

# Immunohistochemical demonstration of different latent membrane protein-1 epitopes of Epstein-Barr virus in lymphoproliferative diseases

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## Abstract

**Aim**—To compare the immunoreactivity of monoclonal antibodies S12 and CS1-4, which recognise different epitopes of the Epstein-Barr virus (EBV) latent membrane protein-1 (LMP-1), in EBV associated benign and malignant lymphoproliferative disorders and control tissues processed using different methods.

**Results**—Both monoclonal antibodies gave comparable results on frozen tissue sections and formalin fixed, paraffin wax embedded samples from cases with Hodgkin's disease and infectious mononucleosis. In all cases S12 stained more cells than CS1-4. For EBV associated B and T non-Hodgkin's lymphomas, frozen tissue sections yielded better LMP-1 staining results than formalin fixed material. Again, in all these cases S12 stained more cells and gave stronger results than CS1-4. For EBV negative tissues, both monoclonal antibodies showed cross-reactivity with melanocytic-like cells in the basal cell layer of the skin, synaptophysin-like staining in layers three and four of the cortex of the brain, and myelin-like staining in peripheral nerves and peripheral ganglion cells. Staining with S12 was always much stronger. Moreover, in contrast to CS1-4, S12 stained pancreatic islets in formalin fixed material but not in frozen tissue sections and sporadically stained solitary epithelial cells in the large bowel especially in formalin fixed tissue sections. CS1-4 also cross-reacted with myoepithelial cells around hair follicles and other adnexa of the skin.

**Conclusion**—The results indicate that for optimal detection of LMP-1, S12 yields better results than CS1-4 and that tissue processing is very important especially when B and T non-Hodgkin's lymphomas are examined.

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Keywords: Epstein-Barr virus, latent membrane protein-1, monoclonal antibody, Hodgkin's disease.

Epstein-Barr virus (EBV) is associated with different lymphoproliferative disorders, both benign and malignant.<sup>1-6</sup> Although the precise role of EBV in cellular transformation is unknown, different viral gene products seem to be

involved, in particular the Epstein-Barr nuclear antigens (EBNAs) and the latent membrane protein-1 (LMP-1).<sup>7</sup> At present, only a few EBV products can be demonstrated immunohistochemically, LMP-1 being one of these. LMP-1 has been detected in EBV associated Hodgkin's disease, B and T cell non-Hodgkin's lymphoma and in infectious mononucleosis.<sup>4,5,8</sup> This protein has transforming potential *in vitro*<sup>9-11</sup> and may be involved in the pathogenesis of EBV linked neoplastic diseases.

LMP-1 expression has been found in typical Reed-Sternberg (RS) cells and their mononuclear variants (Hodgkin; H cells). The frequency of LMP-1 positive H-RS cells may vary considerably, from 10 to 80%.<sup>12,13</sup> To date, it is not known why not all cells infected with EBV in a clonal population express LMP-1. In EBV positive Hodgkin's disease technical reasons, such as the type of fixation and fixation time etc., might explain why not all H-RS cells are stained. In non-Hodgkin's lymphoma arising in patients without an overt immunodeficiency only a few LMP-1 positive neoplastic cells could be detected, even when frozen tissue sections were examined.<sup>5,8</sup> Thus, before a correct interpretation of immunohistochemical findings of LMP-1 expression can be made, one must be certain that the immunohistochemical detection method used is reliable and as sensitive as possible.

LMP-1 expression in lymphomas has usually been investigated using one set of four different monoclonal antibodies: CS1-4.<sup>14</sup> In 1985 another monoclonal antibody against LMP-1, called S12 (OT17), was described.<sup>15</sup> Remarkably, the use of this monoclonal antibody has been only described for the detection of LMP-1 in EBV positive Hodgkin's disease thus far.<sup>16</sup> Both S12 and CS1-4 are directed against the same  $\beta$ -galactosidase fusion protein.<sup>17</sup> At least three different epitopes with marked differences in reactivity on western blots and immunocytochemistry are detected by CS1-4. On the other hand, S12 has a slightly different reaction pattern on western blots and on cell lines, suggesting that the epitope detected is not exactly the same.<sup>14,15</sup>

In the present study we compared the results of immunohistochemical staining with S12 and CS1-4 for the detection of LMP-1 positive cells in infectious mononucleosis, Hodgkin's disease and B and T non-Hodgkin's lymphoma

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in both immunocompetent and immunocompromised patients. Special attention was paid to tissue processing (formalin fixed, paraffin wax embedded versus frozen tissue sections), and cross-reactivity of the monoclonal antibody on normal tissue, EBV negative tissue and EBV negative neoplastic samples.

### Methods

The only tissues used in this study were those which harboured EBV DNA sequences as detected by the polymerase chain reaction (PCR) using primers selected from the large internal repeat of the B95-8 strain and which showed diffuse staining on RNA in situ hybridisation using EBER-1 and -2 probes. The EBV DNA PCR and the RNA in situ hybridisation procedures have been described in detail elsewhere.<sup>4,8,18</sup>

Five different groups of EBV associated lymphoproliferative disorders were included in this study: (1) tonsil tissue samples from four patients with serological and clinical evidence of infectious mononucleosis; (2) lymph nodes from five patients with Hodgkin's disease; (3) lymph nodes, large intestine, pancreas, liver, adrenal gland, and kidney involved in B immunoblastic lymphoma from four bone marrow transplant recipients; (4) three lymph nodes involved in B cell non-Hodgkin's lymphoma and three lymph nodes involved in T cell non-Hodgkin's lymphoma from six immunocompetent patients. Three non-malignant lymph nodes which reacted positively in the EBV DNA PCR and in which sporadic EBER-1 and -2 positive cells were detected were also included in this study.

The following non-malignant tissue samples were used: tonsil, hyperplastic lymph nodes, spleen, thymus, appendix, small and large intestine, stomach, liver, pancreas, lung, urinary bladder, kidney, prostate, thyroid, adrenal gland, pituitary gland, skin, testis, ovary and brain, and peripheral nerves.

On the basis of the reactivity with non-malignant tissue, histiocytosis X, insulinoma, and cutaneous melanoma tissue samples were also investigated.

### IMMUNOHISTOCHEMISTRY

S12 (OT17) IgG2a monoclonal antibody was obtained from Organon Teknika (Boxtel, The Netherlands). S12 was produced in a serum free hollow fibre cell culture system and subsequently purified over a protein A column. For immunohistochemistry, a final concentration of 0.1 µg/ml was used. S12 was detected using biotinylated horse antimouse IgG (diluted 1 in 250; Vector, Burlingame, California, USA), followed by Streptavidin-horseradish peroxidase (HRP) (diluted 1 in 500; Dako, Glostrup, Denmark) and visualised by diaminobenzidine (DAB; Sigma, St Louis, Missouri, USA) or amine-ethylcarbazole (AEC; Sigma) with 0.02% H<sub>2</sub>O<sub>2</sub> in phosphate buffered saline (PBS) (pH 7.4). Alternatively, S12 was detected using goat antimouse IgG2a and HRP (diluted 1 in 100; Southern Biotechnology Ass.,

Birmingham, UK) and directly visualised using DAB or AEC with 0.02% H<sub>2</sub>O<sub>2</sub>.

CS1-4 is a mixture of four different monoclonal antibodies of the IgG1 subclass and was obtained from Dako. Final concentrations used for immunohistochemistry ranged from 0.3 to 0.6 µg/ml. Detection was performed by either biotinylated goat antimouse IgG as described above or by goat antimouse IgG1/alkaline phosphatase (diluted 1 in 20; Southern Biotechnology Ass.) and visualised using naphthol AS-MX phosphate/fast blue BB/0.2 M Tris-HCl (pH 8.5) (Sigma). Endogenous alkaline phosphatase activity was blocked by adding 1 mM levamisol (Sigma) to the reaction mixture.

### TISSUE SECTIONS

Cryostat sections (4 µm) were mounted on poly-L-lysine coated (PLL; Sigma) slides and air-dried for at least one hour at room temperature. After drying, the sections were alternatively fixed for 10 minutes with 4% freshly made paraformaldehyde/PBS, 4% buffered formaldehyde, methanol (absolute), and acetone (absolute).

Formalin fixed, paraffin wax embedded tissue sections (5 µm) were mounted on PLL coated slides and routinely deparaffinised.

### DOUBLE STAINING PROCEDURES FOR THE SIMULTANEOUS DETECTION OF S12 AND CS1-4 AND BLOCKING EXPERIMENTS

For the simultaneous detection of S12 and CS1-4 antigens on tissue sections, five cases of EBV positive Hodgkin's disease were used. Formalin fixed, paraffin wax embedded tissue sections (5 µm) were routinely deparaffinised and incubated with S12 and CS1-4 for one hour. The IgG1 and IgG2a antibodies were detected by simultaneous incubation with goat antimouse IgG1 labelled with alkaline phosphatase (diluted 1 in 20) and goat antimouse IgG2a labelled with HRP (diluted 1 in 100). Thereafter, alkaline phosphatase was first visualised as described above, followed by the visualisation of HRP using AEC. To investigate whether S12 or CS1-4 could block all epitopes of the other anti-LMP-1 monoclonal antibody, tissue sections were pre-incubated with a tenfold excess of one monoclonal antibody whereafter the tissue sections were incubated with the other anti-LMP-1 monoclonal antibody.

Combination of immunohistochemical detection of LMP-1 and RNA in situ hybridisation using EBER-1 and -2 specific probes has been described extensively elsewhere.<sup>4</sup>

### Results

#### REACTION PATTERN OF S12 AND CS1-4 IN NORMAL TISSUE

The cross-reactivity of S12 and CS1-4 is summarised in the table. S12 showed reactivity with different cells in normal skin. Several cells in the basal layer showed cytoplasmic staining. Although the precise nature of these cells is

Reaction pattern of S12 and CS1-4 in normal tissue

Tissue	S12	CS1-4	Reactivity
Large intestine	Positive (P)	Negative	Sporadic epithelial cells
Appendix	Positive (P)	Negative	Sporadic epithelial cells
Pancreas	Positive (P,F)	Negative	Islets of Langerhans
Brain	Positive (P)	Weak (P)	Cytoplasmic staining in layers three and four of the cortex
Peripheral nerve	Positive (P,F)	Weak (P,F)	Myelin-like staining
Skin	Positive (P)	Weak (P)	Resembling dendritic melanotic cells (both antibodies). Myoepithelial cells surrounding hair follicles and other adnexa (CS1-4 only)

P=paraffin wax embedded tissue sections; F=frozen tissue sections. All other tissues tested, as listed in the Methods section, did not react with S12 or CS1-4; no cross-reactivity was observed in EBV negative non-malignant lymph nodes.

not clear, their morphology resembles that of dendritic melanocytes rather than Langerhans' cells, as the positive cells were exclusively found in the basal cell layer of the epidermis. In brain tissue synaptophysin-like staining was observed in layers three and four of the cortex. Peripheral ganglion cells and peripheral nerves showed myelin-like staining. Moreover, pancreatic islets showed cytoplasmic staining (fig 1). The pancreatic islets showed strong reactivity when formalin fixed, paraffin wax embedded material was used. Frozen tissue sections fixed with (para)formaldehyde also showed similar reactivity. No staining of the pancreatic islets was observed when the frozen tissue sections were fixed in acetone or methanol. Otherwise, results were independent of fixation.

CS1-4 also reacted with the basal cells in the epidermis of normal skin, but cross-reactivity in normal brain and peripheral nerve tissue was also observed. Staining with S12 was always much stronger. Moreover, in contrast to S12, myoepithelial cells and smooth muscle cells also showed faint cross-reactivity; no cross-reactivity with pancreatic islets was observed.

#### DETECTION OF LMP-1 IN EBV ASSOCIATED LYMPHOPROLIFERATIVE DISEASES

In EBV positive Hodgkin's disease both monoclonal antibodies stained H-RS cells (fig 2). Both formalin fixed, paraffin wax embedded and frozen tissue sections could be used. Besides the typical H-RS cells, S12 also stained a large number of "intermediate sized" cells (resembling an atypical blastoid morphology) and also a considerable number of small, morphologically non-malignant lymphoid cells. These smaller cells were only observed sporadically when CS1-4 was used. Double staining with S12 (IgG2a) and CS1-4 (IgG1) revealed that large atypical cells frequently showed "true" double staining (3A). Smaller cells reacted exclusively with S12 (figs 3B and 3C), whereas S12 negative/CS1-4 positive intermediate sized and small cells were never observed. Using EBER-1 and -2 probes in combination with S12 and CS1-4, the H-RS cells frequently showed staining for both the EBER-1 and -2 RNA and LMP-1 protein. This co-expression was also observed in a substantial number of the smaller EBER-1 and -2 positive cells (fig 4), but only when S12 was used. In all cases of EBV positive Hodgkin's disease a considerable number of EBER-1 and -2 positive large, intermediate and small cells could be

visualised without any LMP-1 being detected. These EBER positive/LMP-1 negative cells were present more frequently in tissue sections stained with CS1-4 than with S12.

In infectious mononucleosis and in the different EBV positive B and T non-Hodgkin's lymphoma cell sections the staining pattern was different. In all of these cases most of the EBER-1 and -2 positive cells were LMP-1 negative (fig 5). Frozen tissue sections fixed with 4% (para)formaldehyde, methanol or acetone showed much more positive cells compared with formalin fixed, paraffin wax embedded material. Again, S12 positive cells outnumbered the CS1-4 positive cells.

Finally, blocking experiments were performed to investigate whether S12 and CS1-4 recognise different epitopes. Pre-incubation with a tenfold excess of CS1-4 was unable to prevent specific binding of S12 and vice versa, indicating that, at least in part, different epitopes of the LMP-1 molecule were detected.

#### Discussion

The results of this study show that S12 has certain advantages over CS1-4 when EBV associated lymphoproliferative disorders were investigated for the expression of LMP-1; in EBV associated B and T cell non-Hodgkin's lymphoma more cells were stained and the signals were moderately stronger. The number of EBER-1/2 positive neoplastic cells outnumbered the LMP-1 positive cells in all cases. This indicates that despite the clonal character of these tumours, there is variability in EBV gene expression, particularly of LMP-1. This was also true for infectious mononucleosis where only some of the EBER-1 and -2 positive cells expressed detectable LMP-1. Although in Hodgkin's disease both S12 and CS1-4 could be used on formalin fixed, paraffin wax embedded material or frozen tissue sections, S12 stained more H-RS cells and also more smaller EBER-1 and -2 positive cells as observed using double staining techniques for EBER-1 and -2 messenger RNAs and immunohistochemistry for LMP-1 detection.

In previous studies CS3 and CS4 cross-reacted in immunoblots with certain cellular proteins of a molecular weight of 41 kDa and 44 kDa.<sup>14</sup> Although cross-reactivity was never observed on EBV negative/control lymphoid tissue, S12 has a slightly different reactivity pattern on normal human tissues than CS1-4. These differences are remarkable as both sets of monoclonal antibodies are directed against the same 46 kDa fusion protein.<sup>14,15</sup> Our data on double staining using both anti-LMP-1 antibodies and the observation that pre-incubation with S12 or CS1-4 could not prevent specific binding of the other anti-LMP-1 antibody confirm that different epitopes are detected. Similar results were observed in immunoblots: pre-incubation with CS1-4 was unable to prevent specific binding of S12 and vice versa (data not shown). The fact that the staining pattern of S12 in EBV infected cells was slightly different from that of CS1-4 suggests that the epitope of S12 is differentially

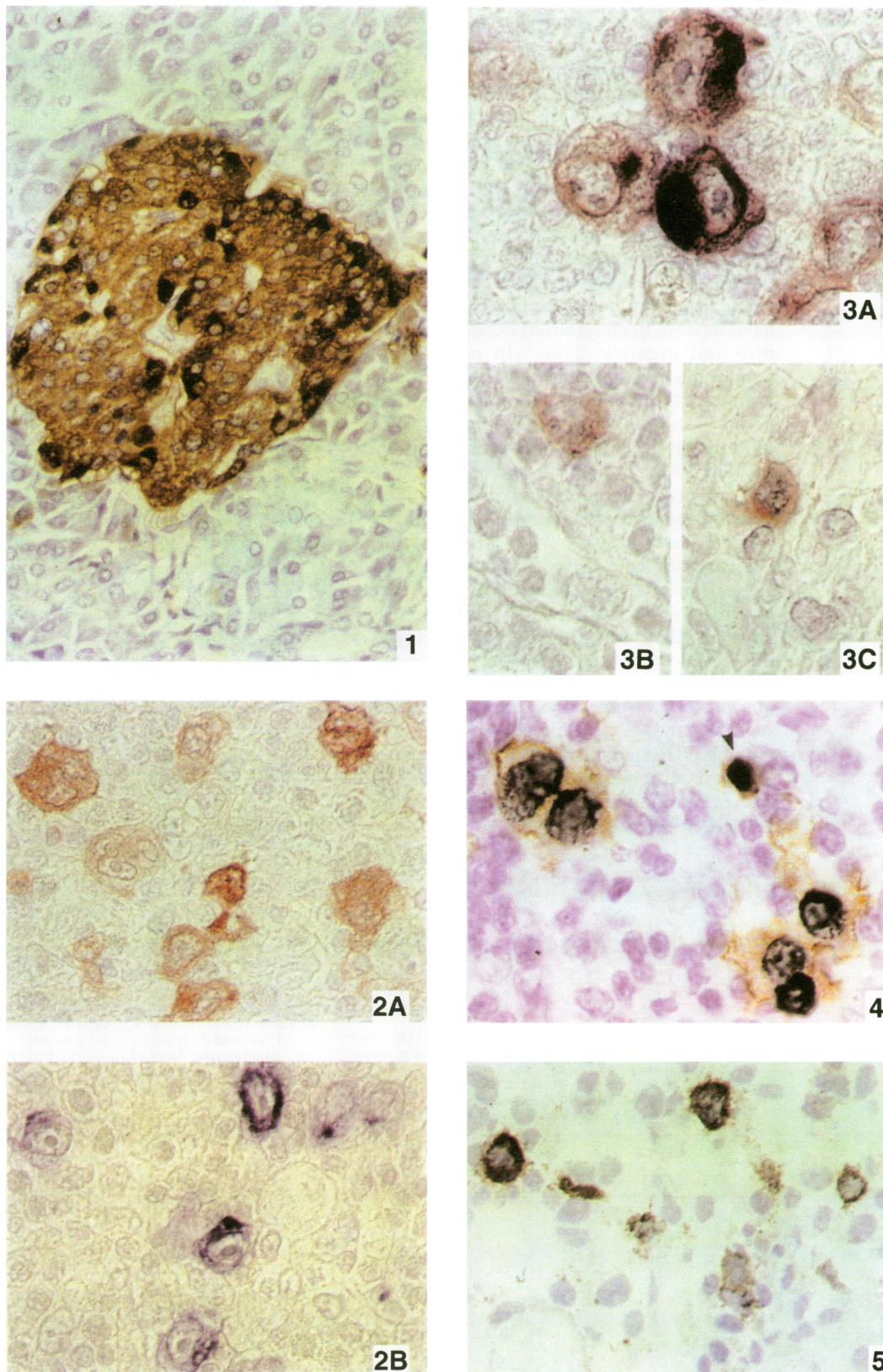


Figure 1 Formalin fixed, paraffin wax embedded tissue sections of normal pancreas stained with S12. Note the non-specific staining of the pancreatic islets by S12. Visualisation using DAB/H<sub>2</sub>O<sub>2</sub> (brown); original magnification × 300.  
 Figure 2 A: Lymph node with Hodgkin's disease. Large atypical cells reacted with S12. Visualisation using AEC/H<sub>2</sub>O<sub>2</sub> (red); original magnification × 520. B: The same lymph node stained with CS1-4. Visualisation using alkaline phosphatase (blue); original magnification × 520.  
 Figure 3 A: Double staining with S12 and CS1-4. S12 (IgG2a) was detected using a subclass specific antibody and was visualised with AEC/H<sub>2</sub>O<sub>2</sub>. Staining with CS1-4 (IgG1) was visualised using alkaline phosphatase (blue). Note that besides the double staining (mixture of red and blue), some red staining is also visible. B and C: Same section as fig 2A. Besides the large atypical cells showing double staining, some small, morphologically "non-malignant" cells were also stained, but only with S12 (red). No staining with CS1-4 was detected in these smaller cells. Original magnification × 825.  
 Figure 4 Combination between RNA in situ hybridisation using EBEB-1 and -2 RNA probes (visualisation using DAB-Ni/H<sub>2</sub>O<sub>2</sub> with silver enhancement; black grains) and LMP-1 detected by S12 (DAB/H<sub>2</sub>O<sub>2</sub>; brown). Note that not only are the large atypical cells stained with both, but also the morphologically "non-malignant" lymphoid cells (arrowhead). Original magnification × 825.  
 Figure 5 Immunoblastic B cell non-Hodgkin's lymphoma in a bone marrow transplant recipient stained with S12. Visualisation using AEC/H<sub>2</sub>O<sub>2</sub> (red); original magnification × 825.

expressed or more easily accessible than the epitopes recognised by CS1–4. In this regard, it is important to note that LMP-1 in its nascent form has a very short half life of two to five hours in vitro, is processed differently at the post-translational level and sub-fragments may localise to different cellular compartments.<sup>19,20</sup>

The clinical importance of LMP-1 expression in the different lymphoma groups is still obscure. It is known that EBV is more frequently present in more aggressive forms of Hodgkin's disease—for example, the mixed cellularity type,<sup>16</sup> whereas no differences in clinical behaviour have been reported between EBV positive and negative cases.<sup>21</sup> In non-Hodgkin's lymphoma little is known about the clinical importance of EBV infection. In one study of nodal T cell non-Hodgkin's lymphoma cases diffusely positive for EBER-1 and -2 also expressed LMP-1 on a limited number of the tumour cells. These cases had a significantly worse prognosis when compared with LMP-1 negative cases.<sup>8</sup>

Histopathological studies concerning LMP-1 expression in vivo might provide more insight into the role of LMP-1 in the pathogenesis of different EBV associated diseases. However, this requires optimal detection methods including immunohistochemistry. In this respect our data show that both S12 and CS1–4 detect specific, yet distinct, LMP-1 associated signals, although S12 detected more EBV positive cells and gave stronger signals than CS1–4. Therefore, we recommend S12 for the detection of LMP-1 in tissue sections.

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