue Specimens

Thirty formalin-fixed, paraffin-embedded surgical specimens of gastric MALT lymphoma were retrieved from the files of the Division of Pathology, Belluno City Hospital. According to the criteria of Isaacson,¹⁵ recently incorporated into the REAL classification and modified as in de Jong et al,¹⁶ 13 cases were diagnosed as low-grade and 17 as high-grade MALT lymphomas.

Immunohistochemical Determinations

Two-micron paraffin sections were cut and mounted on poly-L-lysine treated slides, deparaffined in xylene, rehydrated in graded alcohols, washed three times in phosphate-buffered saline (PBS), and microwaved for two cycles of 8 and 5 minutes at 700 W in 10 mmol/L citrate buffer, pH 6.0, or 0.1 mmol/L EGTA. After cooling to room temperature (15–30 minutes), sections were further washed and treated with 3% H₂O₂ in methanol for endogenous peroxidase activity block. After primary antibody incubations, samples were developed with standard streptavidin-biotin-peroxidase methods and 3',5'-diaminobenzidine (DAB) as a substrate.

Double labelings for CD8/TIA-1, GrB/CD20, GrB/CD8, and GrB/TIA-1 were performed on selected specimens. Labelings with the first and second primary antibodies were sequentially performed as described above, except for the use of streptavidin-alkaline phosphatase staining with Fast Red as a chromogen in the second reaction. The following primary antibodies were used: anti-CD3 (polyclonal, 1:1000), anti-CD8 (clone cd8/144b, 1:25), and anti-CD20 (clone L26) from DAKO (Glostrup, Denmark); anti-CD4 (clone IF6, 1:100), from Novocastra (Newcastle, UK); anti-granzyme B (Clone GrB7, 1:20)

from Monosan (Leiden, The Netherlands); anti-perforin (clone KM585 P1–8, 1:1000) from Kamiya (Japan); anti-TIA-1 (1:1000) from Coulter Corp. (Hialeah, FL); and anti-CD56 (clone 123C3.D5, 1:100) from Neomarker (Fremont, CA). Intratumoral CD3+, CD4+, CD8+, TIA-1+, perforin+, GrB+ and CD56+ cells were evaluated by light microscopy with the support of an eyepiece grid. A minimum of 2000 cells (normal and neoplastic) were counted for each single determination and reported as the percentage of positive cells in total mononuclear cells. For GrB/CD20 double immunostaining, a minimum of 2000 CD20+ and the correspondent GrB+ cells were counted for each case, and expressed as a GrB+/CD20+ ratio. Evaluation was carried out on microscopic fields carefully selected in non-necrotic areas of the deeper portion of the tumor to avoid superficial, nonspecific, inflammatory infiltration.

Detection of Apoptosis-Associated DNA Fragmentation *in Situ*

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to identify DNA fragmentation *in situ* and was based on the protocol derived from Gavrieli,¹⁷ as modified by Migheli.¹⁸ All reagents for the TUNEL reaction were obtained from Boehringer (Mannheim, Germany). Briefly, rehydrated sections were digested with 20 µg/ml proteinase K (Sigma) in Tris-EDTA (TE) for 20 minutes at room temperature. After washing in TE and quenching of peroxidase activity with 3% H₂O₂ in distilled water for 10 minutes, slides were immersed in TdT buffer (25 mmol/L Tris-HCl, 0.2 mmol/L Na-cacodylate, 2.5 mmol/L cobalt chloride, pH 6.6) for 5 minutes and then incubated for 2 hours at 37°C in TdT mixture composed of enzyme solution (TdT) and label solution (fluorescein-dUTP), 1:9 and diluted 1:4 with TdT buffer. After washing with 2× standard saline citrate and Tris-buffered saline (TBS), slides were immersed in 2% bovine serum albumin in TBS-Triton for 15 minutes at room temperature, followed by incubation with Converter peroxidase-labeled antifluorescein antiserum for 30 minutes at room temperature. After washing, peroxidase activity was visualized with a DAB color reaction and slides were counterstained with Harris' hematoxylin, dehydrated, and mounted. For all but one high-grade case, the TUNEL labeling index was determined as the percentage of positive cells in total mononuclear cell number. Double labelings for TUNEL/GrB or TUNEL/CD20 were performed on selected high-grade MALT lymphoma specimens by sequential TUNEL, with DAB as chromogen giving a brown reaction product, followed by immuno-alkaline phosphatase staining with Fast Red as substrate.

Statistical Evaluation

Results are expressed as the mean ± SD percentages of positive cells on total cell count for a given marker. A nonparametric Mann-Whitney *U* test was used to evalu-

Figure 1. Histograms demonstrating the mean percentage ($\pm$ SD) of cells staining for CD3 (A), CD4 and CD8 (B), and CD56 (D), detected in low- and high-grade gastric MALT lymphomas. C: Distribution of CD4/CD8 ratios along histopathological grade.

ate the differences in the distribution of positive cell count between low- and high-grade MALT lymphomas. A Spearman rank order correlation test was used for TUNEL index and CD20/GrB ratio or GrB cell count comparison.

Results

Characterization of T Lymphocytes Infiltrating Primary Gastric MALT Lymphomas

As shown in Figure 1A, the number of infiltrating CD3+ T lymphocytes was similarly distributed in low- and high-grade lymphomas, respectively ($18.4 \pm 6.1\%$ versus $22.4 \pm 6.7\%$; $P = NS$). Analogously, the number of CD4+ ($7.9 \pm 2.1\%$ versus $9.6 \pm 4.2\%$; $P = NS$) and CD8+ lymphocytes ($10.4 \pm 4.4\%$ versus $12.8 \pm 5.1\%$; $P = NS$) was comparable in low- and high-grade lymphomas (Figure 1B). Consistently, the CD4:CD8 ratio was not significantly different between low- and high-grade cases (0.9 ± 0.4 versus 0.8 ± 0.4 ; $P = NS$, Figure 1C). Immunostaining for CD56 showed an inverse relationship between histopathological grade and the proportion of infil-

trating CD56+ NK cells. In fact, as shown in Figure 1D, low-grade lymphomas carried significantly higher numbers of CD56+ NK cells than high-grade cases ($2.1 \pm 0.7\%$ versus $1.2 \pm 0.6\%$; $P = 0.001$).

High-Grade Gastric MALT Lymphomas Have Significantly Higher Numbers of Activated CTLs than Low-Grade Cases

As a first step, we evaluated the prevalence of cytotoxic cell precursors as indicated by TIA-1 immunostaining (Figure 2, A and B). High-grade lymphomas carried a significantly higher proportion of TIA-1+ cells than low-grade cases ($11.6 \pm 3.9\%$ versus $7.8 \pm 1.5\%$; $P = 0.004$, Figure 3A). CD8/TIA-1 double immunostaining demonstrated that cytotoxic precursors were mostly CD8+ T cells (not shown). Of note, also the effectors expressing cytolytic activation markers were markedly more represented in high- than in low-grade lymphomas. In fact, the absolute number of both perforin+ and GrB+ cells detected in high-grade tumors nearly doubled those observed in low-grade cases, respectively ($8.7 \pm 4.3\%$ versus $4.0 \pm 1.9\%$; $P = 0.001$ for perforin, and $8.7 \pm 4.5\%$

Figure 2. Immunophenotypic characterization of intratumoral CTLs in representative cases of gastric MALT lymphoma. **A-D:** Immunostains for TIA-1 (**A** and **B**) and perforin (**C** and **D**) demonstrate that high-grade lymphomas (**B** and **D**) carry a larger proportion of activated CTLs than low-grade cases (**A** and **C**). Original magnifications, $\times 250$ (**A** and **B**) and $\times 400$ (**C** and **D**). **E:** Colocalization of CD8 (red) and GrB (brown), as demonstrated by double immunostaining, confirmed that activated cytotoxic effectors were mostly CD8⁺ T lymphocytes. Double immunolabeling with Fast Red and diaminobenzidine as chromogens. Original magnification, $\times 400$. **F:** Double immunostaining for CD20 (red) and GrB (brown) in a representative high-grade case showing that activated CTLs are frequently located in close proximity to CD20⁺ lymphoma B cells. Of note, activated cytotoxic effectors often show polarization of GrB granules toward lymphoma cells. Double immunolabeling with Fast Red and diaminobenzidine as chromogens. Original magnification (oil immersion), $\times 1200$. **G:** Apoptotic lymphoma cells (TUNEL⁺) in a representative high-grade gastric MALT lymphoma. Original magnification, $\times 400$.

versus $3.0 \pm 1.4\%$, $P < 0.0001$ for GrB, Figures 2C, 2D, 3B, and 3C). CD8/GrB double immunostaining confirmed that activated cytotoxic effectors were prevalently CD8⁺ T lymphocytes (Figure 2E). Consistently, both perforin/CD8 (0.7 ± 0.3 versus 0.4 ± 0.2 ; $P = 0.004$) and GrB/CD8 (0.7 ± 0.3 versus 0.3 ± 0.2 , $P = 0.001$) ratios were markedly increased in high-grade lymphomas (Figure 3D), indicating a more pronounced activation status of CTLs in this group of tumors.

To better understand the meaning of these differences, the ratio between the number of activated CTLs and that of putative target cells (CD20⁺ lymphoma B cells) was directly evaluated in 11 low- and 16 high-grade cases by GrB/CD20 double immunostaining (Figure 2F). The analysis confirmed that, compared to low-grade cases, high-grade lymphomas carried a significantly higher content (about ninefold) of activated CTLs relative to the number of CD20⁺ lymphoma B cells (0.081 ± 0.076 versus 0.009 ± 0.011 ; $P < 0.0001$; Figure 4A). Of note, the large majority of GrB⁺ effectors was in close contact with CD20⁺ lymphoma cells, prevalently showing morphological features of large transformed blast cells (Figure 2F).

The Content of Activated CTLs in Gastric MALT Lymphomas Closely Correlated with the Number of Apoptotic Lymphoma B Cells

Whereas in the majority of CD8⁺ T lymphocytes infiltrating low-grade lymphomas GrB⁺ granules were dispersed throughout the cytoplasm, a large proportion of GrB⁺ cells from high-grade cases showed a polar distribution of cytotoxic granules (Figure 2F), a feature that characterizes cytotoxic effectors actively involved in killing their target cells.^{10,12} Moreover, activated CTLs from high-grade lymphomas were prevalent in close proximity to structures morphologically identifiable as apoptotic bodies. To assess whether the presence of activated cytotoxic effectors correlated with the apoptotic rates, 13 low- and 16 high-grade MALT lymphomas were investigated for the prevalence and distribution pattern of apoptotic cells by the *in situ* end-labeling (TUNEL) method (Figure 2G). As shown in Figure 4B, markedly higher apoptotic rates were found in high-grade compared to low-grade tumors ($1.1 \pm 0.9\%$ versus $5.3 \pm 1.9\%$; $P <$

Figure 3. A-C: Histograms demonstrating the mean percentage ($\pm$ SD) of cells staining for TIA-1 (A), perforin (B), and GrB (C) observed in low- and high-grade gastric MALT lymphomas. D: Histogram demonstrating the mean value of GrB⁺:CD8⁺ and perforin⁺:CD8⁺ ratios ($\pm$ SD) in low- and high-grade cases.

0.0001). Apoptotic death occurred prevalently in neoplastic B cells as shown by the cytomorphologic features of TUNEL⁺ cells and by TUNEL/CD20 double immunostaining (not shown). Furthermore, both the absolute number of GrB⁺ cells and the GrB⁺/CD20⁺ ratios strongly correlated with the number of TUNEL-labeled cells ($r_s = 0.71$, $P < 0.0001$ and $r_s = 0.80$, $P < 0.0001$, respectively; Figure 4, C and D).

Discussion

The present study was undertaken to assess whether cytotoxic immune responses are present in primary gastric MALT lymphomas. Our results show that activated CD8⁺ CTLs are present in gastric MALT lymphomas, suggesting that cell-mediated antitumor immune responses are likely present in this disease. Such a possibility is also supported by the finding of a close spatial relationship between CD20⁺ lymphoma B cells and GrB⁺ CTLs. These effectors, in particular, frequently showed a polar distribution of cytotoxic granules within the cytoplasm, a feature characterizing CTLs actively killing their targets. Moreover, a strong linear correlation between the GrB⁺/CD20⁺ ratio and the apoptotic index was found throughout the whole series, indicating that a significant proportion of apoptotic phenomena affecting

lymphoma cells is a likely consequence of cytotoxic immune responses.

Interestingly, our findings are consistent with a more efficient recruitment and a stronger activation of CTLs in high-grade than in low-grade gastric MALT lymphomas. In particular, these cases showed a GrB⁺/CD20⁺ ratio about ninefold that of low-grade lymphomas, as directly evaluated by GrB/CD20 double immunostaining. Furthermore, high-grade tumors showed a greater prevalence of apoptotic death affecting neoplastic B cells than low-grade lymphomas, similar to previous findings.^{19,20} These differences in activation and recruitment of CTLs observed between high- and low-grade lymphomas could be related to the progressive acquisition of enhanced immunogenic properties by malignant lymphoma cells. It is tempting to speculate that this phenomenon might be related at least in part to the increasing load of genetic changes associated with the evolution to high-grade lymphomas, ie, p53, TGF- β -RII, and *c-myc* alterations.²¹⁻²⁴ Consistently, high numbers of activated cytotoxic T cells were also detected by other investigators in nodal high-grade B-cell non-Hodgkin's lymphomas.²⁵⁻²⁷

Although fewer in number than in high-grade cases, a proportion of T cells phenotypically identifiable as cytotoxic precursors (TIA-1⁺) was also detected in the ma-

Figure 4. A-B: Histograms showing the mean value of GrB+/CD20+ ratio (A) and the mean percentage ($\pm$ SD) of TUNEL+ cells (B) detected in low- and high-grade gastric MALT lymphomas. C-D: Scatter plots demonstrating a strong linear relationship between GrB+ (C) or GrB+/CD20+ ratio (D) and the percentage of TUNEL+ cells in the whole series. Spearman correlation indices (r_s) are reported.

majority of low-grade lymphomas. In these cases, however, only a small number of T lymphocytes with cytotoxic potential expressed GrB or perforin, indicating a globally lower level of activation *in vivo*. It is likely that the peculiar microenvironment characterizing this subset of cases may contribute to this phenomenon. In fact, reactive T cell subpopulations infiltrating low-grade gastric MALT lymphomas were shown mainly to provide survival- and/or growth-promoting signals to the expanding B-cell clones.^{5,7} In particular, previous *in vitro* findings suggested a likely crucial role for local Th2 signaling in the early phases of MALT lymphomagenesis,⁶ a setting that is probably not compatible with the induction of strong cytotoxic responses. The possible role of locally enhanced production of IL-10 and other Th2-type cytokines by *H. pylori*,^{28,29} also found in gastric MALT lymphomas,³⁰ may be relevant in this respect. Moreover, the lower apoptotic indices detected in low-grade cases may be related to the peculiar pathobiological interactions presumably occurring between clonal B cells and reactive T lymphocytes, favoring the rescue of low-grade lymphoma cells from programmed cell death. In fact, it has recently been reported that signaling through CD40

was able to promote the survival of B lymphocytes from low-grade MALT lymphomas *in vitro*.⁶ Besides promoting the proliferation and differentiation of clonal B cells, this microenvironmental setting may also inhibit cytotoxic immune responses.³¹ Accordingly, Th1 responses and CD8+ CTL activity were strongly inhibited in mice with persistent gastric *H. pylori* infection.³² Moreover, loss of *H. pylori* dependence for growth occurring during the evolution to high-grade histology may be associated with microenvironmental changes (ie, a shift toward a Th1 response) that may allow the occurrence of CTL immune responses.

The significantly different distribution of CD56+ NK cells observed between low- and high-grade MALT lymphomas is intriguing. The increased content of these cells in low-grade cases may be the consequence of microenvironmental stimuli, such as IL-10, that are able to enhance the proliferation of NK cells. It is worth considering, however, that recent evidence indicates these cells may have immunoregulatory functions, including the ability to modulate both humoral and cell-mediated immune responses.^{33,34} In particular, the possibility that NK cells may contribute to limit the extent of autoreactive re-

sponses elicited by *H. pylori* in low-grade lymphomas constitutes an attractive hypothesis.³⁴ Whether the increased content of NK cells detected in low-grade MALT lymphomas has a role in the pathogenesis of these disorders remains, however, to be elucidated.

Although the extent of cytotoxic responses specifically directed against lymphoma cells was not directly investigated in the present study, our findings have implications of potential clinical relevance. In fact, they are consistent with the possibility that most patients with high-grade gastric MALT lymphomas may mount antitumor cytotoxic immune responses, which, however, cannot eliminate lymphoma cells completely. Therefore, therapeutic strategies including treatments aimed at potentiating host cytotoxic immune responses could be effective in the clinical management of these lymphomas. With respect to low-grade lymphomas, the limited extent of cytotoxic responses observed in the majority of these cases is probably related to the peculiar pathobiological setting that characterizes these disorders. On these grounds, patients with low-grade lymphomas may probably benefit from treatment modalities that, besides removing the triggering antigenic stimulus (ie, *H. pylori* eradication), can directly inhibit the pathogenically relevant interactions occurring between clonal B cells and reactive T lymphocytes.

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