ABSTRACT

EBV is found to be associated with 100% of poorly or undifferentiated nasopharyngeal carcinomas, a tumor of epithelial origin. The latent membrane protein-1 (LMP1) of EBV, may play a causal role in the development of this disease. The experiments initiated here were designed to examine the activity of LMP1 in vivo in the epidermis of PyLMP1 transgenic mice in relation to its putative role in carcinogenesis. Transgenic positive epidermis showed a 2–3-fold increase in the mitotic index, coupled with an increased level of expression of proliferative cytokeratin markers. In addition, LMP1 has been detected in approximately two-thirds of NPC specimens tested, whereas LMP1 mRNA has been detected by reverse transcription-PCR assays in the great majority of NPC biopsies. (10–12). LMP2 transcripts are also consistently detected in NPC cells (11, 13). In addition, the multiply spliced rightward BamHI-A transcripts (BARTs/CSTs) are abundant in NPC cells (14–17) and capable of encoding several proteins including RPS5, A73, BARFO and RK-BARFO (18–20).

Genetic deletion studies have revealed that LMP1 is one of the latent genes required for in vitro B-cell immortalization by EBV (21, 22). Moreover, LMP1 is oncogenic in nonlymphoid cells, first demonstrated by its ability to induce growth transformation in certain immortalized rodent fibroblast cell lines (23–25). Importantly, this oncogenic action extends to epithelial cells. Expression of LMP1 in the epidermis of transgenic PyLMP1 mice induces hyperplasia, an early step in the carcinogenic process (26). Furthermore, in cultured carcinoma cell lines, heterologous expression of LMP1 leads to reduced serum requirements, loss of anchorage dependence, increased invasive capacity, and, in some cases, inhibition of terminal differentiation (27–31). Moreover, growth characteristics of NPC tumors have been correlated with LMP1 expression levels. Detectable LMP1 protein is linked with the expression of EGFR and Ki67 in NPC biopsies (32), and LMP1-positive NPC tumors appear to grow faster and more expansively than LMP1-negative NPC tumors (33).

In B-cells, LMP1 has been shown to act similarly to a constitutively active CD40 receptor in prolonging cell survival and supporting proliferation (34–36). Like CD40, LMP1 binds to tumor necrosis factor receptor-associated factors 1, 2, 3, and 5 (37–40). In addition, LMP1 associates with the tumor necrosis factor receptor-associated factor-associated death domain proteins TRADD and RIP (41, 42) and to JAK3 (43). Through these interactions, LMP1 causes the activation of NFκB, JNK, and STAT proteins (44–47), which ultimately results in a plethora of changes in cellular gene expression. In epithelial cell lines, LMP1 can induce the expression of CD40 and CD54 (both expressed in NPC cells; Ref. 48, 49), as well as the antiapoptotic gene A20, EGFR, and IL6 (50–52). In vivo, transgenic epidermal expression of LMP1 leads to up-regulation of the proliferative cytokeratin marker, K6 (26).

In transgenic modeling, the accessibility of the mouse skin makes it an ideal tissue in which to study the carcinogenic process. Moreover, the mouse skin model of multistage carcinogenesis has been well characterized with respect to genetic and epigenetic changes (reviewed in Ref. 53). Using chemical carcinogens, the stages of initiation, promotion to a benign tumor (papilloma), malignant conversion, and metastatic invasion have been defined. The most commonly used tumor induction protocol involves a single treatment with the mutagen DMBA and then repeated treatments with the tumor promoter TPA. In virtually all DMBA-initiated tumors, activation of
the H-ras gene has been identified as an initiating event (54, 55), and cyclin D1 has been shown to be a critical target of activated ras in this system (56, 57). TPA treatment then promotes the clonal survival and outgrowth of initiated cells to form an overt lesion. The hyperplastic response of the epidermis to TPA is thought to be mediated in part through sustained direct activation of PKC (58), but, in addition, the enhanced keratinocyte proliferation rate is linked to the accompanying inflammatory skin response (59). Subsequent loss of p53 contributes to malignant conversion, and deletion of the INK4 locus is associated with transformation to a highly invasive tumor phenotype. Several of these steps have been modeled by engineering transgenic strains of mice with activated oncogenes or deleted tumor suppressor genes (60–65).

Transgenic mice expressing LMP1 in the epidermis displayed the phenotype of epidermal hyperplasia with hyperkeratosis that did not progress to tumor formation, demonstrating that LMP1 is insufficient for carcinogenicity (26). The phenotype of LMP1-expressing mice is markedly similar to that displayed by mice expressing activated H-ras, TGFα, or (to some extent) v-fos in the epidermis, all of which demonstrate increased epidermal proliferation (61, 62, 64, 66, 67). These observations raise a number of questions relating to the action of LMP1. First, does LMP1 induce hyperplasia by increasing epidermal proliferation, inhibiting differentiation, or both? Second, can LMP1 act as a classical initiator and elicit tumors similar to those observed by TPA promotion of H-ras transgenic mice (68); or can LMP1 act as a tumor promoter in the manner of v-fos (69). Alternatively, LMP1 may act as either initiator or promoter, depending upon the context, as with TGFα, or possibly act at a later stage to promote carcinogenic progression. To address the role of LMP1 in inducing hyperplasia in vivo, the action of LMP1 in the transgenic mouse epidermis was examined. We show that LMP1 acts by inducing epithelial cell proliferation. Furthermore, to assess the synergistic molecular events associated with LMP1 in carcinogenesis, PyLMP1 mice were treated with a classical regime of chemical carcinogens, revealing that LMP1 can act as a tumor promoter.

**MATERIALS AND METHODS**

**DNA and RNA Preparation, Blotting, and Probing.** Genomic DNA was isolated from mouse tail segments as described previously (26). Transgene status was tested by slot blot or Southern blot using Biodyne nylon membrane (ICN). For Southern analyses, gels were electroblotted in a Hoeffer electroblotting apparatus as described previously (26, 70). Total RNA was extracted from tissues using RNAzol B according to the manufacturer’s instructions (Biogenesis Ltd). Total RNA (20 μg) was electrophoresed and electroblotted essentially as described (26).

All blots were hybridized with [α-32P]dCTP-labeled, randomly primed DNA probe fragments (Prime-it II kit; Stratagene) and washed under stringent conditions [0.1× SSC–0.1% SDS (w/v) at 68°C] before autoradiography. Northern blot signals were quantified by phosphorimage analysis; and a radiolabeled GAPDH probe was used to normalize for loading, dividing sample-specific phosphorimage values with the GAPDH value of the same sample. Short cDNA fragments primarily consisting of the 3′ noncoding region were used as probes for specific murine cytokeratin sequences (K1, K6, K10, and K14 described in Ref. 26). The K17 probe was a 3′–253-bp cDNA fragment (71). For the transgene probe, a 3-kb HindIII–EcoRI LMP1 fragment from the PyLMP1 transgene was used.

**BrdUrd Labeling and Immunodetection.** The protocol used is essentially as described (72). One h before being killed, BrdUrd (50 μg/g body weight) in 0.9% saline was injected i.p. into mice. Dorsal skin, tongue, and tail tissues were collected (as well as tissue for transgene status determination), fixed in 0.9% saline was injected i.p. into mice. Dorsal skin, tongue, and tail tissues were collected (as well as tissue for transgene status determination), fixed in 0.9% saline, and subjected to immunohistochemical staining using a murine anti-BrdUrd antibody (Sigma Chemical Co.), then an antimmune IgG biotinylated second antibody, and finally the Vectastain elite ABC complex (which contains avidin and biotinylated horseradish peroxidase, and which allows the avidin: biotinylated horseradish peroxidase to bind to the biotinylated secondary antibody). Positively stained nuclei (brown) were then detected using DAB (3,3′-diaminobenzidine tetrahydrochloride; Sigma Chemical Co.), and negative nuclei were counterstained with hematoxylin (blue). The number of BrdUrd-labeled nuclei in the interfollicular areas, per field of vision (×312.5) were counted for 10 fields of vision/section, and a mean value for the tissue type was determined. Tissues from at least three different mice in each group were counted in this way, where the mean value of these for the tissue type is referred to as the labeling index. All counts were conducted blind, and transgene status determined subsequently.

**Immunofluorescent Staining.** Immunofluorescent examination of PyLMP1 transgenic epidermis was conducted essentially as described previously (9). Seven-μm sections were collected (as well as tissue for transgene status determination), fixed in 0°C on gelatin-coated slides. Sections were blocked for 30 min in 1× goat wash (1.3 m NaCl; 70 mM NaH2PO4,0.2H2O; 30 mM NaH2PO4·H2O; 0.5% sodium azide; v/v; 10% goat serum, v/v; 2% Triton X-100, v/v; and 0.5% Tween 20, v/v). The primary antibody used was rabbit polyclonal anti-loricrin with secondary FITC-conjugated goat antirabbit IgG (Sigma Chemical Co.). Sections were mounted with Vectashield (Vector Laboratories) and viewed using a Leitz orthoplan microscope at ×312.5.

**Chemical Carcinogen Administration.** The protocol used is essentially as described (73). Mice of PyLMP1 line 53 were backcrossed (from C57Bl/6 strain) into the chemical carcinogen-sensitive FVB strain for this study and mice from a minimum of three backcrosses were used (average, 87.5% FVB at backcross 3). Chemical treatment was initiated with mice 8 weeks of age. Hair from the mouse dorsal region was removed by shaving 2 h before the initial chemical administration. Thereafter, the dorsal skin was carefully and regularly shaved. Twenty-five μg DMBA was applied topically to the dorsal skin in 200 μl of aceton. One week later, mice were treated with the tumor promoter TPA, twice weekly, with 200 μl of 5 × 10−8 M TPA for 20 weeks (regime 1) or, alternatively, not treated further (regime 2). TPA treatment for 20 weeks without DMBA initiation was also conducted (regime 2). DMBA treatment followed by only 4 weeks of TPA treatment constituted regime 3. The number of benign and malignant tumors on each mouse was recorded weekly during treatment and beyond. The benign tumors were categorized according to size, and the number of lesions in size categories 2–4 were counted (category 1, <0.2 mm (not counted); category 2, 0.2–0.5 mm; category 3, 0.5–1.0 cm; and category 4, >1.0 cm diameter). Analysis of the data from each papilloma size category, from the total papilloma count, and from the total malignant carcinoma count was facilitated by use of the Minibab statistical analysis package. Normal distribution of the data were confirmed using Rankits plots, and differences between the two groups were analyzed for significance using the two-sample t test. All mice were of the same background strain, with negative siblings of the transgenic mice used as controls, and housed and fed under the same conditions in a conventional facility. Lesion counts were conducted blind of transgenic status.

Mice were killed if the tumor load became excessive; if any tumor inhibited movement, caused discomfort, or was located at a natural orifice; if any individual tumor exceeded 20 mm in diameter or became ulcerated; or at the termination of the experiment. Decision to remove an animal from study because of these criteria was taken blind of transgenic status. Sections of tumor samples were collected for culture and snap frozen or fixed in 10% neutral buffered formalin (20 mM NaH2PO4; 46 mM NaH2PO4; and 4% formaldehyde, v/v) for paraffin embedding and histopathological analysis.

**Development of Carcinoma Cell Lines.** Primary cells were established from malignant carcinomas by removing the tumor into explant medium (DMEM containing 87 μg/ml pencillin, 117 μg/ml streptomycin, and 2.9 μg/ml Fungizone). The tumor was then transferred to warmed culture medium (DMEM containing 20% FCS, v/v; 2% l-glutamine, v/v; 12 μg/ml pencillin; 97 μg/ml streptomycin; and 1.2 μg/ml Fungizone) in which it was diced with a sterile scalpel. The small pieces were allowed to adhere to a tissue culture system (56, 57). TPA treatment then promotes the clonal survival and biotinylated horseradish peroxidase, and which allows the avidin: biotinylated horseradish peroxidase to bind to the biotinylated secondary antibody. Positively stained nuclei (brown) were then detected using DAB (3,3′-diaminobenzidine tetrahydrochloride; Sigma Chemical Co.), and negative nuclei were counterstained with hematoxylin (blue). The number of BrdUrd-labeled nuclei in the interfollicular areas, per field of vision (×312.5) were counted for 10 fields of vision/section, and a mean value for the tissue type was obtained. Tissues from at least three different mice in each group were counted in this way, where the mean value of these for the tissue type is referred to as the labeling index. All counts were conducted blind, and transgene status determined subsequently.
culture flask until confluent. Thereafter, the immortal cultures were maintained in culture medium with 10% FCS.

**Western Blotting.** Protein was extracted from cells using RIPA buffer [150 mM NaCl, 0.5% NP40 detergent, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM DTA, and 20 mM Tris (pH 7.5)] with protease inhibitors (protease inhibitor cocktail, P2714; Sigma Chemical Co.). Forty μg of protein from each extract was electrophoretically separated by SDS-PAGE (8%) minigels, at 180 V for 40 min. Proteins from the gel were electrobotted onto Millipore Immobilon-P membranes at 65 V for 1 h. The membranes were blocked overnight at 4°C in 15 ml of blocking buffer (0.4% casein, 0.1% Tween 20, and 0.02% NaN3 in PBS), then incubated for 1 h at room temperature with a 1:20 dilution of S12 anti-LMP1 monoclonal antibody (in blocking buffer). The membranes were washed three times for 5 min each in PBS-Tween, and incubated for 1 h at room temperature in secondary antibody (1:10000 dilution of alkaline phosphatase conjugated antimouse IgG (Sigma Chemical Co.) in blocking buffer). Antibody binding was visualized using the CDP-Star detection system (detailed in Ref. 74).

**RESULTS**

**Transgenic Mouse Lines.** In the experiments described herein, transgenic mice expressing LMP1 in the epidermis and tongue epithelium were used. Transgene expression was directed using the py early promoter and enhancer in the construct PyLMP1 and demonstrated by Northern and Western blotting (26). Tissues were taken from mice of two lines (PyLMP1.5 and PyLMP1.53, also referred to as lines 5 and 53 where line 5 was shown to express LMP1 at a higher level than line 53) and to display a more severe phenotype of epidermal hyperplasia with some hyperkeratosis ( likened to the human condition of psoriasis), described previously (26). Line 5 mice suffered from an additional phenotype (lethal in male animals) resulting from a transgenic insertional mutation at a cellular locus (75, 76). As such, all additional in vivo studies have been conducted with line 53. Mice are maintained in two strains, C57Bl/6 and the chemical carcinogen-susceptible strain FVB.

**Expression Levels of Markers of Differentiation and Proliferation.** Up-regulation of K6 protein and mRNA was previously noted in the skin of transgenic LMP1 pups and adults of both lines (26). Surprisingly, suprabasal markers of differentiation, K1 and K10, showed higher levels of expression at day 8 in the transgenic skin samples compared with controls; however this returned to levels equivalent to the controls by day 10. This observation is consistent with the phenotype in that the hyperkeratosis is most marked in the skin of the trunk in mice that are 1 week of age. In addition, loricin (the final differentiation protein to be expressed), revealed an expanded expression pattern in conjunction with increased acanthosis (thickening of the squamous epithelium) in the transgenic skin at 1 week of age (Fig. 2).

These results confirm and extend our earlier observations that markers of proliferation are up-regulated in LMP1-expressing epidermis, detectable by both RNA and protein analyses. Interestingly, markers of differentiation are either equivalent to controls or also up-regulated in the LMP1 transgenic dorsal skin. Thus, no evidence for inhibition of differentiation by LMP1 was observed by these criteria.

**In Vivo Proliferation.** To address directly whether cellular proliferation was affected in the transgenic epidermis in vivo, cellular DNA synthesis was measured by BrdUrd nuclear incorporation in epidermal cells after i.p. injection. Eight transgenic mouse pups of line 53 and eight control siblings were injected at 1 week of age (when the phenotype is most obvious in the dorsal skin). Three transgenic adults, when there is no apparent phenotype in the dorsal skin, and three control siblings were also tested.

Dorsal skin, tongue, and tail tissues were taken from pups, dorsal skin was taken from adults, and several sections of each tissue were analyzed for BrdUrd incorporation (Fig. 2). Ten fields of view were counted for interfollicular positively stained keratinocytes for each tissue section, and the mean value of these for the section was

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6 Laverty and Wilson, unpublished observations.
determined. The section values for each tissue were collated for the groups of mice and the mean for each transgenic or control group calculated as the labeling index. The labeling indices for the sample groups are presented graphically in Fig. 3. In dorsal skin from pups 1 week of age, epidermal cell proliferation is 2-fold higher in the transgenic compared with the control samples. The labeling indices of 22.94 and 11.22, respectively, show a highly significant difference (two-sample *t* test; 95% confidence limit; *P* < 0.0035). As expected, proliferation in the adult epidermis is much lower than in pups; nevertheless the 2-fold difference between transgenic and control samples is maintained, with labeling indices of 4.24 and 2.1, respectively, with a very high significant difference (*P* < 0.0004). Similarly, transgenic animals showed increased proliferation in tail epidermis and tongue epithelium compared with controls (Fig. 3). For 1-week tail epidermis, transgenic and control labeling indices were 56.0 and 17.13, respectively, showing a significant 3-fold elevation of proliferation in the transgenic samples (*P* < 0.026). For 1-week tongue epithelium, where the hyperplastic phenotype is less evident, the labeling indices for transgenic and control samples were 26.75 and 17.16, showing a 1.6-fold elevation in the LMP1 transgenics (*P* < 0.02).

In addition to the 2–3-fold elevation in proliferating cells in the LMP1 transgenic interfollicular epidermis compared with controls, it was also noted that the proliferating compartment was less spatially restricted. In control mice, all stained cells indicative of division were in the basal cell layer of the epidermis, as expected. However, in the LMP1 transgenic epidermis, occasional cells in the suprabasal layers stained positive for BrdUrd incorporation as well (Fig. 2).

**Chemical Carcinogen Treatment.** To ascertain whether LMP1 can act to augment chemical carcinogens or whether it can substitute for either an initiator or a promoter, three treatment regimes were initially conducted using PyLMP1.53 transgenic mice and their negative sibling controls. Treatment 1 involved the classical single initiating treatment with DMBA and then 20 weeks of promoting TPA-treatment (twice/week). Thirty-two transgenic mice and 26 control siblings were treated under this regime and monitored for up to 55 weeks from first treatment. An artificial limit was placed on carcinoma load in view of animal welfare (see “Materials and Methods”), and as such, mice were removed from the study as required, from week 17 onwards. Regime 2 involved 20 weeks of TPA treatment (twice/week) in the absence of DMBA initiation. Transgenic mice and control siblings placed on this regime were monitored for 46 weeks. Regime 3 involved a single DMBA treatment. Transgenic and control mice placed on this regime were monitored for 35 weeks. Lesion formation was counted weekly, and the papillomas were categorized by size (see “Materials and Methods”). Conversion of a papillomatous lesion to a carcinoma was recorded, and confirmation of lesion status by histopathology was conducted after the final sample collection.

All mice in regime 1 developed papillomas (Table 2). The mean numbers of papillomas/mouse/week in size categories 2–4 and the conversion to carcinoma are shown graphically in Fig. 4. Lesions appeared earlier in the transgenic group, at week 5, compared with the control mice, in which lesions first appeared at week 7. Transgenic
mice showed a marked increase in the number of small papillomas (size 2, 0.2–0.5 mm) formed compared with controls (Fig. 4). The difference became significant at week 13 of the study (two-sample test; 95% confidence; 56 degrees of freedom; \( P = 0.017 \) at week 13), showing increasing significance for the remainder of the study (e.g., \( P = 0.0007 \) at week 25 and \( P = 0.0001 \) at week 26).

A proportion of these papillomas grew to the larger sizes with time in both groups. With papilloma size category 3 (0.5–1.0 cm), little difference in the number of lesions developing was observed between transgenic and control groups (Fig. 4), supported by the statistical analysis showing no significant difference in this category. However, surprisingly, control mice developed significantly more papillomas in the size 4 category (>1.0 cm) than the transgenic mice. The difference became significant at week 16 of the study (\( P = 0.013 \)) and continued for the remainder of the study. Combining the opposing sets of data, more lesions in total formed with time in the transgenic group compared with the controls, but this did not show consistent significant difference until after cessation of the carcinogen treatment at week 21.

The mean number of carcinomas formed per mouse (2.69 for transgenic and 2.81 for control siblings) does not show significant difference until after cessation of the carcinogen treatment at week 21. The mean number of carcinomas formed per mouse (2.69 for transgenic and 2.81 for control siblings) does not show significant difference (\( P = 0.86 \)); however, carcinoma load was given an artificial limit in view of animal welfare. Animals were removed from the study because of lesion load from week 17 in the transgenic group and from week 20 in the controls. Consequently, conversion rates of papilloma to carcinoma have been calculated at the week 31 values (allowing time for conversion), when >65% of animals remained under study (in both groups). At week 31, the mean percentage of carcinomas/total lesions was 6% for the transgenic mice and 12% for the controls, without showing significant difference (\( P = 0.14 \)). This indicates that LMP1 did not enhance conversion of papillomatous lesions to carcinomas, although more small papillomas were induced.

After histopathological analysis, lesions that were scored as carcinomas were revealed to be predominantly of the squamous cell type. Two of 37 carcinomas from the LMP1-positive group were of the more malignant spindle cell type, with 1 of 31 in the control group, indicating that LMP1 does not lead to an increased progression to the more aggressive spindle cell carcinoma.

Cell lines were established in culture from several of the carcinomas that developed in the transgenic and control mice. LMP1 expression in the transgenic carcinoma cell lines was confirmed (Fig. 5).

These data show that the PyLMP1 mouse are highly sensitive to chemical carcinogen treatment and that LMP1 appears to promote lesion formation. However, paradoxically, LMP1 inhibits expansion of the benign lesion. Moreover, LMP1 does not affect the rate of conversion of papillomas to carcinomas or promote the progression of squamous carcinoma to spindle cell type. Therefore, LMP1 seems to augment the action of chemical carcinogens only in the early stages of lesion formation.

Because the PyLMP1 mouse phenotype shows some similarities with the phenotype of mice transgenic for oncogenic \( H-ras \), regime 2 (20 weeks TPA treatment only) was conducted to address the ability of LMP1 to functionally substitute for oncogenic \( ras \). After 46 weeks of monitoring (including the 20-week treatment period), no (0 of 11) LMP1-positive mice developed lesions, whereas one control animal (1 of 17) developed a single papilloma, at week 29 of the study, which did not progress to carcinoma (Table 2). Therefore, LMP1 is not able to substitute for the action of oncogenic \( ras \) in this system.

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**Table 2** Papilloma incidence

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<tr>
<th>Regime 1</th>
<th>Transgenic</th>
<th>Control</th>
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<tbody>
<tr>
<td>DMBA + 20 wk PA</td>
<td>32/52</td>
<td>26/26</td>
</tr>
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</table>

**Table 2** Papilloma incidence (continued)

<table>
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<tr>
<th>Regime 2</th>
<th>Transgenic</th>
<th>Control</th>
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</thead>
<tbody>
<tr>
<td>20 wk TPA</td>
<td>0/11</td>
<td>1/17</td>
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<table>
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<tr>
<th>Regime 3</th>
<th>Transgenic</th>
<th>Control</th>
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<tbody>
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<td>DMBA</td>
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<table>
<thead>
<tr>
<th>Regime 4</th>
<th>Transgenic</th>
<th>Control</th>
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</thead>
<tbody>
<tr>
<td>DMBA + 4 wk TPA</td>
<td>7/7</td>
<td>0/16</td>
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</table>

* Two mice in this group developed size 1 (<0.2 cm) papillomas toward the end of the study period, one of which regressed after a few weeks; the cohort remained papilloma-free for the first 52 weeks of the study (depicted in Fig. 6).
LMP1 ACTS AS A TUMOR PROMOTER

To investigate whether LMP1 could substitute for TPA-induced tumor promotion, regime 3 (a single DMBA treatment) was conducted. After 35 weeks of monitoring, four (4 of 14) LMP1-positive mice developed papillomas (1, 1, 2, and 5 papillomas, in each case) whereas no controls (0 of 12) developed lesions (Table 2). This result suggests that LMP1 may be able to act as a weak tumor promoter.

To investigate this further, a new regime was conducted. Weak (or second-stage) promoter activity can be revealed after minimal TPA treatment (77, 78). Regime 4 involved a single DMBA treatment and then 4 weeks of TPA treatment (twice/week). Seven transgenic and six control mice were placed on this regime and monitored for up to 70 weeks (although mice were removed from the study, as described above, from week 31). In this treatment regime, control mice remained papilloma free for 52 weeks; subsequently two mice in this group developed size 1 (<0.2 cm) papillomatous lesions (at the end of the study period), one of which regressed after a few weeks (Fig. 6; Table 2). In contrast, 7 of 7 LMP1-expressing mice formed papillomas after the minimal TPA treatment, some of which converted to carcinoma. Mice in the transgenic group developed papillomas from week 9 of the study period, all mice having lesions by week 12. Expression of LMP1 in the transgenic group because of lesion load (as described in Materials and Methods), at which point the lesion number is recorded as constant from then on for the given mouse.

Paradoxically, expression of LMP1 in the epidermis appears to inhibit the expansion of the benign papillomas (to the larger size lesions), although more small lesions have formed. Similarly, the numbers of carcinomas developing in each group were equivalent, despite the greater abundance of small papillomas in the transgenic group. These seemingly opposite activities may be explained in the ability of LMP1 to activate pathways involved in proliferation (such as the JNK pathway) and in epithelial differentiation (such as the NFkappaB pathway). Whereas LMP1 may provide the proliferative envi-
LMP1 ACTS AS A TUMOR PROMOTER

Ronment which promotes lesion formation, it could simultaneously present a hurdle to lesion expansion by maintaining cell differentiation or senescence. A prediction of this theory would be that the factors (or the loss thereof) which lead to the inhibition of differentiation or senescence could overcome the hurdle and possibly cooperate with LMP1 in lesion expansion or progression.

There are striking similarities between mice expressing TGFα in the epidermis (62, 66) and the PyLMP1 mice. In addition to similarities in pup phenotype and the diminution of phenotype in adults, the mice in both series show an increase in BrdUrd mitotic index in the epidermis (64, 85). However, TPA treatment (in the absence of initiating DMBA) of transgenic mice overexpressing TGFα in the basal layer induced papillomas in the transgenic mice (86, 87), which is in contrast with our findings with the LMP1 mice. However, the inability of LMP1 to induce benign papillomas upon TPA treatment alone may be a function of expression level or the precise site of expression of the transgene. We are currently investigating whether the tumor-promoting activity of LMP1 may be mediated through the TGFα/EGFR pathway.

The importance of the inflammatory response in tumor promotion has been revealed using TNFα-null mice (88). TPA treatment of TNFα-null mice does not result in tumor promotion. Again, it is tempting to suggest that LMP1 may be activating inflammatory signaling pathways through interaction with TRAFs and TRADD to induce lesion formation.

In the viral context in vivo, as a latent and lytic protein, LMP1 may promote cellular proliferation to expand the viral pool and support differentiation to facilitate the lytic cycle. In conjunction with other viral proteins and/or cellular oncogenic mutations that inhibit or redirect differentiation, a potential consequence is tumorigenesis.

NPC has a complex etiology with a possible genetic predisposition, potential contribution by dietary carcinogens, and a viral component. In the transgenic system we have analyzed here, the genetic background of the mice is critical in that different strains of mice show markedly different responses to chemical carcinogens. We have concentrated our efforts on mice bred to a background of genetic predisposition to chemical carcinogens. We have studied one viral component, LMP1, and found that it induces epithelial proliferation in vivo and renders the transgenic mice highly sensitive to chemical carcinogens. Minimizing the carcinogen treatment such that no lesions are formed in control mice nevertheless leads to carcinogenesis in the PyLMP1 mice. LMP1 acts early in the carcinogenic process, increasing the frequency of lesion formation, but can also act to inhibit expansion of the benign lesions, and it does not enhance progression of the lesions. This leads to the hypothesis that LMP1 may be an early factor in the genesis of EBV-associated NPC, which would be consistent with the observation that LMP1 is expressed in preinvasive NPC lesions (89). Moreover, the expression of LMP1 in EBV-infected cells may increase the risk of lesion formation associated with dietary carcinogens (7). Whether LMP1 continues to be a tumorigenic factor in later stages remains to be elucidated.

ACKNOWLEDGMENTS

We acknowledge Jürgen Schweizer for supplying the murine K17 plasmid and Stuart Yuspa for the anti-loricrin antibody. We thank Willie Thomson for assistance in sectioning skin samples, Uta Boeger-Brown for discussion during the progress of the work, and David Stevenson for critical reading of the manuscript.

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Epstein-Barr Virus Encoded Latent Membrane Protein-1 Induces Epithelial Cell Proliferation and Sensitizes Transgenic Mice to Chemical Carcinogenesis

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