Virchows Arch (2000) 436:553–559 

ORIGINAL ARTICLE

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# Immunoreactivity for latent membrane protein 1 of Epstein–Barr virus in nevi and melanomas is not related to the viral infection

Received: 30 September 1999 / Accepted: 9 December 1999

**Abstract** Epstein–Barr virus (EBV) is a human herpes virus with oncogenic potential, associated with several malignancies. The EBV-encoded latent membrane protein 1 (LMP1) is one of nine proteins regularly expressed in virally infected and immortalised B lymphocytes. We now document the consistent immunoreactivity for LMP1 in 90% of 65 nevi and melanomas, using the monoclonal antibody cocktail CS1–4. The immunocytochemical findings, however, were not confirmed using reverse-transcription polymerase chain reaction (RT-PCR) experiments, which failed to demonstrate any actual expression of LMP1 mRNA. In situ hybridisation for EBV-encoded RNAs (EBERs 1 and 2) and PCR amplification of EBV genomic sequences also failed to document any viral infection. Several normal and neoplastic human tissues have also been immunostained for LMP1, without any positive staining, with the exception of a minor percentage of skin melanocytes and of normal blasts of the myeloid and erythroid lineages. We conclude that the vast majority of nevi and melanomas express a still uncharacterised molecule, cross-reacting with anti-LMP1 (CS1–4) antibodies, which may be considered a consistent marker of melanocytic proliferations. The immunoreactivity of normal and neoplastic human tissues for the anti-LMP1 reagent should not be taken as evidence of EBV infection.

**Key words** Epstein-Barr virus · Melanoma · Nevi · LMP1 · EBERs

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## Introduction

The Epstein–Barr virus (EBV) is the causative agent of infectious mononucleosis [14]; it is ubiquitous in the population, more than 90–95% of the adult world population showing serological evidence of past infection [25]. EBV is also related to some human neoplasms, such as Burkitt's lymphoma [24], Hodgkin's disease [43], anaplastic large cell lymphoma [15], peripheral T-cell lymphoma [1], B-cell lymphomas and other tumours in immunocompromised individuals [11, 27] and nasopharyngeal carcinoma (NPC) [30]. EBV is commonly utilised to immortalise B lymphocytes and has been classified as a group-1 carcinogen by the World Health Organization (WHO) International Agency for Research on Cancer [16, 29].

The EBV-encoded latent membrane proteins (LMP1, LMP2A and LMP2B) are three of the viral proteins consistently expressed in EBV-immortalised B lymphocytes. LMP1 is also expressed in the majority of NPCs, but not in Burkitt's lymphoma. LMPs have been shown to transform a human epithelial cell line [6], and to inhibit epithelial differentiation [4]. LMP1 can transform rodent fibroblasts in vitro, rendering them oncogenic in nude mice, thus acting as a viral oncogene [40]. Moreover LMPs can induce B-cell activation markers and adhesion molecules [10, 41]. In EBV-infected B-cells, LMP1 up-regulates Bcl-2, preventing apoptosis [13, 33]. LMP1 also induces expression of the epidermal growth factor receptor [28] and interacts with several tumour necrosis factor (TNF) receptor-associated factors (TRAF1,-2,-3,-5) and the TNF receptor-associated death domain (TRADD) [17, 31]. This interaction leads to the activation of the nuclear factor (NF)-kB and c-Jun N-terminal kinase (JNK) pathways [5, 19, 38], suggesting that LMP1 acts as a constitutively activated member of the TNF receptor family, being similar though not identical to CD40 [9, 20].

Circularised EBV genome is required for the expression of LMP2A and LMP2B, and indeed during EBV persistence the expression of latent genes is restricted to

EBNA1 and LMP2A [22, 39]. LMP2A is important for preventing the switch from latent to replicative infection in response to B-cell activation [42]. The occurrence of anti-LMP2A and anti-LMP2B in sera of NPC patients is highly specific for EBV-associated tumours [21].

In the course of an immunocytochemical survey on the immunoreactivity of different human tissues for a commercially available monoclonal antibody (mAb) to LMP1 (CS1–4), we noticed strong immunoreactivity in the few cases of nevi and melanomas included in the experimental material. The CS1–4 mAb to LMP1 is a cocktail of four mAbs (CS1, CS2, CS3, and CS4) recognising at least three different epitopes on the hydrophilic carboxyl region of LMP1, which is exposed to the cytosol [32].

Prompted by this observation, we have extended our investigation to a large series of nevi and melanomas to ascertain the actual prevalence of LMP1 immunoreactivity and the possible occurrence of EBV infection in these lesions. In addition to immunostaining for LMP1, we have used different molecular biology techniques to evaluate the presence of EBV DNA and of EBV-encoded RNAs (EBERs), which are highly expressed in latent EBV infection [44, 31] but are dispensable for B-lymphocyte transformation in vitro [37].

## Materials and methods

Tissue samples of 55 malignant melanomas from 40 patients [12 superficial spreading melanomas (SSM), 8 nodular melanomas, 28 metastatic melanomas, 3 recurrent melanomas, 2 lentigo maligna and 2 SSM associated with dermal nevi] and of 10 nevi from 10 patients (2 junctional nevi, 1 Spitz nevus, 1 blue nevus, 2 compound nevi, 2 dermal nevi and 2 dysplastic nevi) were fixed in buffered formalin or Hollande solution and embedded in paraffin. Serial sections (5-µm thick) were cut for immunocytochemistry and in situ hybridisation (ISH) experiments. For comparison, a series of normal and diseased human tissues (Table 2 and Table 3) were also investigated for LMP1 immunoreactivity.

### Immunocytochemistry, ISH and Western blotting

Immunocytochemical experiments were performed according to the biotin–streptavidin detection system (Super Sensitive anti-mouse kit, Biogenex, S. Ramon, Calif.), using either peroxidase or alkaline phosphatase as the reporter enzyme. Endogenous peroxidase activity was blocked whenever appropriate with 3% $H_2O_2$ solution; treatment of tissue sections with a microwave irradiation-based antigen retrieval protocol [36] was consistently used; in selected cases, melanin pigments were removed before immunostaining using the potassium permanganate technique [23]. The commercially available mAb to LMP1 (CS1–4) (Dako, Glostrup, Denmark) was used at a 1:50 dilution in Tris-buffered saline (TBS) (pH 7.4); the individual clones of the cocktail (CS1, CS2, CS3 and CS4) were used at 0.75 µg/ml in the same buffer.

ISH for EBERs was performed using a commercially available kit containing a mixture of EBER 1 and 2 oligonucleotide probes (Dako), and following the manufacturer's instructions.

Known positive control tissues for both LMP1 immunostaining and ISH for EBERs included a nodular sclerosis variant of Hodgkin's disease, a Burkitt's lymphoma, a nasopharyngeal carcinoma, and lymphoblastoid and Raji cell lines. Negative control sections for immunocytochemical experiments were probed with an isotype-matched mAb (anti-CD21, Dako), whereas negative controls for EBER ISH were obtained by pre-treating the sections with RNAse or by omitting the specific oligonucleotide probes from the hybridisation mixture. LMP1 immunoreactivity and EBER 1–2 expression were independently evaluated as absent or present by two observers (G.B. and G.V.).

For Western blotting, cryopreserved tissue from three LMP1-immunoreactive melanomas and the Raji cell line were solubilised and separated by Laemmli discontinuous sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) before electroblotting onto nitrocellulose filter for probing with the CS1–4 mAb, as described previously [32]. Specifically bound antibody was detected using rabbit anti-mouse IgG (Dako) followed by incubation for 2 h with $^{125}$I-labelled staphylococcal protein A (Amersham Italia, Milan, Italy) diluted to 0.1 µCi/ml in phosphate-buffered saline (PBS)–milk. The filter was dried and subjected to autoradiography for 1–2 days with an intensifying screen.

### Polymerase chain reaction for EBV DNA

High molecular weight DNA was prepared from frozen tissue samples of seven melanomas by means of SDS–proteinase K digestion, extraction with phenolchloroform and ethanol precipitation [26], and was subjected to amplification by means of polymerase chain reaction (PCR) [34]. Specific primers were synthesised on published DNA sequences corresponding to the *Bam*H1W region of EBV [35]: 5'CCAGAGGTAAGTGGACTT3' and 5'GACCGGTGCCTTCTTAGG3'. Amplification of the BNLF1 gene of EBV [3] was performed by nested PCR, using the following pairs of primers: external 5'AGTTAGAGTC AGATTCATGGC3' and 5'CCTTTGCTCTCATGCTTATAA3', nested 5'CAACCAATAGAGTCCACCAGT3' and 5'TCTTCA GAAGAGACCTTCTCT3'. PCR mixtures contained 100 ng DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 50 pM each primer, 200 µM each dNTP (Pharmacia, Uppsala, Sweden) and 1.25 U *AmpliTaq* DNA polymerase (Perkin Elmer, Foster City, Calif.) in a final volume of 50 µl. The amplification was carried out using a Perkin-Elmer thermocycler (Gene Amp PCR System 2400).

The PCR protocol for the *Bam*H1W region included an initial denaturation step at 94°C for 4 min, followed by 30 cycles (50 s at 94°C, 50 s at 55°C, 50 s at 72°C), and by a final extension step of 7 min at 72°C. For BNLF1 gene amplification, the initial denaturation step at 94°C for 4 min was followed by 35 cycles (50 s at 94°C, 50 s at 50°C, 1 min at 72°C) and by a final extension step of 7 min at 72°C. The reaction product (2 µl) was subjected to a second round of PCR amplification with nested primers for 35 cycles under the same reaction conditions.

**Table 1** Immunoreactivity for CS1–4 in melanocytic lesions

| | CS1–4 immunoreactive cases/total cases tested |
|---|---|
| Nevi | |
| Junctional nevus | 1/2 |
| Spitz nevus | 1/1 |
| Blue nevus | 1/1 |
| Compound nevus | 2/2 |
| Dermal nevus | 2/2 |
| Dysplastic nevus | 2/2 |
| Melanomas | |
| Superficial spreading | 11/12 |
| Nodular | 7/8 |
| Metastasis | 25/28 |
| Recurrence | 3/3 |
| Lentigo maligna | 2/2 |
| Superficial spreading with associated nevus | 2/2 |

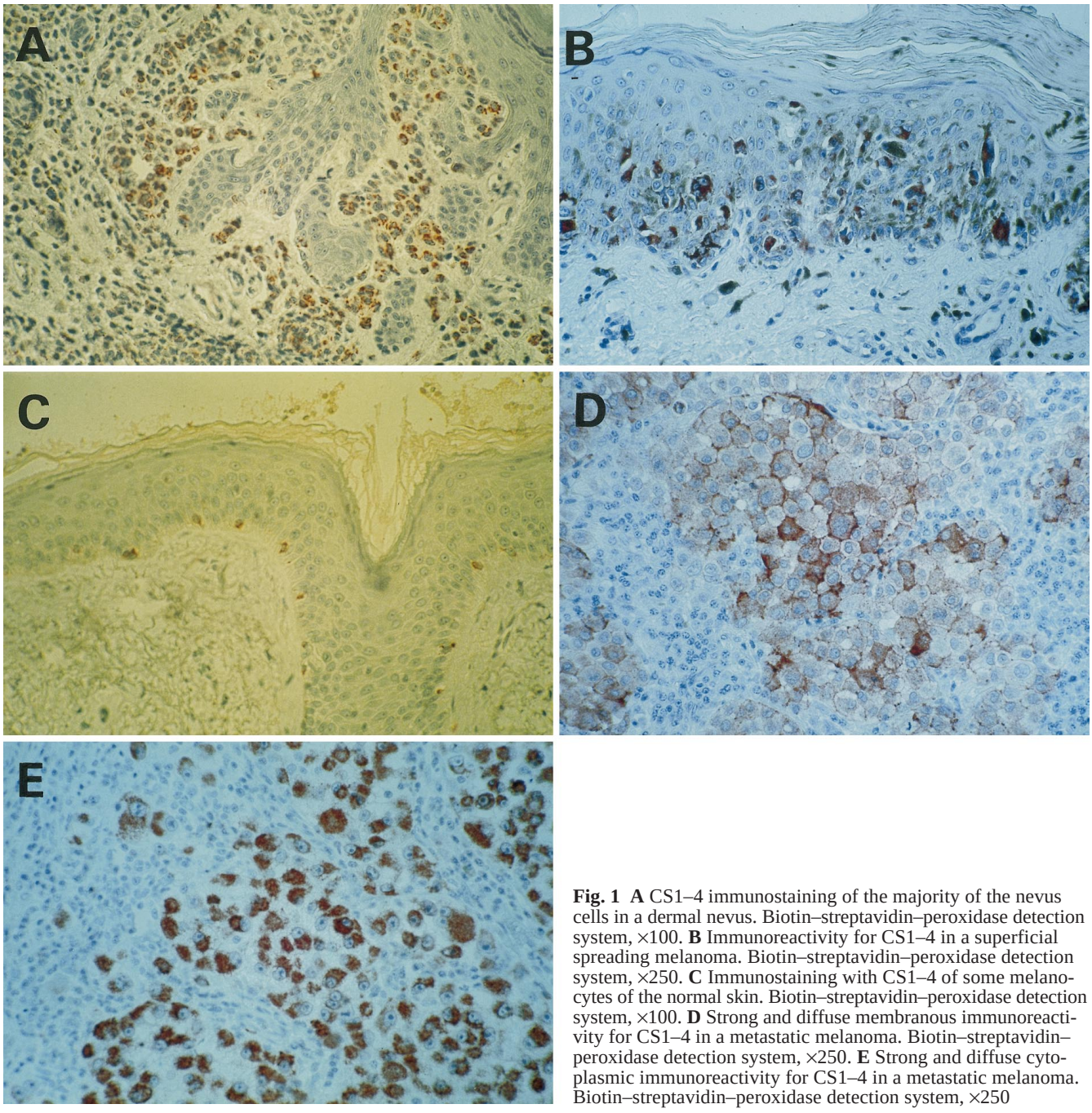


**Fig. 1** **A** CS1–4 immunostaining of the majority of the nevus cells in a dermal nevus. Biotin–streptavidin–peroxidase detection system, ×100. **B** Immunoreactivity for CS1–4 in a superficial spreading melanoma. Biotin–streptavidin–peroxidase detection system, ×250. **C** Immunostaining with CS1–4 of some melanocytes of the normal skin. Biotin–streptavidin–peroxidase detection system, ×100. **D** Strong and diffuse membranous immunoreactivity for CS1–4 in a metastatic melanoma. Biotin–streptavidin–peroxidase detection system, ×250. **E** Strong and diffuse cytoplasmic immunoreactivity for CS1–4 in a metastatic melanoma. Biotin–streptavidin–peroxidase detection system, ×250

Reverse transciptase-PCR for LMP1 mRNA

Total RNA was extracted from frozen tissue of four LMP1-immunoreactive melanomas using the TRIzol reagent (Life Technologies, Gaithersburg, Md.) and stored in RNase-free water at –70°C. Total RNA (3 µg) was denatured at 90°C for 5 min and chilled rapidly on ice, immediately before reverse transcription (RT) in 20 µl 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 2.5 mM $MgCl_2$, 10 mM DTT (Life Technologies), 1 mM dNTPs (Pharmacia), 2.5 µM random hexamers (Perkin Elmer), 200 U MMLV-reverse transcriptase (Life Technologies), 20 U RNAse-inhibitor (Perkin Elmer), at 37°C for 60 min, followed by incubation at 99°C for 5 min. cDNA (2 µl) was then subjected to nested PCR using the same conditions and primers as for the amplification of the BNLF1 gene.

## Results

### Immunocytochemistry and Western blotting

Nine of ten nevi and 50 of 55 (90%) malignant melanomas showed consistent immunoreactivity for the CS1–4 mAb cocktail to LMP1 (Table 1). One junctional nevus, one nodular melanoma, one SSM and three metastatic melanomas were not immunoreactive.

In both nevi (Fig. 1A) and melanomas (Fig. 1B, D, E), immunostaining was both cytoplasmic and membra-

**Table 2** Normal tissues immunohistochemically tested with CS1–4

| Tissues | CS 1–4 positive cases/total cases examined |
|---|---|
| Pleura | 0/3 |
| Lung | 0/2 |
| Peritoneum | 0/2 |
| Oesophagus | 0/2 |
| Stomach | 0/3 |
| Duodenum | 0/2 |
| Liver | 0/2 |
| Pancreas | 0/2 |
| Spleen | 0/1 |
| Appendix | 0/4 |
| Colon | 0/3 |
| Prostate | 0/2 |
| Urinary bladder | 0/3 |
| Kidney | 0/3 |
| Endometrium | 0/2 |
| Ovary | 0/2 |
| Salpinx | 0/3 |
| Placenta | 0/3 |
| Testis | 0/2 |
| Skin | 0/6[a] |
| Thymus | 0/1 |
| Larynx | 0/2 |
| Parotid gland | 0/3 |
| Bone marrow | 1[b]/1 |
| Breast | 0/3 |
| Thyroid | 0/2 |

[a] Some melanocytes were positive
[b] Myeloid and erythroid precursors were positive

**Table 3** The immunoreactivity for CS1–4 of neoplastic and pathological tissue samples

| Tissues | CS 1–4 positive cases/ total cases examined |
|---|---|
| Colorectal adenocarcinoma | 0/2 |
| Breast carcinoma | 0/4 |
| Prostatic adenocarcinoma | 0/2 |
| Gastric carcinoma | 0/4 |
| Medullary carcinoma of the thyroid | 0/2 |
| Carcinoid of the appendix | 0/2 |
| Merkel cell carcinoma | 0/1 |
| Small cell carcinoma of the lung | |
| Oat cell type | 0/2 |
| Intermediate cell type | 0/2 |
| Small cell carcinoma of the stomach | 0/1 |
| Adenocarcinoma with endocrine differentiation of the stomach | 0/2 |
| Sugar tumour of the lung | 0/2 |
| Angiomyolipoma of the kidney | 0/2 |
| Sebaceous nevus | 0/4 |
| Basal cell carcinoma | 0/5 |
| Keratoacanthoma | 0/5 |
| Actinic keratosis | 0/5 |
| Bowen's disease | 0/3 |
| Squamous cell carcinoma | 0/6 |
| Tumour of the follicular infundibulum | 0/1 |

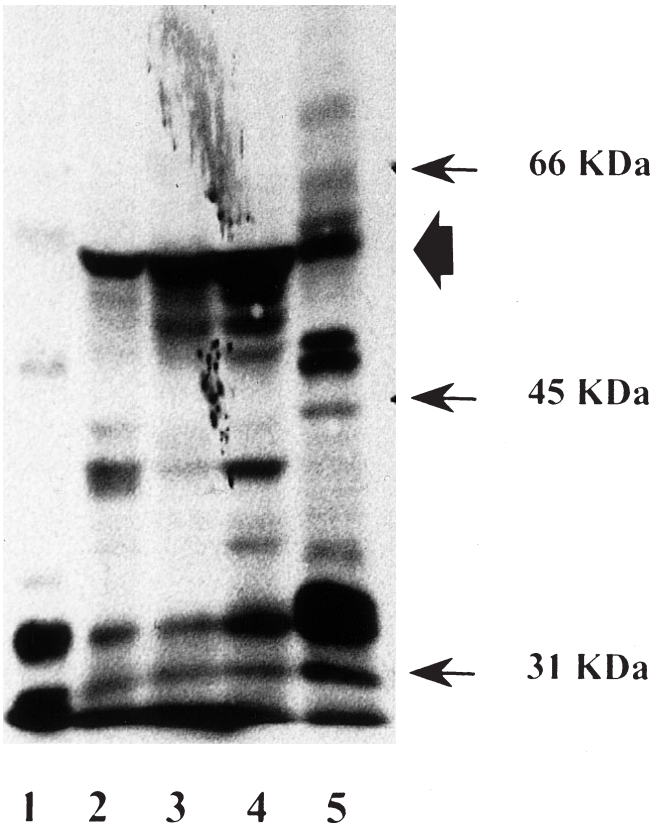


**Fig. 2** Western blotting of three cases of melanoma with radiolabelled CS1–4 mAb. *Lane 1*: amyloid serum protein A, negative control. *Lanes 2, 3, 4:* superficial spreading melanoma, nodular melanoma and metastatic melanoma, respectively. *Lane 5:* Raji cell line, positive control. An immunoreactive band of approximately 60 kDa in *lanes 2, 3, 4,* and *5* is indicated by a *thick arrow*

nous in the majority (consistently more than 50%) of the neoplastic cells. Approximately 30% of the melanocytes of the normal skin surrounding the lesions were also immunoreactive for the CS1–4 antibody (Fig. 1C). The control cells and tissues (lymphoblastoid cell line, Hodgkin's disease and nasopharyngeal carcinoma) were immunostained as expected.

Although the percentage of immunoreactive cells in nevi and primary melanomas was very similar, the staining intensity in melanomas was consistently stronger than in nevi. In the two metastatic melanomas, which could be compared with the corresponding primary tumours, however, the percentage of immunoreactive cells was lower, although the staining intensity was similar to that of primary melanomas.

The isotype antibody used as a negative control for CS1–4 did not stain any cell in nevi and melanomas. All normal and neoplastic human tissues probed with the CS1–4 mAb were consistently devoid of immunoreactivity, except for a proportion (approximately 30%) of the melanocytes in skin samples (Table 2 and Table 3), and for some normal blasts of the myeloid and erythroid lineages.

The four individual clones have been used separately to immunostain ten LMP1 (CS1–4)-immunoreactive

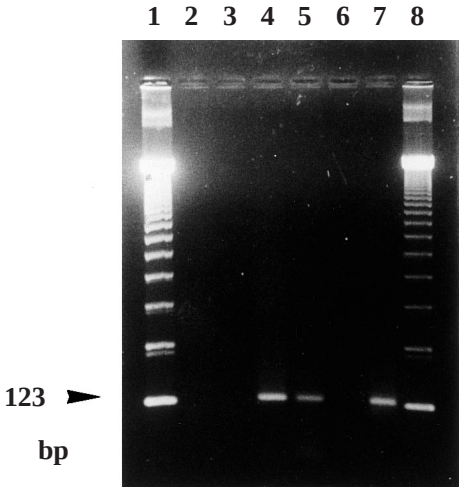


**Fig. 3** Amplification of the *Bam*H1W region of the Epstein–Barr virus (EBV) genome using polymerase chain reaction (PCR). *Lanes 1 and 8:* molecular weight marker ladders. *Lanes 2 and 3:* negative controls (without DNA and with human normal DNA). *Lanes 4, 5 and 6:* nodular melanoma, metastatic melanoma and nodular melanoma. *Lane 7:* Raji cell line, positive control. Single bands of the expected size of 123 bp are shown in *lanes 4, 5* and *7*

melanomas: an individual case showed immunoreactivity for CS1, five cases for CS2, and all ten cases for the CS3 and CS4 clones.

Three melanomas immunoreactive for the CS1–4 mAb were also investigated using the Western blotting technique. A band of constant size of 60 kDa, consistent with the molecular weight of LMP1 (Fig. 2), was detected in all cases.

### ISH, PCR, and RT-PCR

ISH experiments for EBER 1–2 mRNA were consistently negative in all nevi and melanomas, whereas the positive control cases showed the expected reactivity. Two melanomas (one metastasis and one nodular melanoma), with a prominent B-cell infiltration, showed positive hybridisation signals with EBER 1–2 in some lymphocytes.

Only two of the seven melanomas immunoreactive for the CS1–4 mAb showed amplification of the *Bam*H1W region and of the BNLF1 gene in PCR experiments. These were the same cases with EBER 1–2 positive lymphocytes surrounding the neoplastic cells (Fig. 3).

In none of the four melanomas immunoreactive for LMP1 has it been possible to document expression of LMP1 mRNA by means of RT-PCR.

## Discussion

LMP1 is a transforming protein consisting of a 24-amino acid amino-terminal cytoplasmic domain, six membrane-spanning hydrophobic domains separated by short reverse turns and a 200-amino acid carboxy-terminal cytoplasmic domain [7]. The anti-LMP1 mAb CS1–4 is a cocktail of four clones, CS1, CS2, CS3 and CS4, which recognises at least three different epitopes on the hydrophilic carboxyl region of LMP1 exposed to the cytosol [32]. In this study, 90% of melanocytic lesions were immunoreactive for this cocktail of mAbs.

Western-blotting experiments have documented that this mAb cocktail recognises a protein with a molecular weight similar to that of LMP1. CS3 and CS4 clones recognise an epitope (GPQDPDNTD) that occurs three times within the repeat region (AA252–298) of the LMP1 protein (Prof. M. Rowe, personal communication). This repeat sequence gives rise to variations in the size of LMP1 encoded by different EBV isolates [32]. In this respect, it is relevant that the size of the band recognised by the CS1–4 cocktail in three different LMP1-immunoreactive melanomas be of equal size. Indeed, this suggests that the 60-kDa band is not related to an actual EBV infection of the samples under study.

This conclusion is reinforced by the lack of amplifiable LMP1 mRNA in RT-PCR experiments and by the failure to document any EBER1 and 2 expression using ISH. The amplification of the *Bam*H1W region of EBV in two melanomas may well be due to the occurrence of some EBV-infected lymphocytes amongst the inflammatory cells infiltrating the tumours.

At variance with the results presented herein, EBV-correlated human tumours show consistent expression of EBER1–2 and variable expression of the nuclear antigens (EBNA) and of latent membrane proteins, depending on the latency type [42]. Therefore, the immunoreactivity for CS1–4 in nevi and melanomas does not reflect an actual viral infection, but it is most likely due to a cross-reactivity of the antibodies with a still uncharacterised antigen, showing at least partial homology with LMP1. Recently, strong immunoreactivity for CS1–4 in the absence of EBV DNA has been documented in early myeloid and erythroid precursors, and in blasts of leukaemia of both lymphoid and myeloid lineages [12]. Accordingly, immunoreactivity for CS1–4 in normal and neoplastic cells should not be taken as evidence of EBV infection, which is reliably documented by ISH experiments with EBER-specific probes [16].

The antigen identified by the CS1–4 mAb apparently shows a high specificity for melanocytes, nevi and melanomas. Indeed, except for some cells of the myeloid and erythroid lineages, we did not find any other immunoreactive cell in the series of normal and neoplastic human tissues examined. Widely known melanocytic markers, generally used for diagnostic purposes, such as the S-100 protein and the antigen recognised by the HMB-45 mAb, are expressed in several non-melanocytic normal and diseased tissues [2, 8, 18, 45]. In this respect, the more restricted specificity of the CS1–4 cocktail makes this reagent a useful adjunct marker of melanocytic tumours, although further investigations will be necessary to fully characterise this molecule.

**Acknowledgements** We wish to thank Professor M. Rowe for comments and suggestions and Dr. R. Maestro, Dr. B. Muciaccia, Dr. E. Orvieto, Dr. C. Pellegrini, Professor N. Raab-Traub, Professor A. Scarpa and Dr. E. Vicini for their generous help in different experiments. This work has been partly supported by Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.), Milan, Italy.

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