American Journal of Pathology, Vol. 148, No. 1, January 1996

Granzyme B Expression in Reed-Sternberg Cells of Hodgkin's Disease

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Reed-Sternberg (RS) and Hodgkin's (H) cells are considered to be the neoplastic cells in Hodgkin's disease. Although most data suggest a lymphoid origin, the nature of these cells still remains the subject of considerable controversy. Recently, monoclonal antibodies became available, directed against granzyme B, a serine protease specifically expressed by activated cytotoxic T cells (CTLs) and natural killer (NK) cells. Using two granzyme B-specific antibodies directed against different epitopes, we studied the expression of granzyme B in a well characterized group of Epstein-Barr virus (EBV)-positive and EBV-negative cases of Hodgkin's disease. Granzyme B expression was found in part of the H-RS cells in 11 out of 61 tested cases (18%, 9 of 46 cases of nodular sclerosing and 1 of 12 mixed cellularity Hodgkin's disease). In none of these cases did H-RS cells express B-cell markers, whereas in four cases, expression of either the T-cell marker CD3 or CD8 was found in a small minority of H-RS cells. The percentage of granzyme B-positive H-RS cells ranged from <10% to >50%. Granzyme B-positive H-RS cells were present in 6 of 26 EBV-positive cases and in 5 of 35 EBV-negative cases, indicating no relationship with the presence of EBV. Moreover, no significant differences were found regarding either stage at presentation or clinical outcome. We conclude that in a restricted number of cases of Hodgkin's disease, the H-RS cells express granzyme B, and therefore might be considered the neoplastic equivalent of either activated CTLs or NK cells. (Am J Pathol 1996, 148:233–240)

Reed-Sternberg (RS) cells and their mononuclear variants called Hodgkin's (H) cells are considered to be the neoplastic cells in Hodgkin's disease. The nature of the progenitor cells of H-RS cells still remains the subject of considerable controversy (see for extensive review Kadin[1] or Drexler[2,3]).

In summary, immunophenotypical analysis of H-RS cells revealed a variable antigen expression. These cells characteristically express the non-lineage-specific activation markers CD30 and CD15, whereas either T- or B-cell lineage-specific markers can be detected in a minority of cases. Only in lymphocyte-predominant cases of Hodgkin's disease do the neoplastic cells consistently express B-cell-specific markers.[1,2] In studying the phenotypical characteristics of H-RS cells the Epstein-Barr virus (EBV) status is important, because EBV is able to modulate the expression of cellular genes in H-RS cells.[4]

To a certain extent these data are supported by molecular biological studies.[1,2,5,6] Using a recently developed method based on single cell polymerase chain reaction (PCR), the B-cell origin of neoplastic cells in lymphocyte-predominant Hodgkin's disease was recently confirmed by the detection of immunoglobulin (Ig)-rearrangements in individual neoplastic cells.[7,8] Using single cell PCR analysis, Kuppers et al[8] also reported the presence of Ig rearrangements in one case of nodular sclerosing (NS) Hodgkin's disease and in one case of mixed cellularity (MC) Hodgkin's disease, whereas others did not find evidence for either T-cell receptor or Ig rearrangements in NS and MC subtypes of Hodgkin's disease using this method.[9,10] Thus, although morphological and cytochemical studies do not give conclusive evidence as to the true nature of H-RS cells, the evidence on balance favors a lymphocyte derivation.

Supported by a grant from the Dutch Cancer society (VU 94-749).

Accepted for publication October 6, 1995.

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Recently, monoclonal antibodies (MAbs) directed against human granzymes were developed. These serine proteases are major components of the cytotoxic granules found in the cytoplasm of activated cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells.[11–13] Recent studies suggest that these granzymes play an important role in the induction of target cell DNA fragmentation and apoptosis during T-cell-mediated cytotoxicity[14,15]. Because expression of granzymes is restricted to NK cells and activated CTLs,[16] MAbs directed against these proteins can be used to identify activated CTLs or NK cells *in vivo.*[17] Of interest, granzymes might be a better marker for NK cells than conventional NK cell markers, given that conventional markers may be lost from the surface after activation.[18]

In a previous study, granzyme B expression was found in a considerable number of peripheral T-cell non-Hodgkin's lymphomas, especially those that originate from mucosa-associated lymphoid tissue.[19] Among 29 granzyme B-positive peripheral T-cell lymphomas, 4 were diagnosed as large cell anaplastic lymphoma. In some cases, the neoplastic cells in these lymphomas closely resemble the neoplastic cells in Hodgkin's disease. This, in combination with the possibility that H-RS cells may well be of activated lymphocyte origin,[1] prompted us to investigate whether the neoplastic cells present in Hodgkin's disease also express granzyme B and whether these cases had typical clinicopathological characteristics.

Materials and Methods

Patient Selection

Paraffin wax-embedded tissue specimens were selected from the files of the Free University Hospital Amsterdam (Amsterdam, The Netherlands; n = 34) and the Laboratory of Pathology, University Hospital Leiden (Leiden, The Netherlands; n = 27). Cases were classified according to the Rye classification.[20] Staging of the patients was done according to the Ann Arbor classification.[21] Clinical data were obtained from referring specialists. Patient characteristics are summarized in Table 1.

Table 1. *Patient Characteristics*

Characteristics	Granzyme B expression in RS and H cells, number of patients	
	Positive (N = 11)	Negative (N = 50)
Mean age (range)*	39 (17 to 62)	38 (10 to 78)
Sex (M/F)	6/5	26/24
Disease stage		
I	1	10
II	9	28
III	0	2
IV	1	6
unknown	0	4
Mediastinal involvement	2	9

The differences between cases with granzyme B-positive and granzyme B-negative RS and H cells were not significant, as determined by Pearson χ^2 test or Student's *t*-test.*

Immunohistochemistry

The production and characterization of MAbs against granzyme B has been described in detail elsewhere.[17] In summary, MAbs GrB7 (IgG2a) and GrB9 (IgG1) were raised against recombinant human granzyme B. Both MAbs react with isolated granzyme B from activated cytotoxic lymphocytes on immunoblot. Cross-blocking experiments showed that these two MAbs recognized two different epitopes of the granzyme B molecule.[17] In addition, both MAbs detect granzyme B-expressing cells in routinely formalin-fixed paraffin-embedded tissue sections.[22] The GrB9 MAb has also been shown to stain polymorphonuclear (PMN) leukocytes.[22] However, in cell lysates of PMN leukocytes, no granzyme proteins were detected using the GrB9 MAb on Western blot. This staining of PMN leukocytes in tissue sections with GrB9 is probably caused by cross-reactivity with PMN leukocyte-associated serine proteases.[22]

Immunostaining with GrB7 and GrB9 was performed after antigen retrieval of tissue sections by microwave treatment for 15 minutes in a citrate buffer (0.1 mol/L (pH 6.0). Incubation was performed for 1 hour at room temperature. After blocking endogenous peroxidases, immunoperoxidase staining was performed using a biotinylated rabbit anti-mouse antibody and the streptavidin-biotin horseradish peroxidase complex (ABC, DAKO, Glostrup, Denmark) as the second and third steps, respectively. The peroxidase reaction was visualized using diamino-benzidine-/H_2O_2 (Sigma Chemical Co., St. Louis, MO).

All cases were further characterized using a panel of MAbs and polyclonal antibodies on paraffin-embedded tissue sections. Antibodies used were: L26/CD20, polyclonal CD3, OPD4/CD45RO, BerH2/CD30, (all obtained from DAKO), Leu-M1/CD15 (Becton Dickinson, Mountain View, CA), and 144B/CD8 (kindly provided by Dr. D. Y. Mason, Oxford, UK).

The percentage of H-RS cells that stained positive for granzyme B was scored as <10%, 10 to 20%, 20 to

Table 2. *Characteristics of Cases of Hodgkin's Disease with Granzyme B-Positive Neoplastic Cells*

	Granzyme B expression									
	GrB7*	GrB9*	%†	EBV	CD30	CD15	CD3†	CD45RO†	CD8†	CD20†
89–3873	+	±	10–20	−	+	+	0	0	0	0
84–10760	+	+	<10	−	+	−	<10	0	0	0
83–13672	+	±	<10	+	−	+	0	0	0	0
87–2521	++	++	>50	−	+	+	<10	ND	ND	0
90–8059	+	+	10–20	+	+	+	0	0	0	0
86–8642	+	±	<10	+	+	+	0	0	0	0
84–9066	+	++	20–30	+	+	−	0	0	0	0
87–1787	+	+	<10	+	+	+	<10	0	0	0
90–8060	±	±	<10	−	+	+	0	0	0	0
79–5875	±	+	<10	+	+	+	0	0	<10	0
87–9549	±	+	<10	−	+	+	0	0	0	0

*Staining intensity for GrB7 and GrB9, respectively; ± = weak positive staining, + = clear staining signal and ++ = strong staining signal.
†Percentage of positive-staining H-RS cells.

30%, or >50%. The staining intensity was scored as follows (see Table 2); ± indicates weak positive staining, + indicates a clear staining signal, ++ indicates a strong staining signal. As negative control, sections were incubated with an irrelevant primary antibody of the appropriate subclass (anti-AT-III21, IgG1) and (anti-placental alkaline phosphatase, IgG2a) for GrB9 and GrB7, respectively.

RNA in Situ *Hybridization*

To show that granzyme B was produced by H-RS cells, RNA *in situ* hybridization (RISH) was performed to detect granzyme B expression at mRNA level in four cases in which granzyme B was found by immunohistochemistry. The RISH procedure has been described by us previously.[23] The sense and antisense riboprobes used were generated by *in vitro* transcription of a 350-bp *Pst*1 restriction fragment of granzyme B cDNA (nucleotides 1–350)[24] cloned in a pGem 7zf(+) vector (Promega, Madison, WI). The mRNA quality was checked by RISH using β2-microglobulin as control target mRNA.

Detection of the Presence of EBV in H-RS Cells

The presence of EBV in the H-RS cells was determined by RISH using the abundantly transcribed noncoding EBV small RNAs (EBER1 and 2)[23] and by the detection of latent membrane proton-1 using two sets of MAbs (CS1–4, DAKO and S12, Organon, Teknika, Oss, the Netherlands).[25]

Statistical Analysis

Statistical analysis was performed using the Pearson χ^2 test or the Student's *t*-test. Survival rates were calculated according to the method of Kaplan and Meier and differences were analyzed using Mantel-Cox statistics. Survival time was measured from time of initial diagnosis until death from disease or end of follow-up. Patients who died of causes unrelated to the disease were regarded as lost for follow-up in the statistical analysis. Progression-free survival time was measured from time of initial diagnosis until time of disease relapse. In patients without relapse, the progression-free survival time was identical to the overall survival time. *P* values <0.05 were considered significant.

Results

Granzyme B Expression in Control Lymphoid Tissues

Granzyme B expression in normal lymphoid tissues has been described previously by us.[22] Granzyme B-positive cells were detected in all lymphoid tissues tested. In nonneoplastic lymph nodes and tonsillar specimens, granzyme B-positive cells were scarce. When found, they were mostly confined to the sinuses or medullary cords. Phenotypic analysis revealed that most of the granzyme B-positive cells in normal lymphoid tissues and peripheral blood lymphocytes were NK cells.[22] Moreover, we tested three cases of infectious mononucleosis. In all three cases large numbers of granzyme B-positive lymphocytes (20 to 50% of all cells) were observed. As judged by morphology, most granzyme B-positive cells were small lymphocytes, but in all cases also medium-sized blastoid granzyme B-positive cells were found. However, the few cells with an H-RS cell-like appearance were granzyme B-negative.

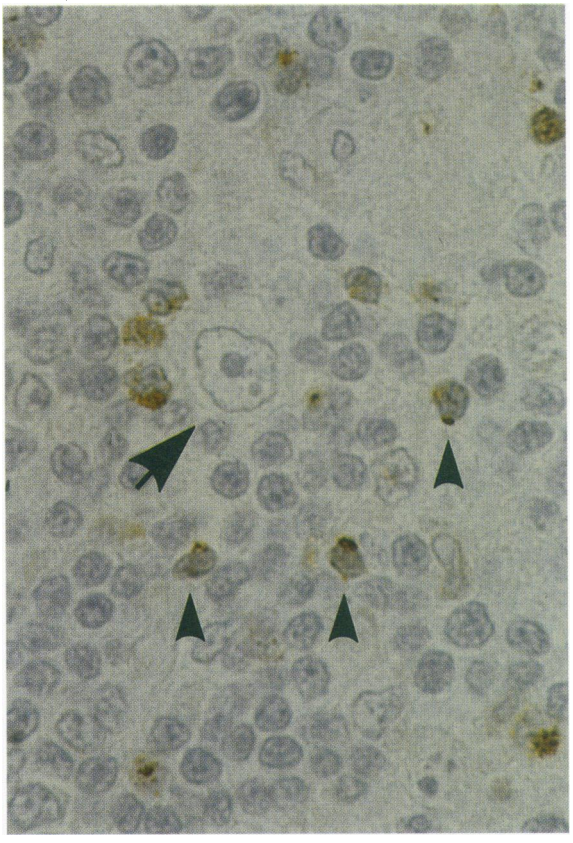

Figure 1. *Sporadic small non-neoplastic lymphocytes express granzyme B* (**small arrowheads**). *In this case no granzyme B expression is detected in the neoplastic cells* (**arrow**). *GrB7 staining, hematoxylin counterstaining, original magnification ×600.*

Granzyme B Expression in Reactive Cells in Hodgkin's Disease

In 57 out of 61 cases tested, granzyme B-positive small reactive lymphocytes were found. As expected, a granular staining pattern was observed in these cells using either GrB7 or GrB9 (Figures 1 and 2a). The percentage of lymphocytes expressing granzyme B ranged from <10% in the majority of cases to >20% in five cases. Although granzyme B-positive small lymphocytes were frequently found in the vicinity of the neoplastic cells, granzyme B expression was detected only sporadically in lymphocytes directly surrounding H-RS cells. Cross-reactivity with PMN leukocytes, as observed using the GrB9 antibody, was not detected using the GrB7 antibody, which is in agreement with previous results.[22]

Granzyme B Expression in H-RS Cells

In the neoplastic cells expression of granzyme B was detected in 11 of the 61 cases (18%; see Table 2). Nine of 46 cases of NS and 1 of 12 cases of MC Hodgkin's disease showed granzyme B-positive H-RS cells. Again a granular staining pattern was observed (Figure 2a). However, considerable differences were found in staining intensity as well as in the percentages of granzyme B-positive H-RS cells, ranging from weak staining in <5% of the H-RS cells to strong staining in >50% of the H-RS cells. Also within individual cases differences in staining intensity of H-RS cells were found. All 11 cases were positive for both GrB7 and GrB9 (Table 2). Staining intensity and number of positive cells were to a large extent comparable for both antibodies. In all cases, cells were negative using isotype matched irrelevant antibodies.

To demonstrate that granzyme B was produced by H-RS cells we used RISH to detect granzyme B-specific mRNAs in H-RS cells in four relatively strong granzyme B-positive Hodgkin's disease cases. Although in these four cases the RNA quality was good as shown by clear RISH signals for β2-microglobulin, granzyme B-specific mRNAs could only be demonstrated in the H-RS cells of one case (Figure 2b). This case (no. 87–2521) also showed the most intense granzyme B staining in the largest number of H-RS cells by immunohistochemistry. However, the number of granzyme B-positive H-RS cells was lower than the number of granzyme B-positive H-RS cells as determined by immunohistochemistry, indicating that RISH is a less sensitive method for the detection of granzyme B-positive cells than immunohistochemistry. This probably explains the absence of detectable granzyme B mRNAs in the other tested cases.

Immunophenotype of Cases with Granzyme B-Positive H-RS Cells

In 10 of 11 granzyme B-positive cases the CD30 antigen was detected in H-RS cells, and in 9 cases expression of CD15 was found. In none of the granzyme B-positive cases were CD20-positive neoplastic cells found, whereas in three cases CD3 expression was detected in a restricted number of H-RS cells (Table 2). No CD45RO expression was observed, whereas a few neoplastic cells in one case expressed CD8. Six of 11 granzyme B-positive cases were EBV-positive as shown by the expression of latent membrane protein-1 in the H-RS cells using the CS1–4 and S12 antibodies and by the presence of EBER1 and 2 in the nuclei of the H-RS cells. There was no significant difference between the percentage of granzyme B-positive cases in

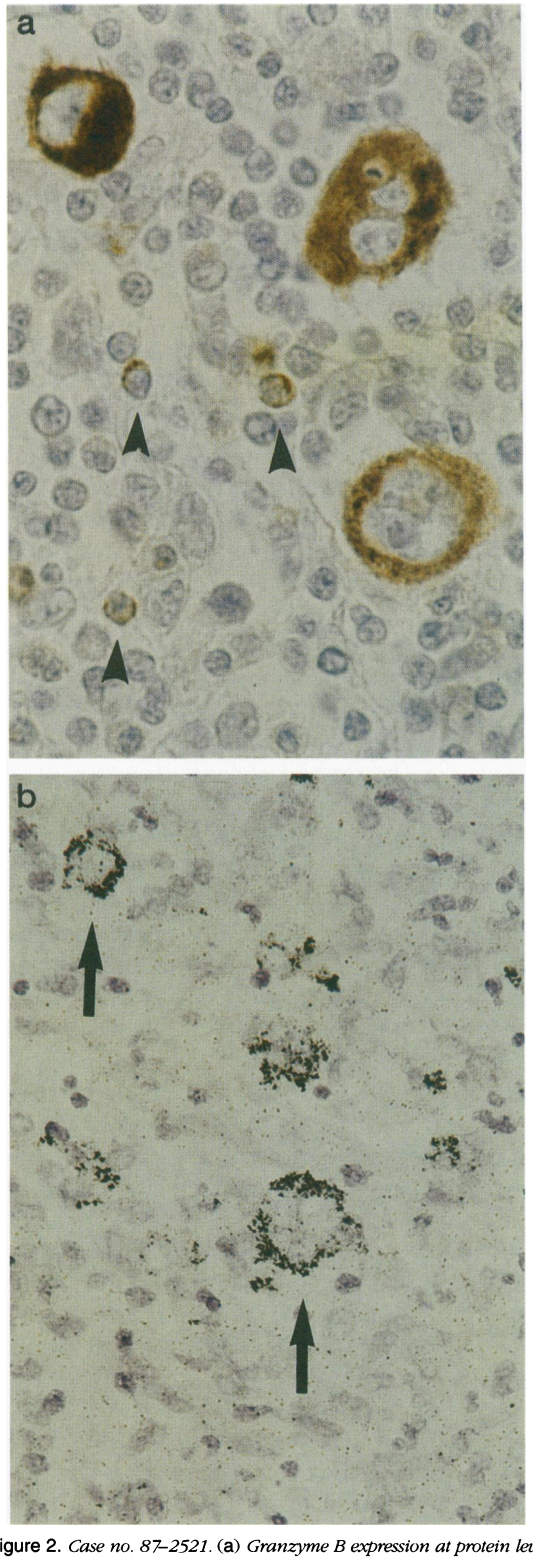

Figure 2. *Case no. 87–2521.* (a) *Granzyme B expression at protein level in neoplastic cells. Staining is relatively strong as compared with granzyme B-positive reactive lymphocytes* (small arrowheads). *GrB7 staining, hematoxylin counterstaining; original magnification ×600.* (b) *Granzyme B expression at mRNA level in neoplastic cells using RISH. A strong black staining is observed in the cytoplasm of the neoplastic cells* (arrows), *indicating the presence of granzyme B-specific mRNAs. Hematoxylin counterstaining, original magnification ×600.*

Table 3. *Tumor and H-RS Cell Characteristics*

Characteristics	Granzyme B expression in RS and H cells (no. of tumors)	
	Positive	Negative
Histology		
NS	9	37
MC	1	11
LP	0	0
LD	0	0
NOS	1	2
EBV status		
positive	6	20
negative	5	30
CD20		
positive	0	7
negative	11	27
CD3		
positive	3	8
negative	8	26
CD45RO		
positive	0	2
negative	11	27
CD8		
positive	1	2
negative	10	27

*The differences between cases with granzyme B positive and granzyme B negative H-RS cells were not significant, as determined by Pearson χ^2 test.

Abbreviations: NS, nodular sclerosing; MC, mixed cellularity; LP, lymphocyte predominant; LD, lymphocyte depleted; NOS, not otherwise classified.

EBV-positive and EBV-negative cases of Hodgkin's disease (6 of 26 *versus* 5 of 35; Table 3).

Clinical Features

No major differences were found in clinical presentation of granzyme B-positive *versus* granzyme B-negative cases (Table 1). In both groups, patients usually presented with stage I or stage II disease at time of diagnosis with tumor localizations in one or several lymph node groups in the neck region. Involvement of the mediastinum was found occasionally in both groups. Follow-up data were available from 55 patients. The mean follow-up times were 69 and 71 months for granzyme B-positive and -negative cases, respectively. Three of 11 patients with granzyme B-positive Hodgkin's disease died of their disease, *versus* 8 of 44 patients with granzyme B-negative Hodgkin's disease. Neither for the overall survival, nor for the progression free survival, was granzyme B status of importance ($P = 0.94$, $P = 0.88$, respectively).

Discussion

We have shown that in 11 of 61 cases (18%) of Hodgkin's disease expression of granzyme B was

detected in the H-RS cells. In none of these cases did the H-RS cells express B-cell markers, whereas in four cases T-cell markers, ie, CD3 and CD8, were expressed. No significant differences were found for EBV status or clinical presentation in relation to granzyme B expression in H-RS cells. To our knowledge, these are the first data indicating that in a restricted number of cases the H-RS cells might be the malignant counterpart of either activated CTLs or NK cells.

Of note, granzyme B positivity was found in 9 cases of NS and in one case of MC Hodgkin's disease. Although some cases of MC Hodgkin's disease are difficult to distinguish from anaplastic T-cell lymphomas, classical NS Hodgkin's disease is not. Therefore, it is very unlikely that erroneous classification of T-cell lymphomas as Hodgkin's disease contributes to our results.

We found heterogeneous expression of granzyme B within individual cases of Hodgkin's disease; not all the H-RS cells were granzyme B-positive, and considerable variation in granzyme B staining was found between the different H-RS cells. The level of granzyme expression in CTLs and NK cells depends on the state of activation.[26,27] Moreover, Chu et al[28] have demonstrated that in different biopsies of the same patient taken during the course of the disease, major phenotypic differences frequently occur, suggesting different stages of lymphocyte activation. Therefore, the heterogeneous expression of granzyme B in H-RS cells within individual cases might be explained by assuming that these cells pass through or are frozen in different consecutive stages of activation.

In principle, granzyme B-positive staining in H-RS cells could also be the result of an accumulation of granzyme B released by tumor cell-specific cytotoxic cells into the cytoplasm of the H-RS cells. However, several reasons indicate that this is probably not the case. 1) A distinct granular expression pattern was observed in H-RS cells, similar to the pattern observed in granzyme B-positive reactive lymphocytes (Figures 1 and 2a). This suggests accumulation of granzyme B within granules. The presence of specific granules in the cytoplasm of H-RS cells has been described before.[29] Moreover, granzyme B-positive small lymphocytes were only sporadically detected in the direct surrounding of H-RS cells. 2) Granzyme B-positive H-RS cells did not show more morphological signs of degeneration than granzyme B-negative H-RS cells in the same cases, or than H-RS cells in cases harboring no granzyme B-positive H-RS cells. Moreover, granzyme B-positive staining as a result of leakage of nonspecific proteins into the cytoplasm of H-RS cells is unlikely, because staining patterns observed using MAbs against serum proteins (eg, IgG and α-1 antichymotrypsine) were entirely different from the patterns observed using both granzyme B-specific MAbs (data not shown). 3) Using RISH, we were able to demonstrate granzyme B-specific mRNAs in H-RS cells in one of four immunohistochemically granzyme B-positive cases tested (Figure 2B), indicating that at least in this case, granzyme B is actually produced by the H-RS cells.

Finding granzyme B expression in Hodgkin's disease-derived cell lines would provide another argument for active granzyme B production within H-RS cells. In the one cell line available at our lab, HDLM-2,[3] no granzyme B expression was found (data not shown). However, given the low percentage of cases harboring granzyme B-positive H-RS cells, a large number of cell lines would have to be tested to find positive cell lines, which is beyond the scope of this article.

In normal lymphoid tissues, low levels of granzyme B expression are consistently detected in NK cells,[22] whereas in CTLs expression of granzyme B is only detected after stimulation with interleukin-2.[26] From our results, we cannot conclude whether H-RS cells represent NK cells or CTLs. Four of our 11 cases showed expression of CD3 and CD8 differentiation antigens, which favors a CTL origin of H-RS cells. CD3 and possibly also CD8 expression may have been down-regulated by the presence of EBV, as previously described by our group.[4] Therefore, the CTL origin might be underestimated. On the other hand, staining for the NK cell-specific CD56 marker could not be performed in paraffin-embedded tissue sections, and also CD56 might be down-regulated in activated NK cells.[18] Moreover, it would be of interest to investigate the expression of two other, more recently described proteins that are primarily expressed in cytotoxic cells, TIA-1[30] and PEN-5.[31] However, antibodies reactive with these antigens are not suitable for paraffin-embedded material.[31] Finally, CD3 and CD8 reactivity of H-RS cells was also observed in granzyme B-negative cases (Table 3).

We conclude that in a restricted number of cases of Hodgkin's disease, the H-RS cells express granzyme B, and therefore might be considered the neoplastic equivalent of either activated CTLs or NK cells.

Acknowledgments

We thank Anja Horstman and Wim Vos for excellent technical assistance, and Gerrit Meijer for assisting

with statistical analysis. We thank Dr. Jacp Middeldorp (Organon Teknika, Oss, The Netherlands for providing the S12 MAb.

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