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Original Paper

Differential Immunogenicity of Epstein–Barr Virus (EBV) Encoded Growth Transformation-associated Antigens in a Murine Model System

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The strong immunosurveillance of humans against EBV transformed immunoblasts, mediated by CD8+ cytotoxic T cells, is based on the recognition of peptides derived from eight of the nine growth transformation-associated proteins, the nuclear antigens EBNA2-6 and the membrane proteins LMP1, -2A and -2B. The ninth protein, EBNA1, required for maintenance of the viral episomes, and expressed in a cell phenotype independent manner, has not been found to generate a cytotoxic lymphocyte (CTL) response in humans. We tested whether EBNA1 has a similar immunologically privileged status in a species that has not encountered the virus in nature, the mouse. Non-immunogenic murine mammary carcinoma cells were transfected with the appropriate viral gene. Rejection responses were assayed in syngeneic mice following repeated immunisation with irradiated cells. Previously, we found that LMP1 expression in S6C, a murine mammary carcinoma of ACA (H-2f) origin, induces high rejectability, whereas corresponding EBNA1 transfectants remained non-immunogenic. In order to test whether this finding could be reproduced on another MHC class I background, we expressed LMP1 and EBNA1 in another non-immunogenic mammary carcinoma, SBfnHd of CBA (H-2k) origin. LMP1 but not EBNA1 transfectants were immunogenic in this system. In order to investigate whether other growth transformation-associated EBV proteins were immunogenic in the mouse, we also transfected the S6C cells with EBNA4, EBNA5, LMP2A and -2B. All four proteins induced strong rejection reactions. These findings are fully consistent with corresponding findings in the human system. They also show that the immunologically privileged status of EBNA1 is not due to some peculiarity of the long-standing co-existence between EBV and the human species, nor to any specific features of the human MHC class I system. They are consistent with the suggestion that EBNA1 may not be properly processed and/or transported, due to specific features of the protein itself. © **1997 Elsevier Science Ltd.**

Key words: EBV, EBNA, antigen, BL, syngeneic, mice, immunogenicity

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INTRODUCTION

EPSTEIN–BARR VIRUS (EBV) can infect B-cells *in vitro* and transform them into immortalised lymphoblastoid cell lines (LCLs). LCLs express nine virally encoded proteins. Six of them are nuclear antigens (EBNA1–6), whereas three are membrane proteins LMP1, -2A and -2B [1]. Phenotypically representative EBV-carrying Burkitt's lymphoma (BL) lines (type I) express EBNA1 only [2]. Upon *in vitro* culturing, most of the type I BLs drift towards a more LCL-like phenotype and express all the EBNAs and LMPs. This difference in viral gene expression is due to differential promoter utilisation [3]. In the immunoblastic LCLs, alternative promoters in the W or C region initiate a giant 112 kb message out of which all six nuclear proteins are spliced (WC programme) [4]. This programme is only used in immunoblasts. In the type I BL cell, a different promoter, located in

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the Q region, initiates a message encoding EBNA1 only (Q programme) [5]. The differential EBV expression of the LCL versus BL lines reflects corresponding phenotype-dependent differences in normal immunoblasts and persistently infected small B-cells, respectively. In acute infectious mononucleosis (IM), a proportion of the B-cell population is transformed into continuously proliferating immunoblasts, resembling LCLs both with regard to cellular phenotype and EBV protein expression. In contrast, latently infected normal seropositive donors express EBNA1 only [6, 7].

T-cells stimulated by autologous EBV-transformed immunoblasts have been found to recognise EBNA2–6 or the LMPs depending on the MHC class I phenotype of the respondent, but not EBNA1 [8–11]. These findings have led to the suggestion that EBNA1 may not induce a cytotoxic T lymphocyte (CTL) response. The recent *in vitro* experiments of Levitskaja and associates [12] have confirmed this idea and further showed that the long glycine alanine (Gly-Ala) repeats within the EBNA1 protein serve as a *cis*-acting inhibitors of ordinary processing and/or transportation. Insertion of the main immunogenic epitope of EBNA4 into the EBNA1 sequence and expression of the construct in HLA A11 positive fibroblasts via vaccinia vectors did not sensitise these targets to the lytic action of the EBNA-4 specific CTLs. Removal of the Gly-Ala repeats lifted this block. Conversely, addition of the Gly-Ala repeats to EBNA4 has prevented the sensitisation of the HLA A11 positive fibroblasts to the cytotoxic effect of the HLA A11 restricted CTLs.

The murine model system has been extensively used to investigate the immunogenicity of human papillomavirus (HPV) encoded proteins. The HPV encoded E6 and E7 proteins are highly immunogenic as indicated by the strong rejection of transfected tumours in pre-immunised syngeneic mice [13, 14]. Furthermore, cytokines and accessory molecules have been used to convert non-immunogenic murine tumours into tumours that are highly immunogenic and rejectable in syngeneic hosts [15, 16].

In a previous study, we have addressed the question of whether a species that does not encounter EBV in nature, the mouse, would show a distinction between EBNA1 and an immunogenic EBV encoded protein, as the human CTLs. We have transfected a non-immunogenic mammary carcinoma, S6C, of ACA (H-2f) origin, with EBNA1 and LMP1 as a positive control. While LMP1 made the cells highly immunogenic (rejectable) in syngeneic hosts, expression of the full-sized EBNA1 had no effect [17].

The present study has extended this work in two directions. To exclude that the non-immunogenicity of EBNA1 was due to some peculiarity of H-2f, we have transfected another mammary carcinoma, SBfnHD of CBA (H-2k) origin, with EBNA1 and LMP1, respectively. The same results were obtained as with S6C. We have also transfected four additional EBV encoded, growth transformation-associated proteins, EBNA4, EBNA5, LMP2A and 2B, into S6C cells. All these transfectants induced rejection responses in immunised syngeneic recipients.

MATERIALS AND METHODS

Cells

SBfnHD and S6C are spontaneous non-immunogenic mammary carcinoma cell lines derived from CBA (H-2k) and ACA (H-2f) mice, respectively [18]. Both were maintained in RPMI supplemented with 10% FCS (fetal calf serum).

Plasmids

Expression vectors for EBNA1 and LMP1 were described elsewhere [17]. The expression vector for EBNA5 was constructed by subcloning the EBNA5 cDNA from the pUC19 vector (a kind gift from Dr Fred Wang) into the BamHI and EcoRI sites of the pBabe retrovirus vector [19] (Figure 1a). The EBNA4 expression vector contained a

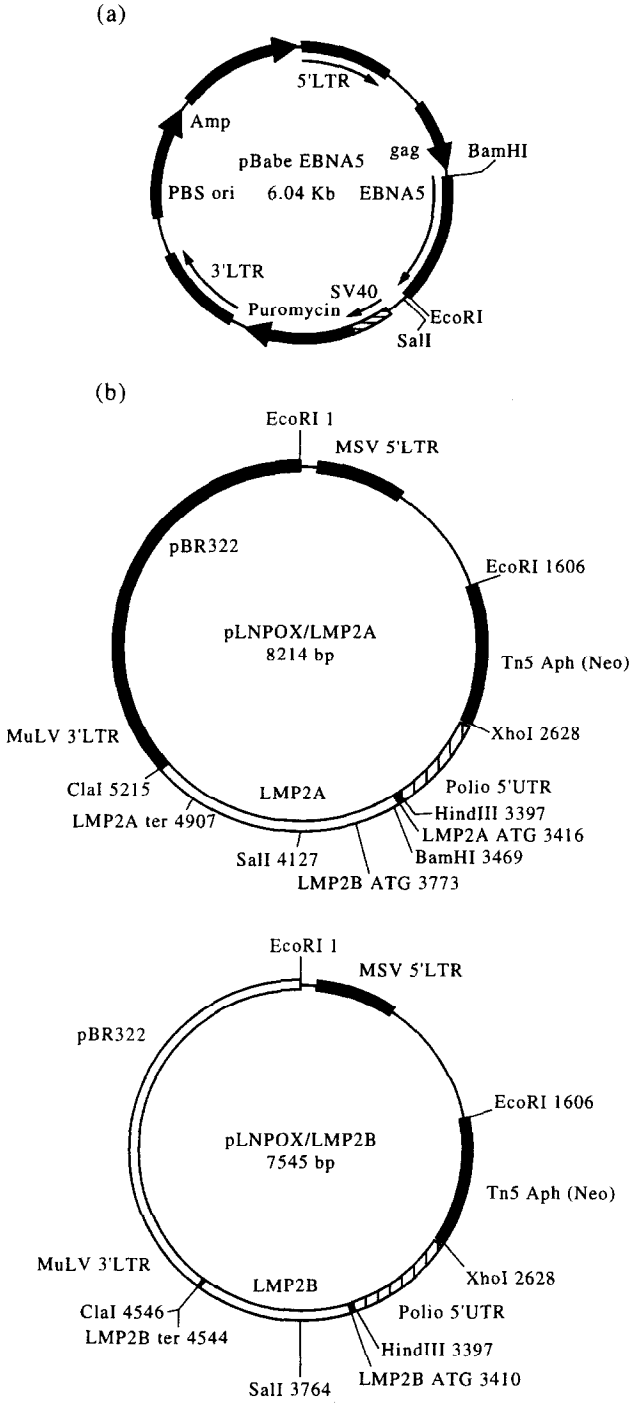

Figure 1. Expression vectors for EBNA5, LMP2A and -2B. (a) EBNA5 cDNA was subcloned in pBabe vector in the BamHI and EcoRI sites. (b) LMP2A and -2B cDNA were subcloned in HindIII and ClaI sites of the pLNPOX vector.

BamHI fragment coding region (B95-8 coordinates 95 239–98 398) cloned in the BamHI site of the pBabe vector. LMP2A and -2B expressing retrovirus vectors were constructed by cloning both genes individually in a base vector pLNPOX (a kind gift from Dr D. Miller, FHCRI, Seattle, Washington, U.S.A.). LMP2A cDNA from a vector pSP6423TP (a gift from Dr P. Farrell) was subcloned in HindIII-ClaI sites (Figure 1b). LMP2B cDNA was made by PCR cloning of the pSP6423TP vector. The 5'(sense) primer was 5'-AGGGAAGCTTCCACCATGA-ATCCAGTATGC-3'. It contained a HindIII site. The 3' (antisense) primer, containing a ClaI site, was 5'-AACCATCGATTTTATACAGTGTTGCG-3'.

Transfection and selection

The EBNA-1 expression vector pBKg130 and the vector control plasmid (10 μg) were electroporated into SBfnHD cells using gene pulser electroporation apparatus (Biorad). The electroporation was carried out in 250 μl PBS at 960 μF, 230 V. The selection was initiated 48 h after the transfection in 1.5 μg/ml mycophenolic acid, 160 μg/ml xanthine and 10 μg/ml hypoxanthine. Drug-resistant cells were cloned and propagated separately. The pBabe EBNA-4, EBNA-5 and the vector control plasmids were transfected into the S6C cells as described above. Selection was carried out in 7 μg/ml puromycin. The LMP1 gene was introduced into the SBfnHD cells by retrovirus-mediated gene transfer. SBfnHD cells were infected with supernatants from the LMP1 retrovirus-producing line PA317 [20]. The tranfectants were selected in histidine-free Iscove's medium supplemented with 0.5 mM histidinol. The selection was maintained for 2 weeks, after which the cells were cloned and propagated separately. LMP2A and -2B and the G418 vector were introduced into the S6C cells by infecting them with 24 h old supernatants from the corresponding virus-producing line PA317. The transduced cells were selected in 500 μg/ml of geneticin (Sigma).

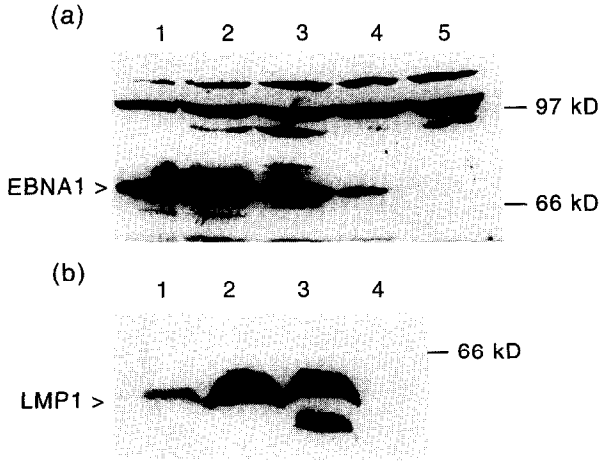

Figure 2. EBNA1 (a) and LMP1 (b) expression in SBfnHD cells. (a)Total extracts from the SBfnHD EBNA1 and vector expressing cells were separated on a 7.5% SDS-polyacrylamide gel. The filter was probed with a polyclonal human serum from an EBV-positive healthy donor. Lane 1: SBfnHD EBNA1 clone 1; lane 2: clone 2; lane 3: clone 4; lane 4: clone 7 and lane 5: SBfnHD vector. (b) LMP1 was detected by S12 monoclonal antibody. Lane 1: SBfnHD LMP clone 1; lane 2: clone 8; lane 3: LCL IARC 137; lane 4: SBfnHD vector.

Detection of EBV antigens in the transfected cells

Expression of EBNA1, EBNA5 and LMP1 was detected by immunoblotting. Briefly, the protein samples were prepared from the parental, vector controls and derived transfectants, separated by discontinuous polyacrylamide gel elctrophoresis, and blotted on to the nitrocellulose filter as described previously [21, 22]. For detection of EBNA1, a polyclonal human serum (HR) was used. LMP1 and EBNA5 were detected with S12 [23] and JF186 monoclonal antibodies, respectively [24]. EBNA4 expression was tested by anticomplement immunofluorescence (ACIF) [25], using a polyclonal human serum (HR). LMP2A and LMP2B expression was examined by RT-PCR. RNA from the LMP2A and -2B transduced cells was extracted using standard procedures [26]. The cDNA was made as previously described [7]. The primer sequences to detect LMP2A and -2B

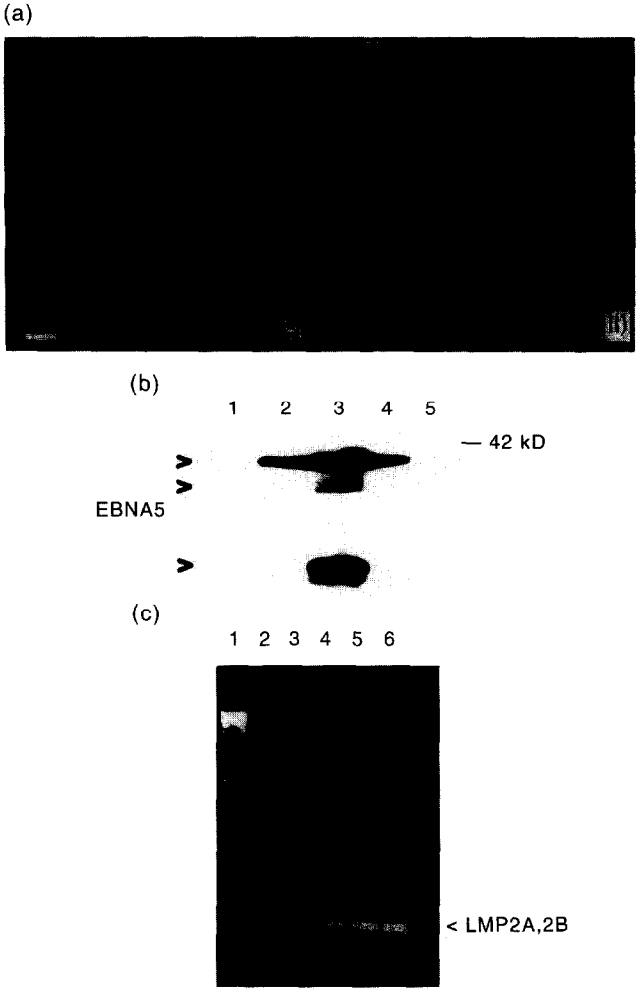

Figure 3. EBNA 4(a) EBNA-5(b) and LMP2(c) expression in S6C cells. (a) Serum from an EBV-positive donor was used to detect EBNA4 expression by ACIF. (i) S6C vector control; (ii) S6C EBNA4 clone 7. (b) EBNA5 expression in the S6C cells was monitored by monoclonal antibody JF186. Lane 1: S6C vector; lane 2: S6C EBNA5 clone 4; lane 3: clone 9; lane 4: LCL IB4; lane 5: EBV-negative line Bjab. A weak non-specific band was detected in the vector transfected cells. (c) LMP2A and -2B expression as detected by RT-PCR. Lane 1: Marker; lane 2: an EBV carrying group III line—Mutu type III; lane 3: H_2O + buffer control; lane 4: LMP2A1; lane 5: LMP2B2; lane 6: LMP2B3. Clones S6C-LMP2A1 and -2B3 were used in the immunogenicity tests.

expression were 5′(sense)-CGG ACT ACA AGG CAT TTA CG-3′ and antisense 5′-CTG CTG CCA ATG TTA AAA GG-3′ (B95-8 coordinates 439 and 171 494, respectively). Three microliters of cDNA were used as template for the nested PCR. The reactions were performed in 50 μl containing 20 mM Tris (pH 8.5), 50 mM KCl, 15 mM $MgCl_2$, 1 μM of primers and 200 μM of each of the four nucleotides dATP, dGTP, dCTP and dTTP. One unit of Taq polymerase (Perkin Elmer) was added to the 50 μl reaction mixture. The samples were covered with 2–3 drops of mineral oil and subjected to 35 cycles of amplification in the thermal cycler (Techne PHC-2). The temperature conditions were denaturation 94°C 45 min, reannealing 61°C 1 min, elongation 72°C 2 min.

Rejection assays

CBA (H-2k) mice were immunised with the untransfected, vector transfected and EBNA1 or LMP1 transfected SBfnHD cells. ACA (H-2f) mice were immunised with EBNA4, 5, LMP2A and -2B transfected and control S6C cells. For immunisation 10^6 cells were irradiated with 10 000 rad and inoculated subcutaneously once a week for 3 weeks. Once a week after the last immunisation, the mice were challenged with graded numbers of viable cells. The mice were irradiated with 400 rad before the live challenge to distinguish between specific immune rejection and non-specific resistance [27]. Tumour growth was followed weekly by caliper measurement in three dimensions.

RESULTS

Expression of EBV encoded antigens in the transfectants

Figure 2a shows that four different EBNA1 transfected SBfnHD clones expressed the full-length EBNA1 (approximately 70 kD). Figure 2b shows the expression of LMP1 in SBfnHD cells. Figure 3 shows the expression of EBNA4, EBNA5, LMP2A and -2B expression in S6C.

Immunogenicity tests

Table 1 shows the immunogenicity of EBNA1 transfected SBfnHD cells in the syngeneic CBA mice. None of the three EBNA1 expressing clones induced any detectable rejection reaction. In contrast, the LMP1 expressing cells were immunogenic in CBA mice.

Table 2 shows rejection tests with EBNA4, -5, LMP2A, -2B and the vector transfected S6C cells. ACA mice pre-immunised with S6C EBNA4 clone 7, EBNA5 clones 4 and 9 rejected the corresponding transfectants at both 10^3 and 10^4 cell challenge doses. Cells carrying the puromycin vector were non-immunogenic. S6C cells expressing LMP2A and -2B were used to pre-immunise the ACA mice. Both LMP2A and -2B induced rejection responses, whereas the vector transfected cells were non-immunogenic (Table 2).

DISCUSSION

Our earlier report [17] showed that EBNA1 transfected S6C were non-immunogenic in ACA mice, in contrast to the strong rejection-inducing ability of the corresponding LMP1 transfectants. This has been fully confirmed in the present study. We also showed that the non-immunogenicity of EBNA1 is not peculiar to the H-2f allele, carried by the ACA strain, but is also valid for H-2k, carried by another non-immunogenic tumour, SBfnHD. Moreover, we found that EBNA4, EBNA5, LMP2A and -2B are also highly immunogenic when expressed in S6C cells.

The high immunogenicity of EBNA4 in the present system is interesting in light of the fact that EBNA4 is the preferential target of HLA A11 restricted CTLs in the human system [8] and has been extensively investigated with regard to immunogenic epitopes and T-cell receptors [28, 29]. It

*Table 1. Rejection tests with EBNA1 and LMP1 transfected SBfnHD (H-2k)**

Cells	Transfected with	No. of viable cells inoculated	Immunised†	Controls	Total takes Immunised	Total takes Controls	% takes Immunised	% takes Controls
SBfnHD	–	10^3	13/15	15/15				
		10^4	15/15	15/15	28/30	30/30	93	100
SBfnHDV1	gpt vector	10^3	3/9	4/10				
		10^4	8/10	10/10				
		10^5	10/10	10/10	21/29	24/30	72	80
SBfnHDE1-1	EBNA1	10^2	0/10	1/10				
		10^3	5/10	9/10				
		10^4	20/25	25/25				
		10^5	25/25	25/25	50/70	60/70	71‡	85
SBfnHDE1-2	EBNA1	10^2	10/10	10/10				
		10^3	5/9	10/10				
		10^4	10/10	10/10	25/29	30/30	86‡	100
SBfnHDE1-4	EBNA1	10^4	15/15	15/15				
		10^5	15/15	15/15	30/30	30/30	100	100
SBfnHDV2	Hist vector	10^3	4/10	6/10				
		10^4	8/10	10/10	12/20	16/20	60	80
SBfnHDL1	LMP1	10^2	2/10	8/10				
		10^3	1/10	10/10				
		10^4	4/24	22/25	7/44	40/45	15§	88
SBfnHDL8	LMP1	10^3	1/10	3/10				
		10^4	7/25	25/25	8/35	28/35	22§	80

* The figures represent the number of mice with progressively growing tumours over the total number of mice inoculated. † The immunisations were performed by 3 weekly inoculations of 10^6 irradiated cells (10 000 rad) from the lines indicated. ‡ $0.02 < P < 0.05$, § $P < < 0.001$.

*Table 2. Rejection with S6C cells expressing EBNA4, EBNA5, LMP2A and -2B**

Cells	Transfected with	No. of viable cells inoculated	Immunised	Controls	Total takes Immunised†	Total takes Controls	% takes Immunised	% takes Controls
S6CV3	Puro vector	10^3	8/10	10/10				
		10^4	10/10	10/10	18/20	20/20	90	100
S6CE4-7	EBNA4	10^3	1/10	10/10				
		10^4	2/20	16/20	3/30	26/30	10§	86
S6CE5-4	EBNA5	10^3	4/30	20/29				
		10^4	11/30	30/30	15/60	50/59	25§	84
S6CE5-9	EBNA5	10^3	1/20	12/20				
		10^4	8/20	17/20	9/40	29/40	22§	72
S6CV4	Neo vector	10^2	6/20	9/20				
		10^3	12/20	20/20				
		10^4	20/20	20/20	38/60	49/60	63‡	81
S6CLMP2A-1	LMP2A	10^2	0/30	9/24				
		10^3	6/30	20/24				
		10^4	11/29	24/24	17/89	53/72	19§	73
S6CLMP2B-3	LMP2B	10^2	0/29	5/30				
		10^3	5/30	5/30				
		10^4	5/30	26/30	10/89	36/90	11§	40

*,†See footnotes to Table 1. ‡$0.02 < P < 0.05$, §$P << 0.001$.

has also been shown that the HLA A11 restricted immunodominant epitope of EBNA4 is mutated in virus strains isolated from populations where HLA A11 is over-represented [30]. It would be interesting to compare these and other viral strains for their immunogenicity in our model system.

Ambiguous findings have been reported concerning the immunogenicity of EBNA5 for human CTLs [31]. Our results clearly show that EBNA5, unlike EBNA1, is highly immunogenic in the mouse system.

We have previously shown that LMP1 is highly immunogenic when expressed in H-2f haplotype-carrying tumour [17]. Here, we show that LMP1 is also immunogenic in H-2k haplotype-carrying tumours. Furthermore, the rejection was LMP1-specific as indicated by the observation that mice immunised with LMP1-expressing SBfnHD cells were able to reject the live challenge only with the same cells and not vector carrying or LMP2A carrying SBfnHD cells (data not shown). In the human system, LMP1 has been shown to serve as the target of HLA A24 and B51 restricted CTL responses [9]. However, the CTL epitopes of LMP1 have not been defined. When expressed in EBV negative BLs, LMP1 induce a variety of phenotypic changes, including the upregulation of adhesion molecules and B-cell activation markers [32]. LMP1 can also induce HLA class II [33] and transport associated protein (TAP) expression [34]. It has been questioned whether LMP1 acts as an ordinary immunogenic protein or as a result of these changes in the cellular phenotype. In our murine carcinoma cells, LMP1 did not significantly alter ICAM1, CD44 and B7 expression (data not shown). Our present findings that LMP1 was highly immunogenic in the absence of any marked phenotypic changes and in both H-2k and H2-f carrying tumours, reinforces the notion that LMP1 can induce rejection reactions like other EBV encoded proteins.

The high immunogenicity of LMP2 is of special interest, because this protein is regularly expressed in NPCs [35]. In a previous study, we have transfected S6C cells with an LMP1 gene derived from a Chinese NPC and found that the transfectants were non-immunogenic, in contrast to their B-cell derived LMP1 transfected counterparts [36]. We have suggested that the NPC from which the gene has been derived may represent a non-immunogenic variant perhaps due to immunoselection. The same question needs to be addressed with regard to LMP2.

The inability of EBNA1 to induce a rejection response is in line with its behaviour in the human system and shows that this is not a peculiarity of the ancient relationship between EBV and the human host. It is consistent with the idea of a specific processing and/or transport problem that prevents the "arrival" of appropriate target peptides to the presenting MHC class I molecule [12].

The good concordance between the mouse rejection tests and the human CTL mediated cytotoxic reactions encourages the extension of the experimental system to parallel *in vitro* experiments. Combined with adoptive transfer of immunity via T-cells, the mouse carcinoma transfection test may provide a useful complement to assess the immunogenic potential of EBV encoded proteins in different tumours.

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