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Expression of CD95 Antigen and Bcl-2 Protein in Non-Hodgkin's Lymphomas and Hodgkin's Disease

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CD95 (APO-1/Fas) is a member of the superfamily that includes the nerve growth factor and tumor necrosis factor receptors, OX40, CD27, CD30, and CD40. Present on a minority of resting blood lymphocytes, CD95 expression is upregulated on activated T and B lymphocytes and natural killer cells, where binding of the antigen by anti-Fas and anti-APO-1 antibodies has been shown to induce apoptosis. This CD95-mediated apoptosis is at least partially inhibited by expression of the Bcl-2 protooncogene. To evaluate possible roles of CD95 and Bcl-2 in growth regulation of lymphoid neoplasms, we studied by immunohistochemistry the expression of CD95 and Bcl-2 in 67 B- and 5 T-cell lymphomas, and 10 cases of Hodgkin's disease. In all, 29 B and 2 T cell lymphomas, and 9 cases of Hodgkin's disease expressed CD95. Compared with diffuse large B-cell and Burkitt-like lymphomas, low-grade B-cell lymphomas more frequently expressed CD95 (52% versus 26%; P < .005). None of the B-cell small lymphocytic lymphomas or mantle cell lymphomas expressed CD95, whereas the majority of follicle center lymphomas, extranodal marginal zone B-cell lymphomas, and immunocytomas were CD95$^+$. Of the 29 CD95$^+$ B-cell lymphomas, only 33% of the high-grade group coexpressed Bcl-2, compared with 87% of the low-grade group (P < .04). Two of three peripheral T-cell lymphomas—including one anaplastic large cell lymphoma—expressed CD95. Staining for CD95 was seen in 9 of 10 cases of Hodgkin's disease. The infrequent expression of CD95 in high-grade B-cell lymphomas suggests an association between loss of CD95 expression/function and a more aggressive tumor grade. Whereas frequent coexpression of Bcl-2 with CD95 may protect low-grade B-cell lymphomas against CD95-mediated apoptosis, in the high-grade group such coexpression is infrequent, and other regulators besides Bcl-2 may be involved in modulating the apoptosis signal delivered by CD95. (Am J Pathol 1996, 148:847–853)

Apoptosis is a distinct form of cell death that is integral to the regulation of cellular proliferation and differentiation and to the maintenance of homeostasis. The decision to die is itself a complex interplay between environmental signals and the ability of the cell to receive and process them. In 1989, Trauth et al[1] and Yonehara et al[2] independently described two monoclonal antibodies, anti-APO-1 and anti-Fas, respectively, whose cross-linking with their respective cell surface proteins resulted in apoptosis in certain APO-1- or Fas-positive cells *in vitro*. Subsequent cDNA cloning has shown that the antigens recognized by the anti-APO-1 and anti-Fas murine monoclonal antibodies are identical,[3,4] and at the 5th International Workshop and Conference on Leukocyte Differentiation Antigens, the APO-1/Fas molecule was assigned the designation CD95.[5] Work by Lichter et al[6] showed that CD95 is encoded by the *APT* gene on chromosome 10q23. Recently, a natural ligand for CD95 has been identified.[7] The mature CD95 antigen has an M_r of 40 to 50 kd and is a type I transmembrane glycoprotein with a 157-amino acid extracellular domain, a 17-amino acid transmembrane segment, and a 145-amino acid cytoplasmic portion. By virtue of the three cysteine-rich segments in its extracellular domain, CD95 is considered to be a member of a superfamily of proteins that includes

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the nerve growth factor receptor, the p55 and p75 tumor necrosis factor receptors, the OX40 antigen, CD40, and the CD27 and CD30 glycoproteins[8–13] (reviewed in ref. 14).

Expression of CD95 is not lineage-specific and has been reported to be constitutive in a variety of epithelial cells. Within the immune system, CD95 is expressed by a minority of resting peripheral blood T and B lymphocytes, but its expression is strongly upregulated on activated T and B lymphocytes, and NK cells.[15–17] In lymphoid tissues, CD95 can be detected immunohistochemically in a subset of follicle center cells (centroblasts)[17–19] and at high levels in monocytoid B-cells[19]; CD95 is not detectable in mantle zone B lymphocytes or mature plasma cells.[17,19]

Incubation of CD95-positive, activated T and B lymphocytes and natural killer (NK) cells with anti-CD95 monoclonal antibodies has been shown to induce apoptotic cell death.[20,21] The mere presence of CD95 on the cell surface, however, is insufficient for the delivery of the apoptotic signal, since the viability of some CD95-positive resting lymphocytes is unaffected by CD95 monoclonal antibody, a finding that suggests lymphocyte activation is a necessary condition to enable the cell to receive the apoptotic signal transduced by CD95.[22,23] In the presence of expression of the Bcl-2 protooncogene, the effect of CD95-mediated apoptosis has been shown to be at least partially inhibited.[24–26]

Previous studies have shown expression of CD95 in many cell lines, including those derived from T-acute lymphoblastic lymphoma/leukemia, Burkitt's lymphoma, and large cell non-Hodgkin's lymphoma.[27] A review of the literature shows only a few published studies of CD95 expression in primary lymphoid neoplasms. These have concentrated on follicle center lymphomas, diffuse large B-cell lymphomas, or B-cell chronic lymphocytic leukemia,[18,19,24,28] with one report including the relatively rare entity of mediastinal large B-cell lymphoma.

We undertook this study to determine immunohistochemically the expression of CD95 in a large group of lymphomas, and to correlate this with histological subtype and grade. In addition, we studied the relationship between the expression of this apoptosis receptor and that of the anti-apoptotic Bcl-2 protein in low- and high-grade B-cell non-Hodgkin's lymphomas.

Materials and Methods

Materials

We studied 72 cases of non-Hodgkin's lymphoma and 10 cases of Hodgkin's disease. These included eight cases of mantle cell lymphoma (MCL) seen in the Department of Pathology at the Massachusetts General Hospital between January 1993 and December 1994 in which there was sufficient tissue for frozen section immunohistochemistry; the remainder represented a random selection of lymphomas diagnosed between January 1992 and September 1993. All diagnoses and subclassification were based upon a combination of routine morphology and immunohistochemistry; these are listed in Table 1 according to the Revised European-American Lymphoma Classification.[29] Four cases were composed of a diffuse infiltrate of centrocytes and centroblasts; three of these cases expressed CD10. These four cases were assigned to the provisional subtype of follicle center lymphoma, diffuse type.[29]

Table 1. *Histological Subtypes of Lymphomas*

Lymphoma	Number of cases
B-cell lymphoma	67
Low-grade	44
CLL	7
MCL	8
IM	2
MALT	12
FCL-follicular	
Grade 1 (predominantly small cleaved)	7
Grade 2 (mixed small cleaved and large cell)	4
FCL-diffuse	4
High-grade	23
DLBCL	17
BK-L	6
T-cell lymphoma	5
Peripheral T-cell lymphoma, unspecified (PTCL)	2
ALCL	1
Precursor T-lymphoblastic lymphoma (T-LBL)	2
Hodgkin's Disease	10
Nodular sclerosis	9
BNLI grade 1	4
BNLI grade 2	5
Mixed cellularity	1

Antibodies

7C11 is a murine monoclonal antibody of immunoglobulin (Ig)M isotype that recognizes the human CD95 (APO-1/Fas) antigen.[5,27] The monoclonal antibody against Bcl-2 protein was from Dako (clone 124; Carpinteria, CA).

Frozen Section Immunohistochemistry

After acetone fixation, cryostat sections were stained with the 7C11 antibody at 12.5 μg/ml, or with the anti-Bcl-2 antibody at 1:40 dilution using the avidin-

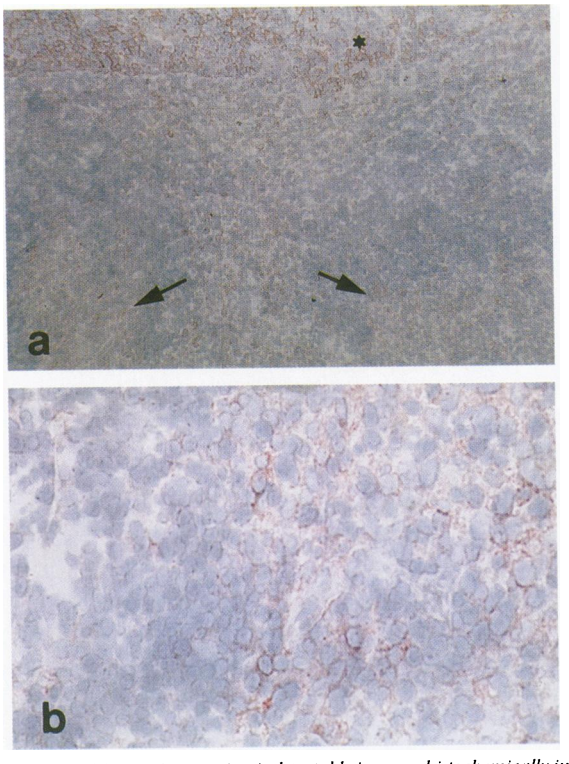

Figure 1. (a) *CD95 expression is detectable immunohistochemically in the basilar epithelial cells* (*) *and in a subset of follicle center lymphocytes* (arrows) *in a hyperplastic tonsil* (*original magnification,* ×40). (b) *The same section of hyperplastic tonsil is shown at higher magnification with follicle center centroblasts staining positively for CD95 on the right; on the left are mantle zone B-lymphocytes which are negative for CD95* (*original magnification,* ×160). (*Immunoperoxidase stain with Gill's hematoxylin counterstain.*)

biotin-peroxidase technique.[30] The sections were developed with H_2O_2 and 3-amino-9-ethylcarbazole (Aldrich Chemical Co., Milwaukee, WI), and examined after counterstaining of the nuclei with Gill's hematoxylin. Membrane and cytoplasmic staining in >20% of the tumor cells with the 7C11 and Bcl-2 antibodies, respectively, was considered a positive result. Staining of the basilar epithelial cells and germinal center lymphocytes with the antibody 7C11 in frozen sections of hyperplastic tonsils was monitored as positive control for CD95 expression (Figure 1). For Bcl-2 protein, staining of interfollicular T lymphocytes and mantle zone lymphocytes in frozen sections of hyperplastic tonsils served as positive control.

Statistics

Comparison of differences was by Fisher's exact test.

Results

Non-Hodgkin's Lymphomas

In all, 31 of 72 (43%) T and B cell lymphomas stained positively for CD95. 29 of 67 (43%) B cell lymphomas and two of five (40%) T-cell lymphomas were positive (Figure 2, a and b). There was no gradation in the intensity of staining with the antibody 7C11.

B-Cell Lymphomas

CD95 and histological grade. In B-cell lymphomas, as shown in Table 2, a significantly higher proportion of the low-grade lymphomas expressed CD95 compared with the high-grade group (52% *versus* 26%; $P < .005$). Within the low-grade B-cell group, immunocytoma (IM) and extranodal marginal zone B-cell lymphoma/low-grade B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) showed the highest frequency of CD95 expression (2/2 and 11/12, respectively), followed by follicle center lymphoma (FCL) (67% overall). In contrast, none of seven cases of B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL) and none of eight cases of MCL showed staining for CD95. In FCL-follicular, all four of four grade 2 cases (mixed small cleaved and large cell) expressed CD95 compared with only three of seven (43%) grade 1 cases (predominantly small cleaved). Although there was a trend, this difference did not reach statistical significance.

Among the high-grade B-cell lymphomas, CD95 expression was seen more frequently in diffuse large B-cell lymphoma (DLBCL) compared with Burkitt-like lymphoma (BK-L) (29% *versus* 17%), but the difference was not statistically significant.

CD95 and Bcl-2 protein expression: As expected, most of the low-grade B-cell lymphomas expressed Bcl-2 (Figure 2c), in significant contrast to the high-grade B-cell lymphomas (93% *versus* 48%; $P < .0005$; Table 2). In particular, whereas 20 of 23 CD95-positive low-grade B-cell lymphomas also expressed Bcl-2, co-expression of Bcl-2 was seen in only two of six CD95-positive high-grade B-cell lymphomas ($P < .04$; Table 2). All of the CD95-negative low-grade B-cell lymphomas expressed Bcl-2, but only 9 of 17 (53%) CD95-negative high-grade B-cell lymphomas expressed Bcl-2 ($P < 0.001$). Within each group, however, there was no statistically significant association between CD95 and Bcl-2 expression.

T-Cell Lymphomas

Two of the three peripheral T-cell neoplasms—including the one case of anaplastic large cell lym-

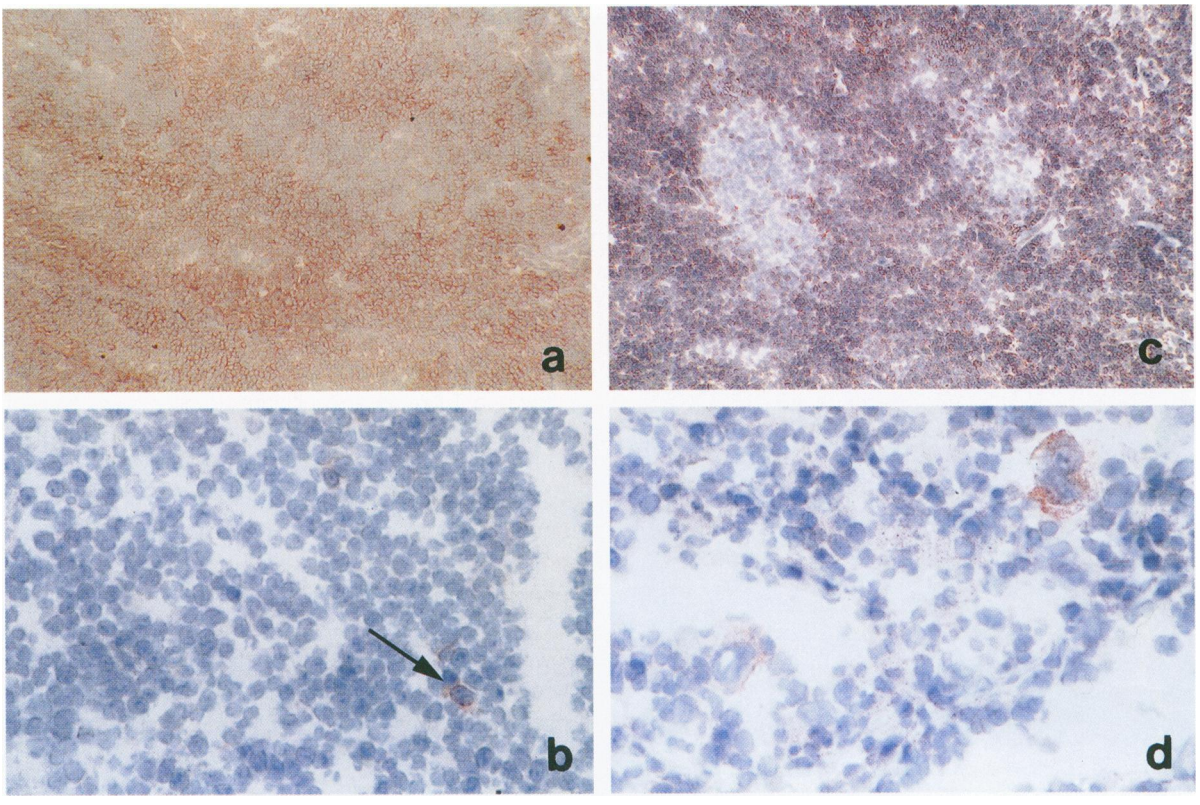

Figure 2. (a) *Positive staining for CD95 in a MALT; the nonstaining nodular areas correspond to residual hyperplastic lymphoid follicles (original magnification, ×40).* (b) *Lack of staining for CD95 in a B-cell CLL; one rare positive-staining cell is most likely an activated lymphocyte (original magnification, ×100).* (c) *The same MALT shown in* (a) *stains positively for Bcl-2 protein; note absent Bcl-2 staining in the hyperplastic lymphoid follicles (original magnification, ×40).* (d) *Positive staining for CD95 is evident in the Reed-Sternberg cells of a case of Hodgkin's disease, nodular sclerosis type, BNLI grade 1 (original magnification, ×160). (All sections are immunoperoxidase-stained, followed by counterstaining with Gill's hematoxylin.)*

phoma (ALCL)—expressed CD95. All expressed Bcl-2 protein. The two cases of precursor T-cell lymphoblastic lymphoma (T-LBL) expressed neither CD95 nor Bcl-2 (Table 2).

Hodgkin's Disease

Ten cases of HD were evaluated. Of these, nine showed CD95 expression in the Reed-Sternberg cells (Figure 2d). The positive cells were relatively frequent in eight cases and rare in one. There was no difference between the cases of Hodgkin's disease, nodular sclerosis type, British National Lymphoma Investigation (BNLI) grade 1[31] and those cases of BNLI grade 2 with respect to CD95 expression.

Eight of nine CD95-positive cases also showed expression of Bcl-2 protein by the Reed-Sternberg cells, as did the one CD95-negative case.

Discussion

We describe the expression of the apoptosis receptor CD95 in a large group of lymphomas, including several subtypes that have not been examined previously such as MCL, MALT lymphoma, and Hodgkin's disease.

First, despite their shared "low-grade" designation, the low-grade B-cell lymphomas display heterogeneity in their frequency of CD95 expression, ranging from 0% in B-cell CLL and MCL, to 43% in FCL-follicular, grade 1, to 92% in MALT lymphoma, to 100% in FCL-follicular, grade 2; both cases of IM expressed CD95. Furthermore, these different entities express CD95 in a manner that is reminiscent of their postulated normal counterparts. Thus, the lack of staining for CD95 in B-cell CLL recapitulates the infrequent and low expression of CD95 in resting B lymphocytes in the peripheral blood and is similar to that reported by other investigators.[15,16,27] Parallel to the absence of CD95 on normal mantle zone B lymphocytes, none of the eight cases of MCL showed staining. In fact, with respect to CD95 and Bcl-2 expression, the tumor cells in MCL appear to share more similarities with B-cell CLL than with other low-grade B-cell lymphomas.

Table 2. *Expression of CD95 Antigen and Bcl-2 Protein in Non-Hodgkin's Lymphomas*

Lymphomas	Number of cases	CD95$^+$		CD95$^-$	
		Total (%)*	BCL-2$^+$(%)†	Total	BCL-2$^+$(%)§
B-cell	67	29 (43)	22 (76)	38	30 (79)
Low-grade	44	23 (52)	20 (87)	21	21 (100)
CLL	7	0 (0)	0 (–)	7	7 (100)
MCL	8	0 (0)	0 (–)	8	8 (100)
IM	2	2 (100)	2 (100)	0	0 (–)
MALT	12	11 (92)	9 (82)	1	1 (100)
FCL-follicular	11	7 (64)	6 (86)	4	4 (100)
grade 1	7	3 (43)	3 (100)	4	4 (100)
grade 2	4	4 (100)	3 (75)	0	0 (–)
FCL-diffuse	4	3 (75)	3 (100)	1	1 (100)
High-grade	23	6 (26)	2 (33)	17	9 (53)
DLBCL	17	5 (29)	1 (20)	12	7 (58)
BK-L	6	1 (17)	1 (100)	5	2 (40)
T-cell	5	2 (40)	2 (100)	3	1 (33)
PTCL-NOS	2	1 (50)	1 (100)	1	1 (100)
ALCL	1	1 (100)	1 (100)	0	0 (–)
T-LBL	2	0 (0)	0 (–)	2	0 (0)

*Numbers in parentheses are percentages of CD95$^+$ cases for each histological subtype.
†Numbers in parentheses are percentages of CD95$^+$ cases which are Bcl-2$^+$.
§Percentages of CD95$^-$ cases which are Bcl-2$^+$.

In contrast to the B-cell CLL and MCL, which are thought to arise from pregerminal center B-cells, expression of CD95 was detected more frequently among the B-cell lymphomas thought to correspond to germinal center and postgerminal center stages of B-cell differentiation. The proportions of FCL-follicular, FCL-diffuse, and DLBCL with CD95 expression in our study are similar to those reported in several recent reports[18,19,24] and range from 37% in DLCBL to 43–100% in FCL of different grades. Interestingly, in FCL-follicular, a greater proportion of grade 2 cases expressed CD95 (four of four, 100%) compared with grade 1 cases (three of seven, 43%), although this difference did not reach statistical significance ($P = 0.2$). Within germinal centers of lymph nodes, CD95 expression is limited to a subset of B-cell blasts, and upregulation of CD95 expression is seen more frequently in activated B and T cells than in resting lymphocytes. It is possible that the higher frequently of CD95 expression in grade 2 FCL-follicular reflects the higher proportion of centroblasts in these tumors.

To our knowledge, this study is the first to examine the expression of CD95 specifically in the low-grade B-cell lymphoma of MALT type. A prior study by Leithauser et al[19] describes immunohistochemical staining for APO-1 in 6 of 12 primary gastrointestinal B-cell lymphoma of "low grade",[19] but it is unclear how many of the 12 are of the MALT type. In our study, 11 of 12 (92%) extranodal marginal zone B-cell lymphoma of MALT type expressed CD95. This high frequency seems to correlate with the high levels of CD95 detectable on the marginal zone B lymphocytes in lymph nodes and correlates with the postulated postgerminal center stage of this neoplasm.

Our results indicate significantly less frequent expression of CD95 in DLBCL and BK-Ls compared with low-grade B-cell lymphomas. Given the hypothesis that clonal expansion of activated lymphocytes may be regulated by the interaction between the CD95 molecule and its natural ligand, the infrequent expression of this apoptosis receptor in the high grade B-cell lymphomas suggests that a defect in the CD95-mediated apoptosis signal delivery system may confer a growth advantage to these subtypes of lymphoma. In particular, although diffuse large B-cell lymphoma and high-grade B-cell lymphoma, BK-L, are neoplasms of the activated germinal center and postgerminal center stages of B-cell differentiation, unlike normal activated B-cells and most cell lines derived from B-cell lymphomas, the high-grade diffuse large B-cell lymphoma and BK-L are largely CD95-negative: this finding suggests that there may be selective pressure *in vivo* against CD95 expression by aggressive B-cell neoplasms and may raise the possibility of CD95 as a tumor suppressor. In support of this possibility is the recent report by Moller et al,[32] in which the expression of CD95 in normal colonic mucosa is compared immunohistochemically with that in colonic adenomas and carcinomas. Their results show an attenuation of APO-1 expression from "regular" in normal colon mucosa, to "diminished" in 2 of 20 colonic adenomas and 101 of 258 carcinomas, to "completely abrogated" in 124 of 258 carcinomas.[32] Furthermore, such a complete loss of APO-1 expression was reported to be more

frequent in metastatic (Dukes classification C and D) than nonmetastatic (Dukes classification A and B) carcinomas. Together with our results, such findings suggest an association between loss of CD95 expression/function and neoplastic transformation or a more aggressive tumor phenotype.

Overexpression of the *Bcl-2* protooncogene has been reported to inhibit the apoptosis mediated by the Fas antigen.[16,17,24–26] Recent studies have shown an inverse association between Fas antigen and Bcl-2 expression in normal germinal center cells[17] and normal peripheral blood lymphocytes, monocytes, and neutrophils.[16,17] The relationship of these two proteins in lymphoid neoplasms appears to be more variable, however. Since Bcl-2 expression may provide protection for those tumors that are CD95-positive, our observation of frequent double expression of Bcl-2 and CD95 among the germinal and postgerminal center low-grade B-cell lymphomas (IM, MALT, FCL-follicular and FCL-diffuse) may explain the relative longevity of these neoplastic cells.

In contrast to low-grade germinal center and postgerminal center lymphomas where Bcl-2 is often coexpressed with CD95, in the high-grade CD95-positive B-cell lymphomas, two-thirds lacked Bcl-2. This finding raises the possibility that in these high-grade lymphomas, other regulators besides Bcl-2 may be involved in the modulation of the apoptosis signal mediated by CD95. Whether or not these CD95-positive, Bcl-2-negative high-grade lymphomas follow a less aggressive course compared with their CD95-negative, BCL-negative counterparts is an intriguing possibility that has not been addressed.

The number of T-cell lymphomas in this study is too small for statistical analysis, but the detection of CD95 in two of three peripheral T-cell lymphomas (PTCLs) (including the case of ALCL) is comparable with the 50% overall frequency reported by Kondo et al[24] for "pleomorphic, medium and large" and "pleomorphic, large" T-cell lymphomas. The lack of CD95 expression in the two cases of precursor T-LBL is consistent with previous reports[27,33,34] and correlates with the higher expression of CD95 on mature, activated $CD45RO^+$ T cells compared with thymocytes.[19,27]

In Hodgkin's disease, we found frequent expression of CD95 antigen by Reed-Sternberg cells, similar to the findings reported by Xerri et al.[34] There were too few cases to permit correlation with histologic subtypes. Our results show no differences in CD95 expression between those cases of Hodgkin's disease, nodular sclerosis type, BNLI grade 1 and those of BNLI grade 2.

In summary, our results indicate a significantly higher frequency of CD95 expression—often with Bcl-2 protein coexpression—in histologically low-grade B-cell lymphomas compared with high-grade tumors. In the low-grade group itself, there is considerable heterogeneity in the frequency of CD95 expression among the different disease entities, and the pattern of reactivity for CD95 in B-cell CLL, MCL, FCL, and MALT shows a close parallel to that in the putative normal counterparts of these lymphoid neoplasms. The infrequent expression of CD95 in high-grade B-cell lymphomas suggests a possible association between loss of CD95 expression/function and more aggressive tumors, and may raise the possibility of CD95 as a tumor suppressor.

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