Expression of c-*myc* and *bcl*-2 oncogene products in Reed-Sternberg cells independent of presence of Epstein-Barr virus

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Abstract

Aims: **To evaluate the expression of c-*myc* and *bcl*-2 oncogene products in Reed-Sternberg cells in Hodgkin's disease, especially in relation to Epstein-Barr virus infection and expression of EBV encoded latent membrane protein (LMP).**

Methods: **Tissues from 33 cases of Hodgkin's disease were studied for the presence of EBV DNA by polymerase chain reaction (PCR) and DNA in situ hybridisation (DISH), for the presence of EBER-1 and EBER-2 EBV RNA by RNA in situ hybridisation (RISH); and for the presence of LMP, *bcl*-2, and c-*myc* proteins by immunohistochemical staining.**

Results: **A substantial number of Reed-Sternberg cells expressed *bcl*-2 in 20 of 29 (69%) and c-*myc* in 30 of 32 (94%) Hodgkin's disease samples. In 18 of the 25 (72%) cases Reed-Sternberg cells expressed both oncogene products. Of these 18 cases, 10 (56%) were EBV-PCR positive; eight (44%) were EBV-PCR negative.**

Conclusions: **Reed-Sternberg cells in Hodgkin's disease frequently express both *bcl*-2 and c-*myc* oncogene products, suggesting that these oncogenes may act in concert in the pathogenesis of the disease. Moreover, the expression of c-*myc* and *bcl*-2 proteins in Reed-Sternberg cells is independent of EBV and LMP status.**

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Several (proto)-oncogenes are expressed in lymphoid malignancies. Recently, particular attention has been paid to the *bcl*-2 oncogene which is present on chromosome 18 and which can be deregulated through juxtaposition with the immunoglobulin heavy chain locus on chromosome 14, typical of most follicular lymphomas.[1 2] Using immunohistochemistry, *bcl*-2 protein expression can be found in a variety of B cell and T cell non-Hodgkin's lymphomas (NHL), including cases in which the translocation t(14:18) is absent, as well as in some cases of Hodgkin's disease.[3-5] The *bcl*-2 protein is localised in mitochondria and prolongs cell survival by inhibiting apoptosis.[6 7]

Expression of other oncogenes, such as c-*myc* and the *ras* family, is also found in both NHL and Hodgkin's disease.[7] The c-*myc* oncogene product is a nuclear protein involved in cell proliferation and differentiation and seems to play an important part in cell cycle control.[8-10] Activation of c-*myc* proto-oncogene, by translocation of the c-*myc* locus on chromosome 8 to the immunoglobulin loci on chromosomes 14, 2, or 22, has been described in Burkitt's lymphoma and other B cell lymphoproliferative conditions.[11-13]

Interplay between oncogenes is thought to be important in the genesis of tumours.[14] In this respect, cooperation between *bcl*-2 and c-*myc* is of particular interest in lymphoma because recent studies have shown that synergy between *bcl*-2 and c-*myc* oncogenes promotes immortalisation of pre-B cells in vitro and the development of high grade lymphoid tumours in transgenic mice.[15-17] Moreover, sequential *bcl*-2 and c-*myc* activation has been associated with the progression of follicular low grade NHL to high grade NHL[18] and with the occurrence of precursor B cell blast crisis of a follicular NHL presenting as "composite" NHL.[19]

Several lymphoid malignancies are associated with EBV.[20] The presence of EBV, shown by PCR, has been shown in about 50-60% of cases of Hodgkin's disease.[21 22] DNA in situ hybridisation (DISH) studies located EBV DNA in Reed-Sternberg cells and its mononuclear variants, the presumed neoplastic cells in Hodgkin's disease.[22 23] Furthermore, EBV has been shown to be active transcriptionally in a substantial number of cases of Hodgkin's disease. Indeed, the EBER RNA of EBV and the LMP protein have been detected in many cases of Hodgkin's disease.[24-26] On the basis of these data, it has been suggested that EBV might have a role in the pathogenesis of Hodgkin's disease.

Interestingly, there is circumstantial evidence that the EBV gene product, latent membrane protein (LMP)-1 can induce the expression of *bcl*-2 in vitro and that *c-myc* expression is upregulated in permissive lymphocytes after an EBV infection.[27 28]

Methods

Lymph nodes from 44 patients with Hodgkin's disease were used in this study. Forty were of the nodular-sclerosing type (NS), one of the lymphocyte predominant type and three were unclassified, because the lymph node showed evidence of partial disease. Six cases of non-malignant, reactive

lymph nodes were used as controls. Representative tissue specimens were fixed in buffered formaldehyde or a sublimate formaldehyde mixture and paraffin wax embedded and/or were snap frozen in liquid nitrogen. Tissue sections (5 μm) were mounted on APES (Sigma) coated glass slides for DISH and immunohistochemical staining or placed in an Eppendor/β tube for PCR analyses.

PCR was performed on all cases. PCR, using primers specific for, globin was performed first. When positive results were obtained, the samples were analysed by an EBV specific PCR. Finally, DISH, using an EBV specific probe, and RISH, using sense or anti-sense RNA probes of a combination of EBER-1 and 2 genes, was carried out on all EBV positive and negative cases.

After dewaxing in xylene and removal of the remaining xylene in methanol, the sections were dried for 10 minutes at 100°C. Double distilled H_2O (100 μl) was added, after which the tissue sections were boiled for 10–15 minutes at 100°C to release the DNA. To see whether suitable DNA was present, 10 μl were used for a β globin PCR (final volume 50 μl), as described previously.[22] The PCR mix comprised 10 mM TRIS-HCl, pH 9·6, 1·5 mM $MgCl_2$, 50 mM KCl, 1 mM dNTPs, 50 pM β globin or EBV specific primer A and B and 1 U Taq DNA polymerase (Perkin and Elmer).

Forty of the following cycles were used: 1 minute at 95°C and 3 minutes at 65°C. After gel electrophoresis (20% of the sample) only those samples were selected for the EBV specific PCR which showed a strong band of 110 base pairs. β globin negative samples were treated with 300 μg/ml proteinase K (Boeringer, Mannheim) for 30 minutes at 37°C, and after boiling them for 5 minutes, to inactivate the proteinase K, another β globin PCR was performed. Negative samples were excluded from this study. EBV specific PCR was performed with primers selected from the *Bam*HI W fragment (large internal repeat) of the 95·8 strain and spanned a length of 240 base pairs. Primer A consists of: 5'-CTCTGGTAGTGATTTGGCCC-3'; primer B of: 5'-GTGAAGTCACAAACAAGCCC-3'. An internal oligonucleotide was used for hybridisation purposes (sequence: 5'-AATCTGACACTTTAGAGCTCTGGAGGACCT-3'). After 40 cycles of 95°C for 1 minute and 65°C for 3 minutes, 20% of the sample was used for gel electrophresis. Thereafter, the samples were transferred on a nylon filter and hybridised with the ^{32}P labelled internal oligonucleotide probe.

DNA IN SITU HYBRIDISATION

The large internal repeat of 3·1 kilobases (*Bam*HI W fragment) of the EBV B95·8 strain was cloned in the plasmid pKUN. Purified EBV insert DNA was labelled with bio-11-dUTP (BRL, Gaithersburg, Maryland) by random primer labelling and purified by Sephadex G-50 filtration. As controls, pBR322, pKUN, and other irrelevant insert probes were used.

The DISH procedure has been described in detail elsewhere.[29] Briefly, endogenous peroxide of dewaxed tissue sections was blocked with 0·3% H_2O_2 methanol for 30 minutes. Thereafter, the tissue sections were treated with a proteolytic enzyme for various amounts of time. The following enzymes were used: 0·1% proteinase K in 0·05 M TRIS-HCl, pH 7·6, containing 5 mM EDTA; 0·25% pepsin (Sigma) in 0·2N HCl at 37°C. After the digestion procedure, the sections were incubated with 0·4% Triton-X 100 (LKB) in phosphate buffered saline (PBS). After dehydration in graded alcohols the sections were incubated in prehybridisation solution (containing 50% formadide, 2 × SSC, 10 × Denhart's solution, 10% dextran sulphate, 250 μg/ml sheared salmon sperm DNA). After washing in water the sections were dehydrated and air dried. Hybridisation mix, (10 μl), containing 1 ng/μl biotinylated EBV insert DNA or a control probe, was applied and mounted with a glass coverslip. After denaturation at 100°–105°C for 5 minutes the tissue sections were hybridised for 18 hours at 37°C. The biotinylated hybrids were detected as follows: incubation with normal rabbit serum (1 in 50; Dako) diluted in PBS for 10 minutes and monoclonal antibiotin (1 in 200; Boeringer) diluted in PBS/3% bovine serum albumin (BSA) for 30 minutes. The tissue sections were washed in 4 × SSC/1% non-fat dry milk. Thereafter the tissue sections were incubated with biotinylated rabbit-anti-mouse (1 in 500; Vector) in PBS/3% BSA. After washing in 4 × SSC/1% NFDM slides were incubated with streptavidin-horseradish peroxidase (1 in 500; Enzo) in PBS. Peroxidase was visualised by incubation for 10 minutes in 0·2 mg/ml diaminobenzidine, 0·002% H_2O_2, 0·07% $NiCl_2$ in 50 mM TRIS-HCl, pH 7·6. All incubation was performed at room temperature.

Silver enhancement of the diaminobenzidine was performed as described previously.[29] Briefly, after thorough washing in double distilled water and 1% sodium acetate, the tissue sections were put in an acid physical developer. This developer consisted of a freshly prepared 80% solution A, 10% solution B, and 10% solution C. Solution A consisted of 80 g sodium acetate/500 ml water, 5·6 ml concentrated acetic acid, 100 ml 1% silver nitrate and 10 ml of 1% cetylpyridinum chloride under vigorous stirring. This solution was kept overnight at 4°C before filtration and the addition of 60 ml of 1% Triton-X 100. Finally, the volume was increased to 800 ml using double distilled water. Stock solution B comprised 5% sodium tungstate. Stock solution C comprised 0·2% ascorbic acid.

RNA IN SITU HYBRIDISATION (RISH)

A mixture of biotinylated EBER-1 and EBER-2 RNA antisense or sense (control) probes were used (kindly donated by Dr LS Young). Paraffin wax sections were dewaxed

in xylol and dehydrated in graded alcohol. The sections were washed in water, treated with 2% lugol (5 minutes, washed in water, and treated with 2% sodium thiosulphate (2 minutes). Endogeneous peroxidase was blocked with 0·3% H_2O_2/methanol (30 minutes). Thereafter, the sections were treated with 0·0001 mg/ml proteinase K in 50 mM TRIS (pH 7·6), containing 5 mM EDTA (15 minutes at 37°C). After washing in PBS (pH 7·6) the sections were fixed in 4% paraformaldehyde/PBS, pH 7·6 (10 minutes). After washing in PBS (pH 7·6), 0·02 ml of hybridisation mix containing 0·002 ml 5 × SSC, 0·008 ml 25% dextran sulphate, 0·001 ml probe, and 0·009 ml formamide with t-RNA, to each tissue section. After denaturation at 60-65°C for 7 minutes the sections were hybridised for two hours in 55°C; washed in PBS (pH 7·6); washed in 0·1 × SSC (68°C for 1 hour), and incubated with 0·2 mg/ml RNase in 10 mM TRIS (pH 8) containing 1 mM EDTA and 0·5 M NaCl (37°C for 15 minutes). The sections were washed in PBS (pH 7·6); in 0·1 × SSC (68°C for 30 minutes), and again in PBS (pH 7·6).

The biotinylated hybrids were detected as follows: preincubation with normal horse serum diluted 1 in 20 in 1% blocking reagent/PBS; incubation with mouse-antibiotin antibody (Boeringer) diluted 1 in 200 in 1% blocking reagent/PBS; washing in 0·1% Tween-20/PBS; incubation with horse-anti-mouse antibody (Vector) diluted 1 in 250 in 1% blocking reagent/PBS; washing in 0·1% Tween-20/PBS; incubation with streptavidin horseradish peroxidase (Enzo) 1 in 250 in 1% blocking reagent/PBS. Peroxidase visualisation and silver enhancement were performed as in the DISH procedure.

Control paraffin wax sections were treated with RQ-DNase (Boeringer) or RNase A (Boeringer) or hybridised with irrelevant anti-sense RNA probes.

RQ DNase did not affect the positive signal obtained with the EBER anti-sense probe. This signal was abolished after treatment with RNase. Hybridisation with irrelevant anti-sense RNA probes gave no signal.

IMMUNOHISTOCHEMICAL STAINING

This was performed as described previously.[22-29] The monoclonal antibody for *bcl*-2 detection was kindly provided by Dr DY Mason. For c-*myc* detection two different monoclonal antibodies were used: 6E10 anti-c-*myc* (Cambridge Research Biochemicals) and anti-c-*myc* from NCI Repository Microbiological Associates, Inc. LMP was detected by use of a mixture of monoclonal antibodies CS 1–4 (Dakopatts). Evaluation of the staining for *bcl*-2 and c-*myc* was carried out as follows: staining intensity of anti-*bcl*-2 mantle-zone B lymphocytes was considered to be 2+ (moderate staining intensity). If the staining intensity was weaker, the staining was scored as 1+; if stronger it was scored as 3+. Staining with the anti-c-*myc* was scored as 1+ (weak staining), 2+ (moderate staining), or 3+ (strong staining). The JY and HL 60 cell lines were used, respectively, as controls for LMP and c-*myc* immunoreactivities. Substitution of the primary antibody with non-immune serum, was used as a negative control.

Results

DETECTION OF EBV-DNA, EBER-1/2 RNA, AND LMP

Positive signals for , globin were obtained in 33 of 44 cases of Hodgkin's disease. These 33 cases were analysed with the EBV specific PCR. Nineteen (57%) out of 33 cases were found positive after hybridisation with the specific oligonucleotide probe. Both positive and negative cases were subjected to the DISH procedure: 10 of 19 PCR EBV positive cases were DISH positive: none of the PCR EBV negative cases gave positive results with the DISH. The hybridisation signal was found in the nuclei of the Reed-Sternberg cells. Sixteen EBV PCR positive and three PCR EBV negative cases of Hodgkin's disease were subjected to the EBV EBER-1/2 RISH procedure; 12 of 16 PCR EBV positive cases were RISH positive, the signal being present in the nuclei of Reed-Sternberg cells and of some small and medium sized lymphoid cells (fig 1). None of the PCR EBV negative cases was RISH EBV positive. Of the 19 EBV PCR positive cases, 12 (63·2%), were LMP positive, the staining being present on the cell membrane and the cytoplasm of the Reed-Sternberg cells (figs 2 and 3). No LMP positivity was found in PCR EBV negative cases. All DISH positive cases were also positive with RISH and LMP detection. Two DISH negative cases were positive with RISH and LMP. All reactive lymph nodes remained negative by PCR, DISH, and RISH and did not stain with anti-LMP antibodies.

DETECTION OF BCL-2 AND C-MYC ONCOGENE PRODUCTS

Cytoplasmic staining of *bcl*-2 was found in small lymphocytes, both in mantle zone and T cell areas, in all cases of reactive lymph nodes. These results were only obtained when frozen tissue sections or sublimate formol fixed tissue sections were used. On formalin fixed tissue sections only weak and less reproducible results were obtained. In 29 cases of Hodgkin's disease (28 nodular-sclerosing type and one LPHD type), *bcl*-2 staining gave reliable results. In 20 of 29 (69%) cases of Hodgkin's disease (all of nodular-sclerosing type), *bcl*-2 positivity was found in the cytoplasm of a variable number of Reed-Sternberg cells (fig 4). The number of positive Reed-Sternberg cells in *bcl*-2 positive cases varied between 20 – >90%. The staining intensity varied from 1+ (7 cases), 2+ (8 cases), to 3+ (5 cases) (fig 3). *bcl*-2 staining (2+) was found in small lymphoid cells in all 29 cases of HD.

No staining with c-*myc* antibodies was obtained in reactive lymph nodes; no staining was observed in germinal centres. In 32 cases of Hodgkin's disease (29 nodular-sclerosing, one lymphocyte predominant Hodgkin's

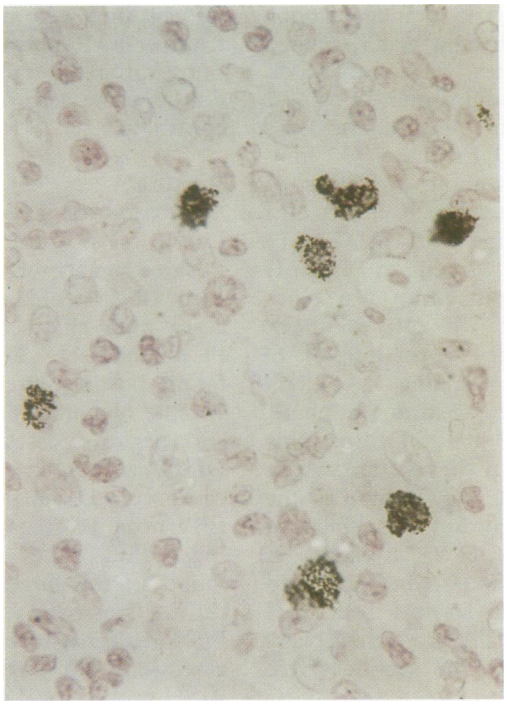


Figure 1 RNA in situ hybridisation using biotinylated EBER-1 and 2 anti-sense RNA probes on a Hodgkin's lymphoma. The signal is visualised using DAB-Ni and silver enhancement. Note that small and intermediate sized cells stain positive. Reed-Sternberg cells also stain positive (see fig 3) (counterstained with haematoxylin).

disease, and two UCL), c-*myc* staining gave reliable results. Best results were obtained when frozen tissue sections or formalin fixed tissue sections were used in combination with the monoclonal antibody from NCI. As with the *blc*-2 antibody, the number of positive Reed-Sternberg cells varied between 20 – > 90% in c-*myc* positive Hodgkin's disease. In 30 of 32 (94%) cases of Hodgkin's disease (29 nodular sclerosing and one lymphocyte predominant Hodgkin's disease), the c-*myc* was present in the nuclei and/or the cytoplasm of a substantial number of Reed-Sternberg cells (figs 5A and 5B). In seven cases some small lymphocytes and histiocytes also showed c-*myc* nuclear staining (mostly 1+). Staining intensity of the Reed-Sternberg cells in 10 cases was 1+, in 17 cases 2+, and in three cases 3+.

RELATION BETWEEN EBV OR LMP STATUS AND BCL-2 OR C-MYC ONCOGENE PRODUCT EXPRESSION IN HODGKIN'S DISEASE

bcl-2 was found in 10 of 17 (59%) cases of EBV positive Hodgkin's disease compared with 10 of 12 (83%) in EBV negative Hodgkin's disease. The intensity of the staining and its relation with EBV status is summarised in table 1. The relation between LMP status and *bcl*-2 is shown in table 2. From the 12 LMP positive cases 10 could be examined for the presence of *bcl*-2. In five LMP positive cases *bcl*-2 expression was one case 1+, three cases 2+, and one case 3+. Comparison between c-*myc* staining results and EBV status is summarised in table 1. Seventeen out of 18 (94%) EBV PCR positive cases expressed c-*myc* compared both 13 of 14 (93%) of the EBV/PCR negative cases. As shown in tables 1, 2, and 3, no correlation between EBV or LMP status and *bcl*-2 or c-*myc* expression was found.

COEXPRESSION OF BCL-2 AND C-MYC PROTEINS IN HODGKIN'S DISEASE

In 18 of 25 (72%) of the cases the lymphomas were positive for both *bcl*-2 and c-*myc*. In relation to the EBV status the data were as follows: 10 of 18 (56%) were EBV/PCR positive compared with eight of 18 (44%) EBV PCR negative cases. In all these cases *bcl*-2 positive Reed-Sternberg cells as well as *bcl*-2 negative RS cells were present. The same was observed for the c-*myc* expression.

Discussion

The c-*myc* immunostaining pattern was mainly nuclear. This is compatible with the expected distribution of c-*myc*, a DNA binding protein.[30] Cytoplasmic c-*myc* staining found may be due to tissue fixation and processing artefacts[31] or it may be related to the variable intracellular localisation of the protein, depending on the degree of cell differentiation.[32] The *bcl*-2 staining was cytoplasmic, as described previously.[3]

We detected the *bcl*-2 protein in Reed-Sternberg cells in 20 of 29 (69%) of Hodgkin's disease cases and in small lymphoid cells in all 29 cases. The c-*myc* protein was observed in Reed-Sternberg cells in 30 of 32 (94%) cases while in only seven cases were some small lymphoid cells and histiocytes

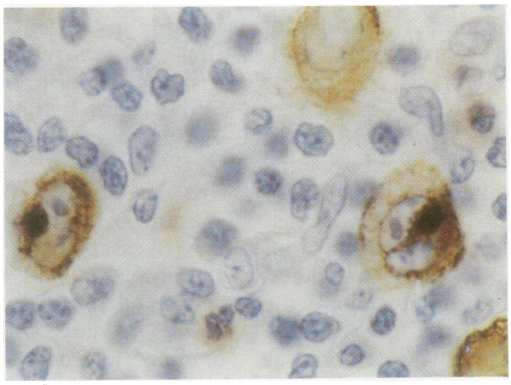


Figure 2 Reed-Sternberg cells stained with monoclonal antibodies against latent membrane protein, visualised with DAB (counterstained with haematoxylin).

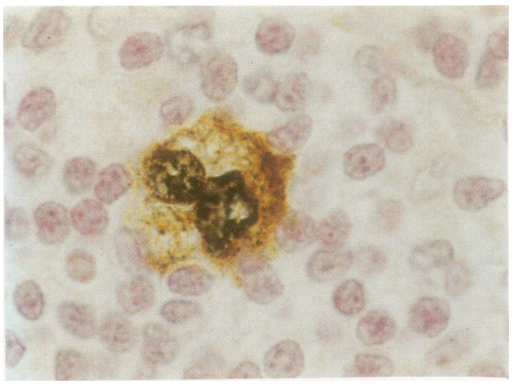


Figure 3 Combination of RNA in situ hybridisation using EBER-1 and 2 probes as described in figure 1, and immunohistochemistry for the detection of latent membrane protein. Reed-Sternberg cell stains clearly positive for EBER-RNA (black) and latent membrane protein (brown) (counterstained with haematoxylin).

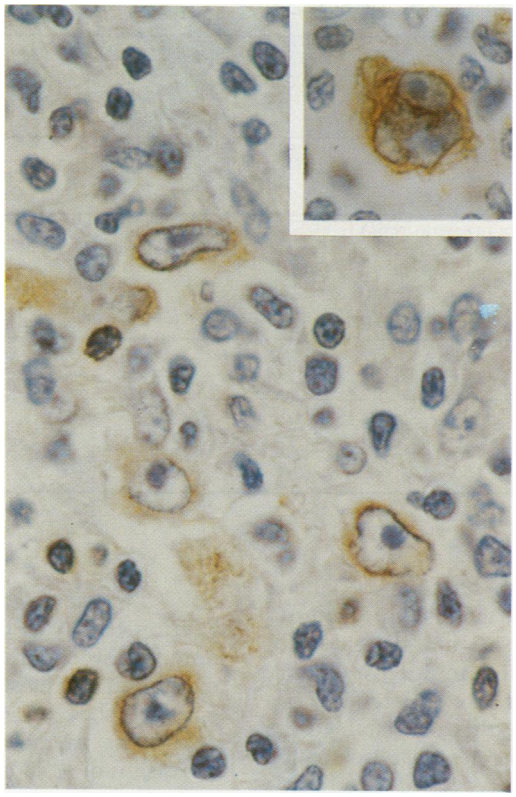


Figure 4 Hodgkin's lymphoma stained with anti-bcl-2 antibody.

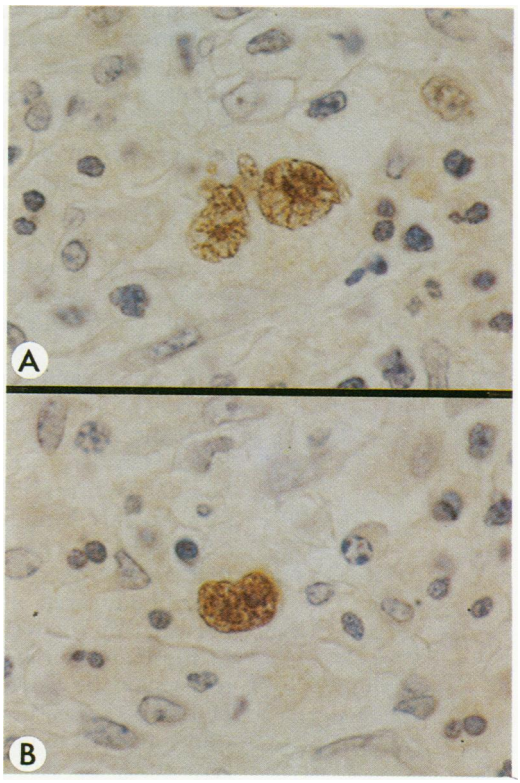
stained. These findings indicate that c-*myc* is expressed more frequently than *bcl*-2 in Hodgkin's disease and it is restricted more to the neoplastic cells of Hodgkin's disease. In addition, c-*myc* expression was generally more intense in Reed-Sternberg cells than in non-neoplastic cells. This agrees with previous findings that tumour cells in NHL express c-*myc* more intensely than non-neoplastic lymphoid cells.[7] Because c-*myc* expression is involved in cell proliferation[8 33] the increased c-*myc* expression in Reed-Sternberg cells in Hodgkin's disease might reflect a higher proliferation rate of these cells when compared with the non-neoplastic cells. The high Ki-67 score in Reed-Sternberg cells also supports this suggestion.[34 35] These data raise the question whether increased c-*myc* expression in Reed-Sternberg cells is also involved in the pathogenesis of Hodgkin's disease. There is growing evidence that oncogene products acting as growth factors, growth factor receptors or intracellular second messengers may play an important part in oncogenesis.[36 37] Recent data suggest that c-*myc* may be involved in the receptor pathway whereby the post-receptor message is transmitted to the nucleus. Activation of c-*myc* could bypass the requirement for the relevant growth factor leading to autonomous cell growth.[38 39] In this regard the increased c-*myc* expression in Reed-Sternberg cells could contribute to the autonomous growth of these cells and thus in the pathogenesis of Hodgkin's disease.

Our finding that Reed-Sternberg cells express the *bcl*-2 protein is of interest because these cells show a high proliferation rate, as evidenced by the high Ki-67 score[34 35]; in non-malignant lymphoid tissue proliferating cells fail to express *bcl*-2 protein.[3]

It could be suggested that Reed-Sternberg cells show aberrant expression of *bcl*-2 protein, reflecting deregulation of the *bcl*-2 gene expression. A potential event causing this deregulation could be the t(14;18) translocation. However, the data from molecular biology studies are contradictory. Whereas Stetler-Stevenson *et al*[40] found *bcl*-2 translocation by PCR in 17 of 53 lymph nodes with Hodgkin's disease, other studies, also using the PCR procedure, failed to confirm these results in 81 Hodgkin's disease cases.[5 41] The latter studies did not have clear explanations for these discrepancies. On the other hand, the data from immunohistological studies are also contradictory. Indeed, Pezzela *et al*[3] and Louie *et al*[41] found no *bcl*-2 staining in Reed-Sternberg cells; Zutter *et al*[4] detected weak *bcl*-2 expression in Reed-Sternberg cells in five of nine cases of Hodgkin's disease, whereas Schmid *et al*[5] found that a minority of Reed-Sternberg cells show moderate or intense staining for *bcl*-2 in all 20 cases of

Table 1 Relation between EBV status, bcl-2 and c-myc expression in HD; EBV positivity was detected by PCR

	Hodgkin's disease	*EBV positive*	*EBV negative*
bcl-2:			
	9	7 (41%)	2 (16%)
1+	7	4 (24%)	3 (25%)
2+	8	5 (29%)	3 (25%)
3+	5	1 (6%)	4 (33%)
Total	29	17	12
c-myc			
	2	1 (6%)	1 (7%)
1+	10	7 (39%)	3 (21%)
2+	17	8 (44%)	9 (64%)
3+	3	2 (11%)	1 (7%)
Total	32	18	14



Figures 5A and B Hodgkin's lymphoma stained with anti-c-myc antibody.

Table 2 Relation between latent membrane protein and bcl-2 expression in EBV PCR positive Hodgkin's disease

bcl-2	Hodgkin's disease	EBV-PCR positive Hodgkin's disease (n = 19*)	
		Latent membrane protein positive	Latent membrane protein negative
+	10	5 (56)	5 (62·5%)
-	7	4 (44)	3 (37·5%)
Total	17	9	8

*Bcl-2 detection was unreliable in two patients due to high background staining.

Table 3 Coexpression of bcl-2 and c-myc in Hodgkin's disease in relation to presence of EBV DNA (detected by PCR)

Epstein-Barr virus	bcl-2 + / c-myc +	bcl-2 − / c-myc +	bcl-2 + / c-myc −	bcl-2 − / c-myc −
+	10	3	0	1
−	8	2	1	0
Total	18 (72%)	5 (20%)	1 (4%)	1 (n = 25) (4%)

Hodgkin's disease tested. These discrepancies may be due to different technical procedures (nature of the tissue, fixation and tissue processing, sensitivity of the immunohistochemical methods, antibodies, etc.). Attention has to be paid to the finding that *bcl*-2 stained the Reed-Sternberg cells in nodular sclerosing and mixed cellularity types of Hodgkin's disease,[4 5] while in lymphocyte predominant Hodgkin's disease the lymphocyte and histiocyte type of Reed-Sternberg cells were *bcl*-2 negative.[42] These data suggest that *bcl*-2 expression might be used as an adjunct in the differential diagnosis between lymphocyte predominant Hodgkin's disease and other subtypes of Hodgkin's disease.

Of particular interest is our finding that Reed-Sternberg cells expressed both *bcl*-2 and c-*myc* proteins in 18 of 25 (72%) cases of Hodgkin's disease. This finding, in view of previous data that *bcl*-2 cooperates with c-*myc* to immortalise B cells in vitro and to promote the development of high grade lymphoid tumors in transgenic mice and in humans,[15-19] suggests that a cooperative action of these oncogenes may be involved in the pathogenesis of a substantial number of cases of Hodgkin's disease. As *bcl*-2 can prolong cell survival by blocking programmed cell death,[6] it could be hypothesised that in Hodgkin's disease *bcl*-2 gives a distinct survival signal to a cell clone and may contribute to lymphomagenesis by allowing this clone to persist until other signals, such as c-*myc* activation further propagates it to malignancy. However, the lack of detectable *bcl*-2 protein in Reed-Sternberg cells in some cases of Hodgkin's disease of this study does not conform to this hypothesis. *bcl*-2 protein might be present but has undergone mutations so that the epitope recognised by the monoclonal antibody used in this study is no longer present. Alternatively, mechanisms unrelated to the inappropriate expression of *bcl*-2 protein may be involved in a minority of Hodgkin's disease cases. Further investigation is needed to clarify this issue. In the present study we also focused on the question whether in Hodgkin's disease there is a correlation between *bcl*-2 or c-*myc* expression and EBV, or latent membrane protein status determined by PCR, DISH, RISH and immunohistochemistry.[22 26] In vitro studies on lymphoid cells showed that EBV infection increases the expression of c-*myc* oncogene and latent membrane protein induces the expression of *bcl*-2 oncogene product, thereby preventing programmed cell death.[27 28] Our findings show, however, that *bcl*-2 or c-*myc* are expressed in Reed-Sternberg cells in Hodgkin's disease independently of the EBV DNA, RNA or LMP status. In addition, we observed in some cases that EBV and latent membrane protein positive Reed-Sternberg cells do not express c-*myc* and *bcl*-2 proteins, respectively. This does not conform to the in vitro data[27 28] and suggests that further investigation is required to elucidate the regulation of the expression of *bcl*-2 and c-*myc* in EBV infected cells in vivo.

In summary, this study shows frequent expression of both *bcl*-2 and c-*myc* proteins in Reed-Sternberg cells in Hodgkin's disease. In view of the synergy between these oncogenes in the immortalisation of lymphoid cells in vitro and the development of high grade lymphoid malignancies in transgenic mice and in man, our data suggest that the cooperation of *bcl*-2 and c-*myc* may be involved in the pathogenesis of a substantial number of Hodgkin's disease cases. In addition, we show that *bcl*-2 and c-*myc* are expressed in Hodgkin's disease independently of EBV-DNA, RNA and/or latent membrane protein status.

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