Differences in the Immunogenicity of Latent Membrane Protein 1 (LMP1) Encoded by Epstein-Barr Virus Genomes Derived from LMP1-positive and -negative Nasopharyngeal Carcinoma

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ABSTRACT

We have previously shown that an EBV-encoded latent membrane protein 1 (LMP1) gene derived from a nude mouse-propagated nasopharyngeal carcinoma (NPC) tumor and expressed in nonimmunogenic murine mammary carcinoma S6C cells failed to convey immunogenicity (rejectability) in syngeneic mice, whereas the corresponding B-cell derived LMP1 gene made the mice highly immunogenic. This raised the question of whether LMP1-expressing NPCs have been selected for low immunogenicity at the viral gene expression level. If so, LMP1-negative tumors that carry highly methylated LMP1 regulatory sequences may not have been exposed to a similar immunoselection. In the present study, we have compared LMP1 genes derived from two LMP1-positive NPCs and two LMP1-negative NPCs. All four genes were expressed in S6C cells in parallel with the previously tested isolates from a B-cell (B95-8)-derived and a nude mouse-propagated NPC (Cao)-derived gene. As in the previous study, we have found that the B-cell-derived LMP1 isolate was highly immunogenic. LMP1-positive tumor-derived isolates were poorly immunogenic, whereas the isolates from the LMP1-negative NPC tumor had intermediate immunogenicity. Sequence data revealed that LMP1 genes from LMP1-expressing NPC had 16 amino acid substitutions, whereas LMP1 from non-LMP1-expressing NPC had only 9 amino acid changes in the coding region. Three of the changes were at shared sites, but with different modifications. The fact that the gene from non-LMP1-expressing NPC mutated at a low frequency but was more immunogenic than the LMP1 gene derived from LMP1-expressing NPC, which was highly mutated but less immunogenic, favors the idea that LMP1-positive tumors escape immunosurveillance in immunocompetent hosts by either a selective down-regulation of LMP1 expression, methylation in the LMP1 promoter sequence, or mutation of LMP1 in LMP1-expressing samples.

INTRODUCTION

Immunological control of EBV-transformed B-cell proliferation is carried out by specifically sensitized CD8+ CTLs and, to a lesser extent, by some nonspecific effectors such as natural killer cells. These CTLs are responsible for the self-limiting nature of infectious mononucleosis (1). After acute mononucleosis, EBV persists as a latent and asymptomatic infection for life. Persistent infection is thus achieved in the face of continued host immune response. The mechanisms of this successful coexistence are of great interest for the understanding of both the viral strategy and host immune surveillance (2).

NPC1 shows a conspicuous geographical distribution, with a very high incidence in Southeastern China, North Africa, and Alaska. EBV genomes have been detected in 100% of undifferentiated NPC patient samples.

NPC patients have higher anti-EBV antibody titers than healthy EBV carriers (3, 4). Elevated antibody titers can be found before the onset of clinical NPC symptoms and in patients with primary or recurrent carcinoma (5–7).

In contrast to EBNA1, which is consistently expressed in both BL and NPC tumors, LMP1 is expressed in 65% of NPCs as detected by immunoblotting (8, 9) but is not expressed in BLs. We have shown that LRS is highly methylated in the 35% of NPC patients who are non-LMP1 expressors, in contrast to those who are LMP1 expressors, in whom LRS is never methylated (10). NPC develops in immunocompetent individuals, suggesting that the tumor cells are able to escape immune surveillance.

We have previously shown that B-LMP1, but not EBNA1, can convert nonimmunogenic mouse mammary tumor S6C cells derived from ACA (H-2f) mice into immunogenic, rejection-inducing tumor (11). Furthermore, a NPC-derived LMP1 clone (C-LMP1) makes an immortalized but non tumorigenic human keratinocyte cell line (RHEK1) more clonable in soft agarose and increases its ability to give rise to tumorigenic variants as compared with its B-LMP1 counterpart (12). The same two LMP1 clones were also compared for their immunogenicity in a mouse model system. B-LMP1 but not C-LMP1 made the cells rejectable (13). The two LMP1 clones also varied in their DNA sequence (14).

This suggests that immune surveillance may be avoided by mutating potential immunogenic epitopes to nonimmunogenic ones in LMP1 gene of the LMP1-positive NPC cells (13). Alternatively, immunogenic LMP1 may be down-regulated by methylation of the promoter (10).

If there are indeed two mechanisms whereby LMP1-dependent immunogenicity can be avoided in NPCs (mutation or down-regulation), a comparison between LMP1 isolates from LMP1-expressing and non-LMP1-expressing NPCs could be relevant. As an approach to this question, we have compared LMP1 genes derived from two LMP1-positive and two LMP1-negative NPCs. All four genes were expressed in nonimmunogenic S6C murine mammary carcinoma cells in parallel with the previously tested isolates one from a B-cell (B95-8)-derived and one from a nude mouse-propagated, NPC (Cao)-derived gene.

We have found that LMP1 isolates from non-LMP1-expressing NPCs mutated at a low frequency but were more immunogenic than LMP1 isolates derived from LMP1-expressing NPCs, which were highly mutated but less immunogenic.

MATERIALS AND METHODS

Tumors and Cell Lines. NPC tumor biopsies were collected from the Ear, Nose and Throat Department, The Guangzhou Provincial Hospital, China, before treatment. Biopsy specimens were snap-frozen within 1 h of surgical removal and stored at −70°C. Diagnosis was based on a histopathological examination performed by the hospital pathologist according to the WHO classification (15). Cao is a nude mouse passaged tumor from a Chinese NPC carrying type A EBV.
The S6C cell line was derived from a spontaneous mammary adenocarcinoma in an ACA (H-2f) mouse. This tumor is not immunogenic in syngeneic mice (16).

**Construct.** The LMP1 fragments were introduced into expression vectors by cloning a 1671-bp Smal fragment (B95-8 coordinates, 169589–167918) covering the coding region of LMP1 from LMP1-expressing NPC and non-LMP1-expressing NPC into the DraI site of the pSHIS4 retroviral expression vector as described previously (13).

**Immunization.** For immunization, LMP1-transfected and control S6C cells suspended in PBS were irradiated with 10,000 rads and injected s.c. once a week for 3 weeks into syngeneic 4–6-week-old ACA mice. One week after the last immunization, mice were whole body irradiated with 400 rads. Within 24 h of irradiation, mice in each group were challenged s.c. with graded doses of viable cells. Tumor growth was followed weekly for up to 8–10 weeks by three-dimensional caliper measurements.

**LMP1 Expression.** LMP1 expression in the biopsies and LMP1 transfectants was detected by immunoblotting as described previously (17). Briefly, frozen tissues were homogenized in a small volume of radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% sodium deoxycholate, 0.5% NP40, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride] in a conical glass homogenizer with a tight-fitting pestle and sonicated (3 × 15 s) on ice. Extracts were heated at 90°C for 5 min and clarified by centrifugation for 20 min at 10,000 × g. Aliquots of the supernatant were used for SDS-PAGE on 7.5% acrylamide gels in Mini-gel (Bio-Lab) at 15 V/cm until the bromphenol blue marker reached the bottom.

LMP1 was detected using monoclonal antibody S12 and an alkaline phosphatase-conjugated secondary antibody (Sigma). EBNA1 expression was verified by immunoblotting with polyclonal, polyvalent PG serum as described previously (7).

**Methylation.** Methylation of the LMP1 promoter region was analyzed by digesting high molecular weight cellular and viral DNA with either HpaII (specificity, CCGG) or HhaI (specificity, CCGG). Ten units of enzyme per milligram of DNA were added to complete digestion. The HpaII digests were controlled by parallel digestion with MspI, an isoschomer that is not inhibited by methylation. The digested DNAs were separated on a 1.5% agarose gel, and the blot was hybridized with a probe covering the promoter and 5′-flanking sequence of LMP1 as described previously (10).

**Sequencing.** Sequencing was done using the ALF sequenator (Pharmacia) according to manufacturer’s protocols.

**RESULTS**

**LMP1 Expression.** NPC biopsies were assayed for EBNA1 and LMP1 expression by immunoblotting using monoclonal antibody S12 for LMP1 (Fig. 1A) and polyclonal serum for EBNA1 (Fig. 1B).

Three tumors (T123, T124, and T125) express LMP1 and are designated as LMP1-expressing NPCs (LMP1+). Three tumors (T127, T128, and T131) did not express LMP1, as judged by immunoblotting, and are designated as non-LMP1-expressing NPCs (LMP1−). All of these tumors expressed EBNA1.

The methylation study was performed in these LMP+ and LMP− tumors. The LRS region containing the LMP1 promoter was unmethylated in two of the three LMP1 expressors (T123 and T125) and showed a mixed methylation pattern in the third (T124). The LRS region was methylated in the three LMP1− tumors (T127, T128, and T131; Fig. 1C). The mixed pattern of methylation and the two different fragments of LMP1 produced by PCR-based amplification with one dominant fragment (data not shown) suggest the presence of two different strains with different LMP1 fragments. One strain contains a deletion, and the other is mutated.

We selected LMP1+ tumors T123 and T125 and LMP1− tumors T127 and T128 for further study. The LMP1 gene from each of the LMP1+ and LMP1− tumors was cloned and transfected into S6C cells. Two transfectants from each tumor, each with a comparable level of LMP1 expression, were used for the immunogenicity study.

**LMP1 from LMP1-expressing NPCs Is Nonimmunogenic, Whereas LMP1 from non-LMP1-expressing NPC Is Immunogenic.** The growth of LMP1-transfected S6C cells in preimmunized and control mice is summarized in Table 1. The vector-transfected S6C cells grew equally well in preimmunized and control mice.

The B-LMP1 cells were rejected in a significant proportion of the preimmunized mice compared with vector-control-immunized mice. In contrast, Cao-LMP1 grew well in both immunized and nonimmunized mice. Thus, these clones were used as negative and positive controls, respectively.

S6C clones T123-3 and T123-10 from the T123 tumor and clones T125-17 and T125-8 from the T125 tumor showed a range of tumor take from 79% at a low challenge dose (100 cells) to 100% at a high challenge dose (10,000 cells). The clear-cut result was seen in clones at high challenge dosages resulting in 100% tumor take. The growth pattern was similar to that of Cao-LMP1 and was nonimmunogenic. In contrast, clones T127-1 and T127-3 from the LMP1− T127 tumor and clones T128-12 and T128-35 from the LMP1− T128 tumor cell line were frequently rejected in preimmunized mice. All clones were immunogenic, and tumor take ranged from 40% at a low challenge dose to 52% at a high challenge dose. Tumors growing in...
the immunized mice were equally as positive for LMP1 expression by immunoblotting as the original clone (data not shown).

The difference in immunogenicity was even more visible after pooling data from clones of T123 and T124 (the LMP1+ group) and from clones of T127 and T128 (the LMP1− group; Fig. 2). LMP1 from non-LMP1-expressing NPCs showed less tumor take and was immunogenic. In contrast, LMP1 from LMP1-expressing NPCs showed a high tumor take and was nonimmunogenic.

The average of percentage tumor take for Cao-LMP1 is 80% compared with 98% for the vector control and 22% for B-LMP1 (Fig. 3). Whereas LMP1+ from T123 and T125 was basically nonimmunogenic with a 92% tumor take (Figs. 2 and 3), the LMP1− from T127 and T128 showed immunogenicity with a 47% tumor take. Tumors were rejected at all three challenge doses tested. The differences in the immunogenicity of the LMP1+ and LMP1− S6C cells were also reflected by the s.c. growth pattern of the tumors and the extensive skin damage (e.g., necrosis) around the tumor site in the LMP1− clone as compared with the LMP1+ clone (see “Discussion”).

Mutation Pattern Is Different between LMP1 Genes from LMP1-expressing NPCs and Those from Non-LMP1-expressing NPCs. LMP1 genes derived from LMP1-expressing NPCs and non-LMP1-expressing NPCs were sequenced. In total, there are 23 frequent changes in LMP1 (22 aa changes and 1 deletion). Three are changed at the same position but have different substitutions in LMP1+ and LMP1− NPC-derived isolates. LMP1 genes from LMP1-expressing NPCs had 13 different substitutions, whereas LMP1 from non-LMP1-expressing NPCs had 6 different changes (Table 2).

LMP1− NPC biopsies had a deletion in the region of aa 343–352. LMP1+ NPC samples did not have this deletion but had two mutations in this region (G344D and G354A) instead. Together with the observation that a 10-aa deletion can be detected in almost 90% of NPC biopsies and 60% of LMP1+ NPC biopsies in this laboratory,4 these observations indicate that this deletion is thus associated with neither LMP1 expression nor immunogenicity. Because all samples are from NPC biopsies, we cannot judge whether this 10-aa deletion is associated with higher tumorigenicity (18).

Thus, the difference in the sequence of LMP1+ and LMP1− may be associated with different immunogenicity.

DISCUSSION

In our first experimental series, we transfected S6C, a nonimmunogenic mouse mammary carcinoma of ACA (H-2f) origin, with B95-8 cell-derived EBNA-1 and B-LMP1, respectively, and compared the tumor take incidence and growth rate of the transfectants and their controls in preimmunized syngeneic and semisyngeneic hosts. B-LMP1-expressing transfectants induced strong rejection responses, whereas EBNA-1 transfectants were nonimmunogenic in syngeneic hosts and only weakly immunogenic in some semisyngeneic F1 hybrids (11).

4 B. Troyanovsky, I. Ernberg, F. Chen, X. N. Zhang, and L. F. Hu. The two predominant EBV variants in China had different effects on NF-κB activation, but this did not relate to expression in tumor, submitted for publication.

Table 1 Rejection of B- and C-LMP1-transfected S6C cells in the syngeneic ACA (H-2f) mice

<table>
<thead>
<tr>
<th>Transfectant cells</th>
<th>LMP1 status in tumor</th>
<th>No. of viable cells inoculated</th>
<th>Tumor take</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>−</td>
<td>10²</td>
<td>8/9</td>
</tr>
<tr>
<td>B-LMP1</td>
<td>+ in cell line</td>
<td>10³</td>
<td>13/13</td>
</tr>
<tr>
<td>Cao-LMP1</td>
<td>?, but + in mice</td>
<td>10²</td>
<td>2/25</td>
</tr>
<tr>
<td>T123-3</td>
<td>+</td>
<td>10²</td>
<td>3/6</td>
</tr>
<tr>
<td>T123-10</td>
<td>+</td>
<td>10³</td>
<td>5/6</td>
</tr>
<tr>
<td>T125-17</td>
<td>+</td>
<td>10²</td>
<td>2/5</td>
</tr>
<tr>
<td>T125-8</td>
<td>+</td>
<td>10²</td>
<td>8/10</td>
</tr>
<tr>
<td>T127-1</td>
<td>−</td>
<td>10²</td>
<td>2/7</td>
</tr>
<tr>
<td>T127-3</td>
<td>−</td>
<td>10²</td>
<td>2/8</td>
</tr>
<tr>
<td>T128-12</td>
<td>−</td>
<td>10²</td>
<td>1/6</td>
</tr>
<tr>
<td>T128-35</td>
<td>−</td>
<td>10²</td>
<td>2/6</td>
</tr>
</tbody>
</table>

Fig. 2. Comparison of the percentage of tumor takes of different transfected LMP1-expressing S6C cells in immunized (dot column) and nonimmunized (grid column) ACA mice at all of the challenge doses tested. The numbers above each column indicate the number of mice inoculated.

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Using the same model system, we previously found that LMP1 derived from Cao NPC was unable to induce rejection of the transfected tumors in preimmunized, syngeneic ACA mice (13). In the present study, we have cloned, sequenced, and examined four different LMP1 isolates to examine their immunogenicity. Moreover, we asked whether the LMP1 isolates from non-LMP1-expressing NPCs would be immunogenic. We confirm our previous findings that LMP1 from the LMP1-expressing NPCs is nonimmunogenic and further show that LMP1 isolates derived from non-LMP1-expressing NPCs are more highly immunogenic. The present results would thus argue that LMP1 isolates from the LMP1-expressing NPCs are rendered low or nonimmunogenic by mutating the potential immunogenic epitopes, whereas LMP1 isolated from the non-LMP1-expressing NPCs is immunogenic, but its expression is silenced in vivo by methylation in the LRS region. These findings suggest that NPC tumors may have been immunoselected at the level of the viral genome.

We have shown that B95-8-derived EBNA5 and both LMP2A and LMP2B made the cells immunogenic (relebale) (19). As far as LMP2 is concerned, this is important because both the 2A and 2B forms of LMP2 are regularly expressed in NPC (20). This raises the question of how NPCs escape immune surveillance despite expression of LMP2A and LMP2B. Could it be that LMP2A and LMP2B expressed in the NPCs are nonimmunogenic, just as LMP1 derived from the LMP1-expressing NPC tumors? Clinical and follow-up data from 74 cases of NPC have shown that the LMP1+ tumors grew faster and more expansively than LMP1− tumors but nevertheless had a better prognosis. LMP1− tumors recurred at a higher frequency and showed an increased tendency to form metastases (21). These seemingly paradoxical observations may be related to the following two experimentally demonstrated facts:

(a) LMP1 can transform immortalized strains of rodent fibroblasts (22). We have shown (12), moreover, that NPC-derived C-LMP1 increases the agarose clonability and (22). We have shown (12), moreover, that NPC-derived C-LMP1 increases the agarose clonability and (22). We have shown (12), moreover, that NPC-derived C-LMP1 increases the agarose clonability and (22).

(b) Our earlier work, showing a correlation between the lack of LMP1 expression in NPC biopsies and methylation of the regulatory LRS, indicates that LMP1 negativity may represent a secondary change, representing a regulatory response of the tumor to immunoselection. By the time this response occurs during tumor progression, the NPC cells may have become independent of the proliferation-driving effect of LMP1 by secondary cellular changes. Our findings indicate that the loss of LMP1 expression may nevertheless reduce the expansiveness of the tumor in vivo to some extent.

Our findings are consistent with the experience that EBV+ BLs are treatable by chemotherapy and more localized than EBV− BLs (23). Another analogy can be found in the better prognosis of human papillomavirus-positive anogenital carcinoma, as compared with their human papillomavirus-negative counterparts (24–26).

It has been demonstrated that EBV can mutate its highly immunogenic epitopes in the EBNA4 gene under strong immune pressure due to the high prevalence of HLA A11 in Southeast Asia (27). Such epitope variation under immune selection is also observed in HIV and lymphocytic choriomeningitis virus (28, 29).

NPC LMP1+ shares 3 of 16 mutations with Cao LMP1 but does not have the aa deletion. The shared sites of mutation involve different aa residues. Given the lack of overlap between nonimmunogenic Cao and NPC LMP1+ mutations relative to the immunogenic B95-8 LMP1, it seems that there are multiple routes of immune evasion through the modification of LMP1 coding sequences. With a readout of the different immunogenicity of LMP1 isolates from the NPCs, it will be possible to localize the region within the LMP1 sequence responsible for the difference in immunogenic potential.

Another possible mechanism of escape could be as in Hodgkin’s disease (HD), where active EBV-specific CTLs are found in the lymphoid infiltrate of EBV− but not in EBV+ HD (30, 31). This and the reactivation of EBV-specific CTLs from the blood of HD patients independently of the EBV status of their tumors suggest that local suppression of EBV-specific CTL responses may also be an important pathogenic factor.

LMP1 expression has been shown to induce the production of IL-10 (32) and IL-6 (33). Both human IL-10 and viral IL-10 are up-regulated in NPC (34). IL-10 is able to inhibit the activation of Th1 cells and appears to modulate MHC class I expression in lymphoid and epithelial cells (35). Expression of MHC class II is higher than that of MHC class I in NPC. Thus, the role of IL-10 and IL-6 in LMP1-expressing malignancies and their contribution to tumor growth are unclear.

In summary, EBV must escape immunosurveillance in immunocompetent patients to allow tumor development. CTL escape mutants of EBV would have a greater chance of being reactivated to produce infectious virus in the oropharynx and to proliferate within the host.

<table>
<thead>
<tr>
<th>Codon</th>
<th>LMP1+</th>
<th>LMP1−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change in the same position with different substitution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Leu to Met cons.</td>
<td>Leu to Ile cons.</td>
</tr>
<tr>
<td>84</td>
<td>Cys to Val cons.</td>
<td>Cys to Gyl cons.</td>
</tr>
<tr>
<td>338</td>
<td>Leu to Pro cons.</td>
<td>Leu to Ser cons.</td>
</tr>
<tr>
<td>Change in different position</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>Leu to Ile cons.</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>Val to Ile cons.</td>
<td></td>
</tr>
<tr>
<td>57</td>
<td>Ser to Ala non.</td>
<td></td>
</tr>
<tr>
<td>91</td>
<td>Thr to Ile non.</td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>Gly to Ala cons.</td>
<td></td>
</tr>
<tr>
<td>119</td>
<td>Val to Ala cons.</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>Asp to Ala non.</td>
<td></td>
</tr>
<tr>
<td>178</td>
<td>Leu to Met non.</td>
<td></td>
</tr>
<tr>
<td>189</td>
<td>Gln to Pro non.</td>
<td></td>
</tr>
<tr>
<td>245</td>
<td>Pro to His non.</td>
<td></td>
</tr>
<tr>
<td>252</td>
<td>Gly to Asp cons.</td>
<td></td>
</tr>
<tr>
<td>298</td>
<td>Asp to Asn non.</td>
<td></td>
</tr>
<tr>
<td>322</td>
<td>Gly to Gln non.</td>
<td></td>
</tr>
<tr>
<td>331</td>
<td>Gly to Asp non.</td>
<td></td>
</tr>
<tr>
<td>334</td>
<td>Gln to Arg cons.</td>
<td></td>
</tr>
<tr>
<td>335</td>
<td>Gly to Asp cons.</td>
<td></td>
</tr>
<tr>
<td>339</td>
<td>Met to Ile/Lys cons./non.</td>
<td></td>
</tr>
<tr>
<td>344</td>
<td>Gly to Asp cons.</td>
<td></td>
</tr>
<tr>
<td>354</td>
<td>Gly to Ala cons.</td>
<td></td>
</tr>
<tr>
<td>343–352</td>
<td>Deletion</td>
<td></td>
</tr>
</tbody>
</table>

* cons., conservative change; non., nonconservative change.
Our results indicate that mutation may be favorable to reduce immunogenicity in LMP1-expressing tumors and affect the activity of cellular factors, leading to development of NPC.

This switch may be responsible in part for tumor development and escape from host immune system surveillance.

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