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Epstein–Barr Virus (EBV)-encoded Membrane Protein LMP1 from a Nasopharyngeal Carcinoma is Non-immunogenic in a Murine Model System, in Contrast to a B cell-derived Homologue

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Epstein–Barr virus (EBV)-encoded LMP1 gene derived from a nude mouse passaged nasopharyngeal carcinoma (NPC) of Chinese origin (C-LMP1) and its B cell (B95-8 prototype)-derived counterpart (B-LMP1) were compared for their ability to induce tumour rejection in a mouse mammary adenocarcinoma system. Each of the two LMP1 genes was introduced individually by retroviral vectors into a non-immunogenic mammary carcinoma line, S6C, that originated in an ACA (H-2^f) mouse. Syngeneic ACA mice were immunised for 3 consecutive weeks with irradiated B- or C-LMP1 expressors or control cells. The immunised and control mice were then challenged with graded numbers of viable cells from the corresponding cell line. Only the B-LMP1 expressing cells were highly immunogenic. Up to 10^5 cells were rejected in pre-immunised mice, whereas at least 10^2 cells grew in non-immunised controls. No rejection response was detected against the C-LMP1 expressing cells which grew equally well in control and immunised mice, with a minimum inoculum of 10^2 cells in the majority of the clones. In a previous study, we found numerous sequence differences between B- and C-LMP1. The question of whether any of these differences is related to the non-immunogenicity of C-LMP1 needs further investigation. Meanwhile, our findings raise the possibility that the NPC cells may escape host rejection by the development of a non-immunogenic LMP1 variant under the impact of immunoselection.
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INTRODUCTION

EPSTEIN–BARR VIRUS (EBV)-transformed lymphoblastoid cell lines (LCL) of normal B cell origin express six EBV-encoded nuclear proteins (EBNA1–6) and two membrane antigens, LMP1 and 2 [1]. These cells are highly immunogenic in autologous hosts. During acute mononucleosis, EBV-infected B cells proliferate in the blood and lymphoid tissues, but are eliminated by the host immune response during convalescence. At least four of the EBV-encoded nuclear antigens (EBNA2, 3, 4 and 6) and both membrane antigens can serve as cytotoxic T cell (CTL) targets, whereas to date EBNA1 has not been found to be detectably immunogenic for T cells [2–4]. Using a murine model system, we have shown previously that LMP1 but not EBNA1 can convert a non-immunogenic mouse mammary carcinoma, S6C, derived from an ACA (H-2^f) mouse, into a rejectable tumour [5].

EBV carrying Burkitt's lymphoma (BL) cells that have retained the characteristic (type I) tumour phenotype during *in vitro* passage, express only EBNA1, not EBNA 2–6, LMP1 or LMP2 [6]. This may be partly responsible for their escape from immune surveillance, in conjunction with other BL cell phenotype-associated features, such as low expression of adhesion molecules [7] and certain major histocompatability complex (MHC) class I antigens [8], and reduced antigen processing [9].

Most of the immunoblastic tumours that appear in congenitally or iatrogenically immunodefective patients express the full set of EBV-encoded LCL-associated proteins, affirming that the proliferation of such cells is normally limited by the immune system [10].

The immunogenicity of the third, paradigmatic EBV-carrying cell, nasopharyngeal carcinoma (NPC) is not known. NPCs develop in immunocompetent individuals, suggesting that the tumour cells have escaped immune surveillance. NPC cells express EBNA–1 but not EBNA2–6 [11, 12]. Approximately two thirds of the NPC tumours express LMP1 protein while one third are negative [11, 12]. It has been suggested that the LMP1-negative tumours have lost or suppressed expression of the

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membrane antigen as a consequence of immunoselection, but then how did the LMP1-positive tumours survive? In an earlier study [13], we compared the LMP1 gene sequence, cloned from a nude mouse passaged NPC tumour (CAO) of Chinese origin, with a corresponding B cell-derived clonal prototype (B95-8, carrying a virus of mononucleosis origin). Numerous sequence differences were found in the coding part of the gene, most frequently at the N- and C-terminal ends. Some of these differences may have immunological consequences.

In order to test the possibility that the NPC-derived LMP1 gene (designated as C-LMP1) may encode a less immunogenic protein than its B cell-derived counterpart (designated B-LMP1), we have compared the two genes for their ability to convert the non-immunogenic mammary carcinoma S6C into an immunogenic tumour, rejectable by syngeneic mice. We were able to confirm the strong immunogenicity of the B-LMP1 and to demonstrate, in addition, that C-LMP1, expressed at an equally high level, is non-immunogenic. This raises the interesting question of whether NPC tumours may have been immunoselected at the level of the viral genome.

MATERIALS AND METHODS

Cell line

The S6C cell line was derived from a spontaneous mammary adenocarcinoma that has originated in an ACA (H-2^f) mouse [14]. The cells were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin.

LMP1 expression vectors

To generate the B-LMP1 retroviral expression vector, a HindIII-*Eco*RI fragment containing the herpes simplex virus-1 (HSV-1) thymidine kinase (TK) promoter and the LMP1 gene (B95-8 coordinates 169482-167918 [15, 16]) was cloned in the *Sna*BI site of a retroviral vector, PSHIS4 (Fig. 1), as described previously [5]. The C-LMP1 retroviral expression vector was constructed by cloning a 1671 base pair (bp) *Sma*I fragment (B95-8 coordinates, 169589-167918) covering the coding region of C-LMP1 [13] into the *Dra*I site of the pSHIS4 vector (Fig. 1).

Retrovirus infection of S6C cells

The supernates of B-LMP1 and C-LMP1 retrovirus-producing PA317 cells [17] were used as the sources of helper virus-free transducing virus. The supernates were centrifuged at 5000 RPM for 10 min and filtered through a 0.45-μm filter to remove contaminating cells and debris. The S6C cells were grown in 10 ml of the retrovirus-containing supernates for 24 h in the presence of 8 μg/ml polybrene (Sigma, St Louis, Missouri, U.S.A.). The supernates were then removed and fresh RPMI 1640 supplemented with 10% FCS was added. After 24 h, transfected S6C cells were selected in histidine-free Iscoves medium supplemented with 0.5 mmol/l histidinol (Sigma). Growing colonies were picked and propagated separately after 2 weeks of selection. The vector transfectants were generated by electroporating (Gene Pulser, Bio-Rad, Richmond, California, U.S.A.; 240 V, 960 μF capacitance) the S6C cells with 10 μg of the pHIS4 vector in 200 μl of phosphate buffered saline (PBS) buffer. The vector transfectants were selected as described above.

Detection of LMP1 protein

For immunoblotting, 10^7 cells were lysed in 1 ml lysis buffer containing 65 mmol/l Tris pH 6.8, 2% sodium dodecyl sulphate

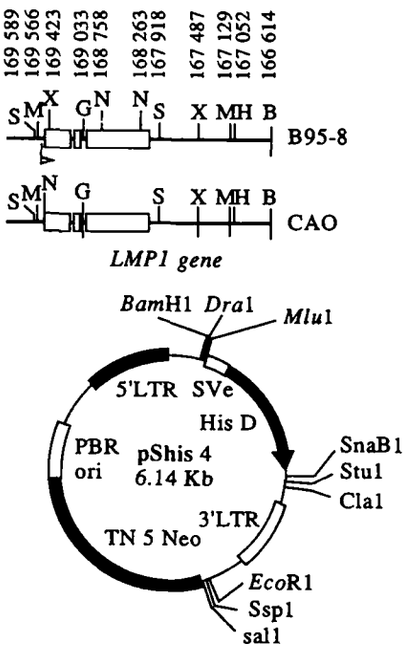

Fig. 1. The retroviral vectors for LMP1 expression. The B-LMP1 gene is linked to the HSV-1 TK promoter and cloned at the *Sna*BI site of the PSHIS4 vector. A 1671 bp *Sma*I fragment covering the NPC-derived CAO-LMP1 coding sequences is cloned at the *Dra*I site in the same vector. The flag shows the location of the EDL1 promoter. His D, *S. typhimurium* histidinol dehydrogenase gene, S-*Sma*I, M-*Mlu*I, G-*Bgl*II, N-*Nco*I, X-*Xho*I, H-*Hind*III, B-*Bam*HI.

(SDS), 10% glycerol and 5% mercaptoethanol. The proteins were separated on a 7.5% polyacrylamide–SDS gel [18] and blotted on to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) [19]. The filters were stained with Ponceau S (Sigma). After 1 h of incubation in 5% dried skimmed milk dissolved in PBS, the filters were incubated overnight with LMP1 monoclonal antibody S12 [20] at 4°C. After three washes in PBS containing dried skimmed milk, the filter was incubated with alkaline phosphatase-conjugated secondary antibody for 2 h at room temperature. The filters were washed three times with balanced salt solution (BSS) containing 0.5% Tween 20. Specifically-bound second antibody was detected by fast red salt precipitation in the presence of alpha-naphthyl phosphate (Sigma).

Rejection assays

For immunisations, concentrated cell suspensions of B-LMP1, C-LMP1, vector transfected and untransfected S6C cells were prepared in PBS by pressing the corresponding *in vivo* grown tumours through fine meshed stainless steel nets. The cells suspended in PBS were then irradiated with 10 000 rad and inoculated subcutaneously in syngeneic ACA (H-2^f) mice of 4–6 weeks of age, once a week for 3 weeks. One week later, the mice were subjected to 400 rad whole body irradiation. We have shown previously [21] that this does not inhibit the established, specific rejection responses but minimises the boostering of non-specific resistance by the challenge inoculum. Within 24 h of irradiation, the mice were inoculated subcutaneously with graded doses of viable cells, suspended in 200 μl PBS. Non-immunised controls were irradiated and inoculated in parallel. Tumour growth was followed weekly for up to 8–10 weeks by caliper measurements in three dimensions. The mean tumour

load was calculated by adding the tumour diameter of individual tumours, and dividing the sum with the total number of mice with and without tumours.

A progressively-growing tumour with a minimum tumour size of at least 5 mm diameter by caliper measurements was scored as positive. A caliper reading score of zero or subcutaneous nodules that did not grow progressively, and did not exceed 5 mm in diameter were scored as negative.

RESULTS

LMP1 expression in transduced S6C cells

LMP1 expression was determined in three clones, each from B-LMP1 and C-LMP1 virus-infected S6C cells. All of these expressed high levels of LMP1 (Fig. 2). In agreement with our previous findings [13], the C-LMP1 protein had a higher molecular weight (66 kD) than B-LMP1 (63 kD). This is consistent with the 18 amino acid difference deduced from the sequence. As expected in cell lines with a stably integrated gene, periodic testing showed no decrease of LMP1 expression on serial culturing.

Immunogenicity of B-LMP1-expressing S6C cells

The growth of B-LMP1-expressing and control cells in pre-immunised and control mice is summarised in Table 1. The unmanipulated and the vector-transfected S6C cells grew equally well in control and pre-immunised mice. The B-LMP1-expressing clones S6C B3 and B5 which were chosen as positive controls, were rejected in a significant proportion of the pre-immunised mice. The growth curves are shown in Fig. 3 and the total tumour take incidence with all cell doses is illustrated in Fig. 4. Tumours of approximately 10–15 mm diameter, growing in the immunised and control mice, were sampled for LMP1 expression by immunoblotting. As shown in Fig. 2, the B-LMP1-expressing clone, S6C B3, expressed similar levels of LMP1, whether harvested from the immunised or from control mice. LMP1 expression levels were comparable to the corresponding *in vitro* passaged line.

C-LMP1 expressing S6C cells induce no rejection response

S6C C1 and S6C C7 clones grew equally well in immunised and non-immunised mice (Table 1, Figs 3, 4). Clone S6C C8 was weakly immunogenic in comparison to clone S6C C1. However, this difference disappeared as the size of the inoculum increased, in contrast to the B-LMP1-expressing clones, which were strongly rejected at all the doses (Table 1). As demonstrated in Fig. 4, this interclone variation at low challenge dosages was less significant than the clearcut difference between the B- and C-LMP1 groups of clones. Likewise, the growth delay of approximately 1 week between the vector controls S6C V and S6C C7 (Fig. 3) is within the range of experimental fluctuations due to non-specific factors, such as viability of the inoculated cells or clonal variations within the S6C line. The two clones eventually reached the same tumour load with the same growth kinetics (Fig. 3).

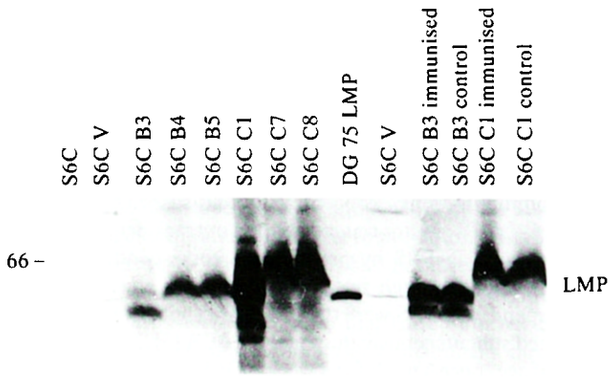

Fig. 2. B- and C-LMP1 expression in S6C cells. Clones S6C B3, B4 and B5 express B-LMP1 and S6C C1, C7 and C8 express C-LMP1. Total cell extracts from the parental, vector control, B-LMP1 and C-LMP1 virus-infected S6C and positive control DG75TKLM cells [29] were separated on a 7.5% polyacrylamide–SDS gel. The expression of LMP1 was determined using the anti-LMP1 monoclonal antibody S12. The tumours arising in the immunised mice (S6C B3 immunised and S6C C1 immunised) and in the control mice (S6C B3 control and S6C C1 control) were also analysed for LMP1 expression. A non-specific band was detected in S6C and S6C V.

Table 1. Growth of LMP1-transfected and control S6C in pre-immunised and control ACA (H-2^f) mice†*

Cells	Transfected with	No. of viable cells inoculated	Immunised	Control
S6C	—	10^3	9/10	10/10
		10^4	10/10	10/10
		10^5	10/10	10/10
S6C V	Vector	10^2	9/10	8/8
		10^3	10/10	10/10
		10^4	10/10	10/10
S6C B3	B-LMP1	10^2	2/17	15/20
		10^3	2/20	17/20
		10^4	1/20	19/19
S6C B5	B-LMP1	10^3	0/8	1/10
		10^4	0/10	8/10
		10^5	1/10	10/10
S6C C1	C-LMP1	10^2	10/20	10/20
		10^3	16/20	15/20
		10^4	19/19	20/20
S6C C7	C-LMP1	10^2	10/10	10/10
		10^3	10/10	10/10
		10^4	7/10	10/10
S6C C8	C-LMP1	10^2	4/10	10/10
		10^3	5/10	10/10
		10^4	10/10	10/10

*Immunisations were performed with three weekly inoculations of 10^6 irradiated cells (10 000 rad) of the same type as used for viable challenge. All mice received 400 rad whole body irradiation before the live challenge. †The figures represent the number of mice with progressively growing tumours divided by the total number of mice inoculated.

The differences in the immunogenicity of the B- and the C-LMP1-expressing S6C cells were also reflected by the subcutaneous growth pattern of the S6C B5 and S6C C7 cells in non-immunised syngeneic mice. As shown in Fig. 5, the B-LMP1-expressing S6C B5 clone became necrotic in the centre around 3–4 weeks after inoculation. It also caused extensive skin damage in contrast to the C-LMP1 expressing S6C C7 clone, that grew progressively without necrosis or skin damage. In SCID mice, both clones grew progressively without necrosis or skin damage (not shown).

DISCUSSION

Earlier studies have shown that LMP1-specific human CTLs can readily be generated *in vitro* [4, 22] and that LMP1-transfected S6C cells can be rejected in pre-immunised syngeneic mice [5]. In contrast, EBNA1 does not appear to be immunog-

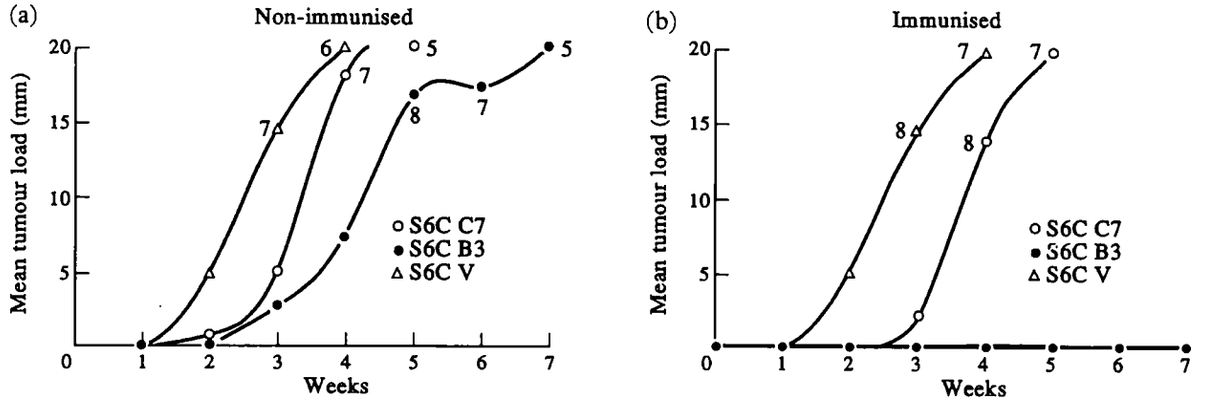

Fig. 3. Growth of the S6C clones expressing B-LMP1, C-LMP1 and vector control in (a) non-immunised and (b) immunised mice. The challenge dose was 10^4 cells. The figures at different time points show numbers of live mice. The time points without figures represents 10 live mice. The mean tumour load was calculated by adding the individual tumour diameters and dividing the sum by the total number of mice inoculated (with or without the tumour).

enic for T cells and attempts to generate EBNA1-specific CTLs have failed in several laboratories [2–4]. EBNA1-transfected S6C cells were shown to be non-immunogenic in syngeneic mice in our earlier studies [5].

All cells that carry the EBV genome express EBNA1. EBV-transformed immunoblasts express LMP1 (together with EBNA1–6), but they cannot grow progressively *in vivo*, unless the host is immunosuppressed. BL cells grow in immunocompetent hosts, but express neither LMP1 nor EBNA2–6. NPCs do not express EBNA2–6, but the majority of NPCs are LMP1-positive. In view of the fact that the NPCs develop in immunocompetent hosts, it is of interest to explain how LMP1-positive cells can escape immune rejection. Our present findings are consistent with the possibility that they may do so due to a mutation in the viral gene. From the functional point of view, this would be analogous to the non-immunogenicity of EBNA1 for T cells, even though the mechanism responsible for the lack of immunogenicity may be quite different.

To test the validity of this hypothesis, the immunogenicity of additional NPC-derived LMP1 genes will have to be compared with their B cell-derived counterparts in S6C cells and in tumour cells of different haplotypes. Our previous studies have shown that 35% of the NPC tumours failed to express the protein. The possibility that this was an artefact of proteolysis was ruled out when we found that the LMP1 regulatory sequences (LRS) were highly methylated in the LMP1-negative tumours but

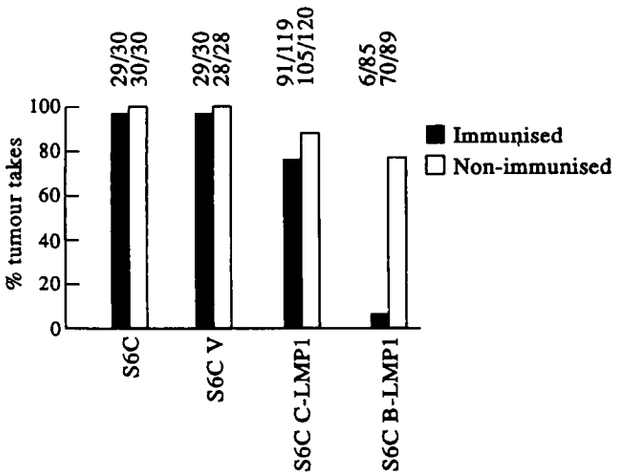

Fig. 4. The summary of percentage tumour takes of non-transfected, vector-transfected, B-LMP1- and C-LMP1-expressing S6C cells in immunised and non-immunised ACA mice at all the challenge doses tested. The numbers above each column indicate the number of mice with progressively growing tumours over total inoculated.

Fig. 5. *In vivo* growth of B-LMP1-expressing S6C B5 and C-LMP1-expressing S6C C7 clones in non-immunised syngeneic mice. 10^4 cells were inoculated subcutaneously into syngeneic ACA hosts. S6C B5 grew as a highly necrotic tumour (left) and S6C C7 grew without any skin damage (right).

unmethylated in the LMP1-positive tumours [23]. This suggests that the immunoselection of NPC tumours may have favoured the growth of two alternative variants: on the one hand, non-immunogenic mutants, like C-LMP1, and on the other hand, tumours where the transcriptional activity of the LMP1 gene has been suppressed by regulatory changes associated with methylation. The question of whether these are true alternatives can be tested by comparing LMP1 isolates from LMP1-positive and LMP1-negative tumours in the present test system. Will proteins expressed from the LMP1 genes of LMP1-negative NPC tumours be equally immunogenic after expression in appropriate vectors, as B-LMP1 isolates, and will LMP1 isolates from LMP1-expressing tumours prove to be non-immunogenic as C-LMP1 in this study?

Viral mutations converting potentially immunogenic into non-immunogenic proteins in relation to a particular host have been described for other viruses, like human immunodeficiency virus (HIV) [24] and lymphocytic choriomeningitis virus (LCMV) [25]. In the EBV system, a human leukocyte antigen (HLA) A11 immunodominant epitope of the EBNA4 protein [26] was found to have mutated in several viral isolates from regions of Papua New Guinea with a high frequency of HLA A11-positive individuals [27].

In conclusion, the sequence variations between B-LMP1 and C-LMP1 are associated with two functional differences: a higher propensity to predispose immortalised human keratinocytes of the Rhek-1 line to a tumorigenic change [28], and a marked difference in immunogenicity. Further studies are required to define specific sequences responsible for these differences, and the possible generalisability of our findings.

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