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Short Communication

Epstein–Barr Virus Infection and *bcl-2* Proto-Oncogene Expression

Separate Events in the Pathogenesis of Hodgkin's Disease?

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The present study was undertaken to investigate whether Epstein-Barr virus- (EBV) encoded latent membrane protein (LMP) induces the expression of BCL-2 in Hodgkin and Reed-Sternberg (HRS) cells of Hodgkin's disease (HD) and thereby provide a possible mechanism for the role of EBV in the pathogenesis of this disease. Fifty-three cases of HD were studied for the presence of EBV using EBV-encoded RNA in situ hybridization and LMP immunohistochemistry. Immunostaining for BCL-2 on paraffin material was performed using microwave treatment of tissue sections before the application of the primary monoclonal antibody. EBV was located in HRS cells in 16 cases (30%). All cases that were EBV-encoded RNA in situ hybridization positive, also expressed LMP. BCL-2 expression in HRS cells was detected in 16 cases (30%), but only two of these were also EBV-positive. In both of these cases, only occasional HRS cells expressed BCL-2, in contrast to LMP, which was detected in nearly all such cells. BCL-2 staining was predominantly cytoplasmic with some membrane pattern. These results demonstrate that BCL-2 expression can be detected in HRS cells in routinely processed HD tissue and that whereas EBV does not induce the expression of BCL-2 in HD, BCL-2 may have a role in the pathogenesis of EBV-negative cases of HD. (Am J Pathol 1993, 143:1270–1274)

The etiology of Hodgkin's disease (HD) remains elusive. However, an association has been shown between some cases of HD and the presence of Epstein–Barr virus (EBV). Thus, raised antibody titres to EBV are present before the onset of the disease,[1] and monoclonal EBV genomes can be found in tissues from patients with HD,[2,3] indicating that viral infection was an early event in the disease process. The virus has also been localized to the Hodgkin and Reed–Sternberg (HRS) cells by *in situ* hybridization in a proportion of cases[3–6] and has been shown to persist throughout the course of the disease.[5] In addition, the virus has been shown to express at least one gene product with oncogenic potential, the latent membrane protein-1 (LMP-1), in HRS cells.[7] Despite this evidence indicating a causal relationship between EBV and HD in some cases, it is clear that EBV is not associated with all cases of HD.[1–7] Furthermore, the precise role of EBV in the pathogenesis of HD is far from clear.

Recent studies into the mechanisms of EBV-induced lymphocyte transformation point to a role for the virus in prolonging the lifespan of lymphoid cells, through the expression of viral-encoded latent gene

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products.[8] In particular, it has been shown that expression of LMP-1 protects lymphoid cells from apoptotic cell death, and that this action is mediated through the induction of the *bcl*-2 proto-oncogene.[9] Thus, one hypothesis for the role of EBV in the etiology of HD would be an induction of BCL-2 protein expression by LMP-1, leading to an increase in the lifespan of the affected cell. In addition, the demonstration of *bcl*-2 gene rearrangements in a proportion of cases of HD using the polymerase chain reaction[10,11] and the cytogenetic identification of the characteristic t(14;18) translocation[12] suggest that induction of BCL-2 protein expression in Hodgkin's disease could also occur independently of EBV infection. This study was designed to determine the relationship between EBV infection, LMP-1 expression, and BCL-2 in Hodgkin's disease. The results are discussed in the context of the proposed role of EBV in the pathogenesis of HD.

Materials and Methods

Formalin-fixed, paraffin-embedded material from 53 cases of HD was retrieved from the departmental files. Some of these cases have been studied previously.[6,11] Cases consisted of 37 males and 16 females, age range 14 to 67 (mean 34 years). On histological review, cases were classified as nodular sclerosis (n = 30), mixed cellularity (n = 16), lymphocyte predominant (n = 4), lymphocyte depleted (n = 2), and interfollicular (n = 1). EBV-positive controls consisted of a case of HD known to be positive[13] and EBV-transformed lymphoblastoid cell line. Ramos cell line (EBV-negative) was used as a negative control. Sections from a case of follicular lymphoma and reactive tonsil were used as controls for BCL-2 immunostaining.

The presence of EBV in HD tissue was examined by *in situ* hybridization using digoxigenin-labeled oligonucleotide probes targeted to EBV-encoded RNAs (EBERs), as previously described.[13] Immunostaining for EBV-encoded LMP was performed using a cocktail of four monoclonal antibodies (CS1–4 at 1/20 dilution) (Dako Ltd., High Wycombe, England), followed by avidin-biotin complex-peroxidase detection system (Vector Laboratories, Peterborough, England).

All cases were immunostained for BCL-2 protein expression using monoclonal antibody Bcl-2-124 (Dako). The procedure was carried out essentially as described by Cattoretti et al,[14] with some modifications. This technique has been shown to render paraffin sections suitable for immunostaining with some antibodies that are otherwise only applicable on cryostat sections.[14,15] Briefly, deparaffinized sections were blocked for endogenous peroxidase activity (0.5% H_2O_2 in methanol), rinsed in phosphate-buffered saline, and subsequently boiled in 0.01 mol/L citrate buffer (pH 6) for 10 minutes in a microwave oven at 750W (Toshiba, model ER 8650). Sections were cooled to room temperature and rinsed in PBS and primary antibody applied at 1/20 dilution in PBS. Sections were incubated in a moist chamber for 16 hours (overnight). The avidin-biotin complex peroxidase detection system was used.

Results

Detection of EBV

Strong *in situ* hybridization signals were seen in EBV-positive controls, but no such staining was detectable in the negative control (Ramos cell line). Of the 53 cases of HD, hybridization signals were observed in HRS cells in 16 cases (30%) (Table 1). In these cases, the virus was not exclusive to HRS cells, and occasional small lymphocytes were also positive. In a further nine cases (17%), the virus was exclusively localized to occasional small lymphocytes. EBV positivity varied according to different histological subtypes. A strong association between EBV and mixed cellularity subtype was observed; 69% of the cases in this group contained EBV in HRS cells compared to only 17% of nodular sclerosing type. The hybridization signal was intense and localized to the nucleus, with sparing of the nucleolus.

All the cases that were EBER *in situ* hybridization positive in HRS cells were also found to be immunoreactive for LMP. There was a complete correlation between the two methods. However, no LMP expression was observed in cases in which the virus was localized only to small lymphocytes. LMP immunostaining in HRS cells was generally very

Table 1. *EBV and the Expression of BCL-2 in Hodgkin's Disease*

Histological subtype	Number of cases	Staining in HRS cells		
		EBV +	Bcl-2 +	EBV & BCL-2 +
LP	4	0	2	0
NS	30	5	10	0
MC	16	11	4	2*
LD	2	0	0	0
IF	1	0	0	0
Total	53	16	16	2

* Less than 5% of the HRS cells expressed Bcl-2, but almost all were positive for LMP. NS = nodular sclerosis; MC = mixed cellularity; LP = lymphocyte predominant; LD = lymphocyte depleted; IF = interfollicular.

strong, with both cytoplasmic and membrane staining in almost all morphologically malignant cells (Figure 1a).

Detection of BCL-2

Immunostaining for BCL-2 protein in the case of follicular lymphoma, showed strong staining predominantly localized to the neoplastic follicles (Figure 2a). Some small lymphocytes scattered in the interfollicular regions also expressed BCL-2. In the reactive tonsil, BCL-2-positive cells were seen predominantly in the interfollicular region, with only occasional germinal center lymphocytes positive. Similarly, BCL-2-positive small lymphocytes were also seen in all 53 cases of HD examined (Figure 1b). Thus, these served as internal positive controls. However, of the 53 cases, BCL-2 expression in HRS cells was seen in 16 cases (30%) (Table 1, Figure 2b), and in five of these cases, only occasional BCL-2-positive HRS cells were seen. Of the 16 BCL-2-positive cases, EBV was found in the HRS cells in two (Table 1). In both of these cases, less than 5% of HRS cells were positive for BCL-2, but nearly all were positive for LMP.

Discussion

The finding that EBV-encoded LMP induces the expression of BCL-2 in cell lines[16] is of considerable interest given the possible involvement of this virus in the pathogenesis of Hodgkin's disease.[1–7] The product of the *bcl*-2 proto-oncogene is a 25-kd protein, which has been located to the inner mitochondrial and the nuclear membranes[16,17] and which acts to inhibit apoptosis in B cells, increasing the chances of further genetic changes.[16–19] Previous immunohistochemical studies have identified BCL-2 protein in the HRS cells of a proportion of cases of HD,[20–22] although others have failed to find such expression.[23,24] One report studying both EBV in-

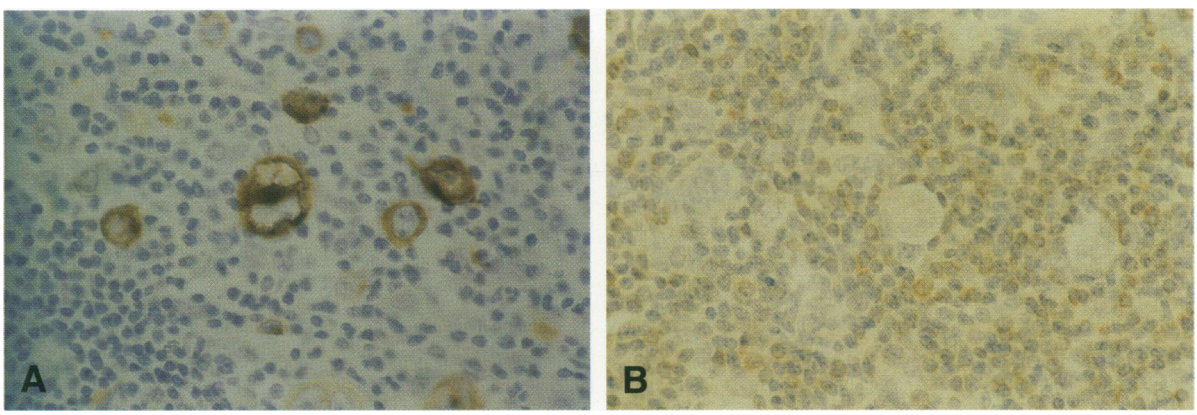

Figure 1. a: *LMP immunostaining in a case of mixed cellularity Hodgkin's disease. Strong cytoplasmic and membrane staining can be seen in virtually all HRS cells.* **b:** *Same case stained for BCL-2. Although strong BCL-2 staining can be seen in small lymphocytes, the malignant cells are clearly negative.*

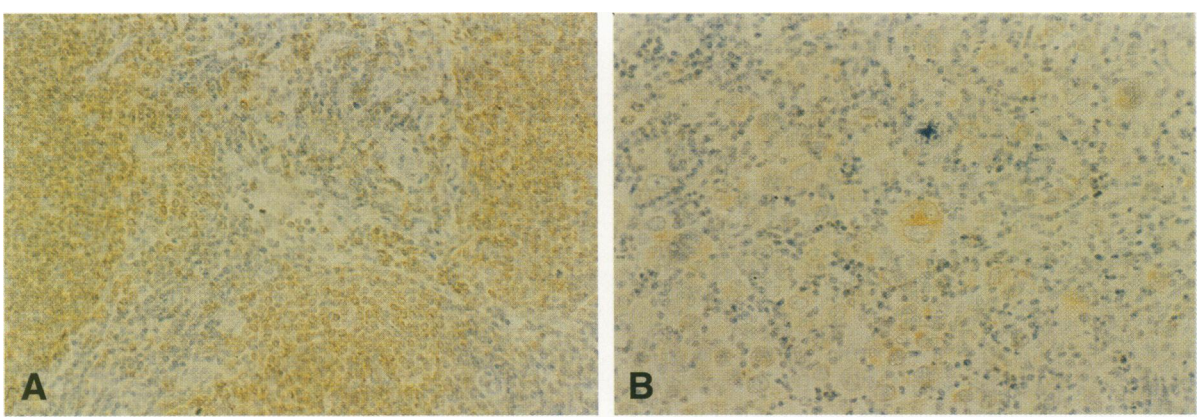

Figure 2. a: *Low power of a section of follicular lymphoma stained for BCL-2. Staining is predominantly localized to the neoplastic follicles. Some small lymphocytes scattered in the interfollicular regions also express BCL-2.* **b:** *A case of EBV-negative nodular sclerosing HD demonstrating cytoplasmic BCL-2 staining in HRS cells.*

fection and BCL-2 protein found no correlation between the two variables.[21] These authors performed BCL-2 immunostaining on fresh frozen material. In the present study, the expression of EBERs, LMP, and BCL-2 in 53 cases of HD has been investigated, using formalin-fixed, paraffin-embedded archival material. For BCL-2 immunostaining, sections were heat-treated in a microwave oven before the application of the BCL-2 monoclonal antibody. This technique has recently been shown to render paraffin sections suitable for immunostaining with some antibodies that are otherwise only applicable on cryostat sections.[14,15]

EBV was demonstrated in HRS cells in 16 of the 53 cases HD examined using EBER *in situ* hybridization. All of the positive cases were subsequently found to express LMP, although only two of these showed BCL-2 staining in HRS cells. Furthermore, in both of these cases, only occasional HRS cells expressed BCL-2, but almost all these cells were LMP-immunopositive. The absence of BCL-2 expression in HRS cells in the majority of the EBV-positive cases cannot be attributed to methodology or lack of sensitivity of the detection system, because BCL-2-immunopositive small lymphocytes were seen in these cases. These results indicate that in contrast to data obtained from *in vitro* cell culture studies on lymphoid cells[9,25] LMP does not induce detectable levels of BCL-2 in HRS cells. Recently, Lu et al[26] reported similar findings in undifferentiated nasopharyngeal carcinoma. These authors found no significant correlation between the pattern of BCL-2 expression and EBV involvement. Whether EBV infection causes an up-regulation of BCL-2 in other situations *in vivo* therefore requires further study. It would be of particular interest to investigate the expression of BCL-2 in posttransplant lymphomas, because these lymphomas are also EBV-associated and express viral latent products, including LMP.[27]

Evidence of BCL-2 expression was also found in 14 cases of HD that showed no expression of LMP-1. In some of these, it is possible that a t(14; 18) may be responsible for the increased expression of this oncoprotein.[10–12] However, this is unlikely to be the only mechanism involved because many BCL-2-positive cases do not contain a demonstrable translocation.[22] In this series, a t(14;18) was only found in two of seven cases BCL-2-positive cases that were studied by polymerase chain reaction.[11] Furthermore, recent cytogenetic data show that t(14;18) is a rare event in HRS cells and the t(14;18) detected by polymerase chain reaction may be due to infiltrating lymphocytes.[28]

Our results have important implications for the possible roles of EBV and BCL-2 in HD.EBV does not cause an appreciable BCL-2 protein expression in HRS cells, and this result confirms the suggestion that EBV infection and BCL-2 protein levels are not correlated in HD.[21] It also suggests that EBV infection and BCL-2 expression may be independent events in the pathogenesis of HD. Thus, EBV may be involved in about one-third of all cases of HD, BCL-2 may be involved in another one-third, and as yet unknown factor(s) in the remaining cases. Whereas the role of EBV remains unclear, it is possible that the virus may inhibit apoptosis through the expression of other viral-encoded genes. For instance, the EBV-encoded BHRF1 gene has significant homology to *bcl-2* and is expressed transiently in some latently infected cells.[27,29] The expression of BCL-2 in HRS cells would be expected to prolong the survival of these cells, thereby contributing to the disease process.

In conclusion, we have demonstrated that EBV-encoded LMP-1 does not induce the expression of BCL-2 protein in HRS cells. Moreover, when BCL-2 is expressed at detectable levels in HRS cells, generally it is in cases that are EBV-negative. These findings suggest that EBV infection and BCL-2 expression may each have a role in the pathogenesis of HD, but they represent different events in a multistep pathway of oncogenesis. Further studies will be required to investigate the possibility that each is involved in the inhibition of apoptosis in HRS cells and to study possible synergies with proliferation-associated oncogenes, such as c-*myc*.[30,31]

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