Expression and Tumorigenicity of the Epstein-Barr Virus BARFI Gene in Human Louckes B-Lymphocyte Cell Line

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ABSTRACT

We previously showed that the Epstein-Barr virus, which encodes the BARFI gene, could transform rodent fibroblasts. In this work, the expression of the BARFI gene was studied in the human Louckes B-lymphocyte cell line. Introduction of the BARFI open reading frame under the control of the Mo-MuLV LTR promoter into nontumorigenic Louckes lymphoid cells led to the activation of the c-myc protooncogene and increased expression of the B-cell surface proteins, the transferrin receptor, CD21, and CD23. BARFI-expressing cells induced a diffuse lymphoma-like tumor in newborn rats treated with anti-thymocyte serum that was, however, transient and regressed after 3–4 weeks as the immune system recovered. The tumor induction was similar to that observed with lymphoid cell lines in vitro generated by infection with the B95–8 virus strain, in which lytic antigens are expressed at low levels. After long-term culture, Louckes cell clones lost expression of the BARFI gene and were unable to induce tumors.

INTRODUCTION

EBV, an ubiquitous human herpes virus which causes infectious mononucleosis, is closely associated with two human cancers: BL and nasopharyngeal carcinoma. This virus is also associated with the development of lymphoma induced in patients with acquired immune deficiency syndrome. EBV infects and immortalizes human B-lymphocytes with the viral genome being maintained as an autonomously replicating episome in a latent state. However, in some cases, particularly in the presence of chemical-inducing agents, the in vitro immortalized human B-lymphocytes can enter a lytic viral cycle.

At least three viral genes and a specific viral fragment are currently thought to be relevant to the oncogenic function of EBV. LMP1 (1, 2) can transform rodent fibroblasts or human keratinocytes immortalized by both oncogenic SV40 and adenovirus (3). The expression of this gene induces hyperplastic dermatosis in transgenic mice (4) and may be involved in the differentiation of an immortalized nontumorigenic human squamous cell carcinoma line (5). The latent nuclear proteins, EBNA2, EBNA3A, and EBNA3C, also seem to play an important role in the immortalization of B-lymphocytes (6, 7, 8). Both of these genes (EBNA2 and LMP1) up-regulate the B-cell functions, ICAM-1 and LFA1, or the B-cell activation marker, CD23 (9, 10). A 40-kilobase pair viral fragment situated in the right side of the viral genome is capable of immortalizing primary human and mouse epithelial cells and of expressing an Mr 31,000 protein recognized by EBV-positive sera (11, 12). We recently showed that the BARFI ORF encoded in this 40-kilobase pair fragment has transforming activity in rodent fibroblast cell lines (13). Its transcript is expressed in early stages of the viral cycle, and its gene product, which has an Mr, 33,000 (p33), can be recognized by EA-positive sera from patients with nasopharyngeal carcinoma (14).

Since human B-lymphocytes are natural host cells for EBV, we wished to examine the consequences of expression of the BARFI gene in such cells. For this purpose, we chose two EBV-negative B-cell lines, Louckes (15, 16) and BJAB (17), which have previously proved useful as EBV-negative controls. The tumorigenicity of both cell lines was examined. One from the clones of BJAB was found to be tumorigenic in newborn rats and thus could not be used in our study, while the Louckes cell line was nontumorigenic. The BARFI gene, under the control of the Moloney murine leukemia virus LTR promoter, was introduced into Louckes cells, and tumor formation was observed after cell injection into newborn rats. Here we examine the properties of these tumors compared with tumor found by three LCL lines immortalized in vitro by B95–8 virus. We also examined the expression of c-myc mRNA in BARFI-positive and -negative cell clones and show that the presence of BARFI enhances expression of this oncogene. The presence of the BARFI sequence also activated the expression of cell surface proteins, CD21, CD23, and the transferrin receptor.

MATERIALS AND METHODS

Cell Culture. Louckes is a EBV-negative American Burkitt’s lymphoma B-cell line (15, 16) which is nontumorigenic in newborn rats. The two BJAB cell lines used here are derived from EBV-negative B-lymphoma cells either frozen in 1980 (BJAB-1) or subcloned (BJAB-2: from M. Vuillaume, International Agency for Research on Cancer, Lyon, France). The polyclonal lymphoid cell lines, LCL-1, LCL-2, and LCL-3, were established in vitro by B95–8 virus infection of cord blood B-cells (from Dr. Pelloquin, Pasteur-Mérieux) and maintained in culture for 4 years. Expression of EBV antigens is about 2 and 8% of early antigen and 1 and 4 of VCA for LCL-2 and LCL-3, respectively, while LCL-1 expressed only latent and no lytic antigens. All cell lines were cultured in RPMI 1640 medium supplemented with 10% FCS and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin), in a 5% CO2 moist atmosphere with feeding every 3–4 days.

DNA Transfection and Isolation of Cell Clones Expressing BARFI or Neomycin Genes. The 1.1-kilobase Smal fragment which contains the whole BARFI ORF, and the corresponding cDNA, was cloned into the pZip-Neo-SV(X) 1 vector, as previously described (13). The construction with the genomic sequence is called pZAR1 and that with the cDNA sequence is called pZ55. Transfection was performed using the electric field-mediated DNA transfer method (electroporation) (18). Briefly, 2 × 107 log-phase growing cells were washed and suspended in 0.8 ml cold PBS without Ca2+ and Mg2+.

Cells and DNA (20 μg) were mixed and allowed to stand for 10 min on ice, then placed in a gene pulse cuvette, and subjected to a single electric pulse delivered by a Bio-Rad gene pulser (3000 V/cm, with a capacitance of 25 μF). The cell-DNA mixture was then left for 7 min on ice before being added to 1 ml warm growth medium. Three days after electroporation, the cells were counted and plated into 96-well plates at 1000 or 2000 cells in 0.25 ml growth medium/well in the presence of 3 μg/ml neomycin (GIBCO) for selection. The cells were maintained under these conditions for 3–4 weeks with medium changes once a week. Neomycin-resistant cell populations were harvested.

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[3] The abbreviations used are: EBV, Epstein-Barr virus; BL, Burkitt’s lymphoma; ORF, open reading frame; VCA, viral capsid antigen; FCS, fetal calf serum; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; LMP, latent membrane protein; ICAM, intercellular adhesion molecule; LFA, lymphocyte function-associated molecule; EA, early antigen; LCL, lymphoblastoid cell line; cDNA, complementary DNA; Neo', neomycin resistant.

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from independent wells, replated into 6-well plates with 4 ml medium/well, and then expanded into distinct lines.

**Indirect Immunofluorescence.** Log-phase growing cells were washed twice in PBS and dried on slides at room temperature before being fixed for 10 min in acetone. WC serum (EA* VCA*) (from Dr. E. Kieff, Harvard Medical School, Boston, MA) or North African serum (EA* VCA*) was diluted in PBS (1:200) and added to cells for 45 min at 37°C. The slides were washed three times with PBS and incubated for 30 min at 37°C with fluorescein-conjugated anti-human immunoglobulin diluted in PBS (1:40). After two washes with PBS and one with water, the slides were then viewed under fluorescence microscopy (19).

**Immunofluorescence and Flow Cytometry.** Live cells were stained with saturating amounts of monoclonal antibodies and fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin. One million log-phase growing cells were pelleted gently, washed once with PBS, and then analyzed with a flow cytometer (FACS 4; Becton Dickinson and Co.). The results are expressed as mean fluorescence/volume ratio of 10,000 cells. Antibodies used for this experiment were CD21, LFAx, CD40 (Mab 89), LFA3, CD10, ICAM-1, and transferrin receptor from Immunotech, CD23 (Mab 5) from Sherling Plough (Dardilly, France), CD23 (MMH6) from Dr. Rowe, and LFA1β (MIM23) from Dako.

**Tumorigenicity Analysis.** The cell clones containing the pZip-Neo-SV(X) vector or bearing either the genomic sequence PZAR1 or cDNA sequence p255 were grown in log phase for 24 h after feeding. Cells were then counted, and 10 x 10⁶ cells were washed twice with medium without FCS, then resuspended in 100 µl medium without FCS, and injected s.c. into newborn rats. Six animals were injected per each cell clone, and tumor development was monitored twice/week for 8 weeks.

**RNA Analysis by Northern Blot Hybridization.** RNA was extracted using an RNA isolation kit (Stratagene). For Northern blot analysis, electrophoresis of 2.5 µg polyadenylated RNA was performed in 1% agarose-2.2 M formaldehyde gel, followed by transfer to reinforced nitrocellulose filters (Schleicher & Schuell). Probes (BARFI, c-myc, transferrin, and actin sequences) were labeled using a random primed DNA labeling kit (Boehringer Mannheim), and hybridization (1-3 x 10⁶ cpm/ml hybridization solution) was carried out as previously described (14). Transferrin, c-myc, and actin sequences are from Sherling Plough, from Dr. O. Brison (Institut Gustave Roussy, Villejuif, France), and from Dr. E. Kieff (Harvard Medical School, Boston, MA), respectively.

**BARFI Sequence Analysis by Southern Blot Hybridization.** Cellular DNA was extracted in buffer (10 mM Tris-HCl, pH 7.4-50 mM EDTA-150 mM NaCl-1% Sarkosyl-20 mg/ml proteinase K). The mixture was incubated first for 20 min at 55°C, followed by 1 h at 37°C, and then extracted twice with 1 volume phenol and chloroform before precipitation. For Southern blot analysis, 10 µg cellular DNA were digested with the restriction enzyme EcoRI and separated by electrophoresis in 1% agarose gel, followed by transfer to reinforced nitrocellulose filters (Schleicher & Schuell). The probe (1.1-kilobase Smal fragment containing the entire BARFI sequence) was labeled with random primers (as above), and hybridization (1 x 10⁶ cpm/ml hybridization solution) was carried out for 15 h at 65°C.

**RESULTS**

**Expression and Effect of BARFI Gene in Louckes Cell Line.** Both cDNA (0.7 kilobases) and genomic (1.1 kilobases) sequences corresponding to BARFI ORF and pZip vector alone were introduced by electroporation (18) into Louckes B-cell line, using the pZip-Neo-SV(X) eukaryotic expression vector (20). Three days after transfection, 1-2 x 10⁵ cells/well in 96-well plates were grown under selection in the presence of 3 mg/ml neomycin. Neo' cells were harvested from independent wells and replated as distinct cell lines.

The Neo' population of cells in one well was considered clonal. Two (genomic sequence) and three (cDNA sequence) Neo' clones were isolated for Louckes cell lines. Two clones with the pZip vector alone were also isolated from each cell line.

The expression of the BARFI-encoded p33 protein in Neo' clones of Louckes cells was examined by immunofluorescence using high-titer EA and VCA-positive human sera (Fig. 1). The sera revealed positive immunofluorescence on whole cells as well as on some membranes and/or cytoplasm (Fig. 1B). This positive immunofluorescence was not observed with EA- or VCA-negative human sera. Some cells (about 5%) in the Neo' population (Fig. 1B) had no significant immunofluorescence, probably because of genetic variation among cloned cells. The cell size of p33-expressing clones measured using a FACS was not significantly different from that of parental cells (data not shown). BARF1-positive clones grew faster than parental clones (range, 1.2- to 1.5-fold). The level of mRNA expression in BARF1-positive Louckes Neo' clones was measured by Northern blots using the entire BARFI gene (1.1-kilobase Smal fragment) as a probe: equal quantities (2.5 µg) of polyadenylated mRNA were loaded into each well of an agarose gel, separated by electrophoresis, transferred onto nitrocellulose filters, and hybridized. Northern blot analysis revealed a major RNA of 1.8 kilobase in BARF1-transfected cell clones, whereas neither the nontransfected Louckes nor Louckes cells bearing pZip vector alone demonstrated any detectable BARFI RNA transcripts. Based on molecular weight predictions (0.8 kilobase; Ref. 14), a higher molecular weight of BARFI-positive RNA is most likely a spliced viral RNA transcript (20). In Neo' Louckes cell clones, the expression level of BARFI mRNA on some clones is slightly higher than others (Fig. 2A, lanes 5-7) relative to that of actin mRNA (Fig. 2A). BARF1-positive cell clones were examined for the transcription of c-myc protooncogene which is activated in Burkitt's lymphoma biopsies and in B-lymphocytes infected by EBV. Therefore, we hybridized the same blot (used for BARFI expression) with the third exon of the c-myc sequence as a probe. As shown in Fig. 2A (top), all clones bearing the BARFI sequence expressed high levels of c-myc mRNA (lanes 5-8) com-
BARF1 (middle), or actin (bottom) sequences as a probe. The second blot (ß) was hybridized for the Louckes cells (lane 1) or parental Louckes cells (lane 2). Clone LouZARI-l (lane 3), clone LouZ55-4 (lane 4), clone LouZ55-5 (lane 5), and clone LouZ55-7 (lane 6). Lane 1, Louckes cells; lane 2, pZIP-transfected Louckes cells; lane 3, pZIP-transfected Louckes cells; lane 4, clone LouZARI-l; lane 5, clone LouZ55-4; lane 6, clone LouZ55-5; lane 7, clone LouZ55-7.

pared with those of the clone containing only the pZip control vector (lane 2) or parental Louckes cells (lane 1).

BARF1 Induces CD21, CD23, and CD71 (Transferrin Receptor) Expression. Since the transforming gene, LMP, induced the expression of cell surface proteins, CD23, LFA1, LFA3, transferrin receptor, and ICAM-1 (9, 10) and increased CD23 and transferrin receptor in B-cell growth regulation (21, 22), we examined the expression of CD21, CD23, CD40, LFAe, LFA3, ICAM-1, and transferrin in the BARF1-positive cell clones. As shown in Table 1, there was 3- to 4-fold higher expression as mean fluorescence intensity of CD71 (transferrin receptor molecule) and CD21 in almost all BARF1 transfectants than in parental or pZip vector-transfected Louckes cells. However, CD23 expression varied among BARF1 clones from 1.5- to 7-fold. The profile of fluorescence intensity by cell sorter analysis is shown in Fig. 3A. A significant displaced profile of BARF1 transfectants was observed for three cell surface molecules [CD21 (Fig. 3A), CD23 (Fig. 3B), and CD71 (Fig. 3C)]. No significant expression of the other cellular markers, CD40, LFAe, LFAβ, LFA3, or ICAM-1 was observed in BARF1 transfectants except for LFA3 for which two of four BARF1 transfectants had 2- to 3.7-fold more expression. We then analyzed the expression of transferrin mRNA in the BARF1 transfectants and the controls. Polyadenylated RNA was prepared from each cell clone and hybridized with probes for transferrin or actin (Fig. 2B). We obtained a faint positive response with more intense 4.7-kilobase transferrin mRNA hybridization signals for the BARF1 transfectants (Fig. 2B, lanes 3-7) compared with those of the control pZip transfectants (Lou-pZip-1 and Lou-pZip-3, lanes 1 and 2, respectively).

Tumorigenicity of BARF1-expressing Human B-Cell Lines in Newborn Rats. One of the key aspects of this work involved examining the tumorigenicity of BARF1-expressing human B-cells, which are the normal host cells for EBV. First, we tested the potential tumorigenicity of both parental cell lines (Louckes and BJAB) in newborn rats, since these cell lines were established from Burkitt's lymphomas. We injected 1 X 10^7 cells/animal into newborn rats treated with anti-thymocyte serum.

In the case of the parental Louckes cells (Table 2) and BJAB cell line (BJAB-2: early passage frozen in 1980), no tumor formation was observed over 8 weeks. However, the BJAB-1 cell line, a subclone of BJAB, was tumorigenic, similar to an EBV-converted BJAB line (BJAB-B95-8). The BJAB-1 cell line proved highly resistant to transfection experiments, whereas non-tumorigenic BJAB-2 was not. After these initial experiments, we focused our attention on BARF1 gene expression in Louckes cell line, comparing tumor induction by the BARF1-positive (cDNA sequence or genomic sequence) Louckes cell clones with parental or control vector-bearing Louckes cells. Table 2 shows that no tumor formation was observed with parental Louckes (24 newborn rat assays) or with three control lines bearing the pZip vector (24 newborn rat assays). However, four Louckes cell clones containing BARF1 yielded tumors, which appeared on the 9–21th day after injection, reached their maximum size over the next 7–14 days, and regressed by 27–30th days. One clone (LouZ55–5) showed two peaks of tumor development (9th and 21st days).

Tumor biopsies were stained with m-cresol purple and analyzed by

Table 1. Summary of cell surface molecule expression on Louckes transfectants

<table>
<thead>
<tr>
<th>Clones</th>
<th>CD21</th>
<th>CD23</th>
<th>CD40</th>
<th>CD71</th>
<th>LFAe</th>
<th>LFAβ</th>
<th>LFA3</th>
<th>ICAM-1</th>
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<tbody>
<tr>
<td>Louckes</td>
<td>22</td>
<td>15</td>
<td>66</td>
<td>94</td>
<td>6</td>
<td>8</td>
<td>10</td>
<td>91</td>
</tr>
<tr>
<td>L-pZip 1</td>
<td>28</td>
<td>18</td>
<td>60</td>
<td>88</td>
<td>7</td>
<td>7</td>
<td>12</td>
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<td>L-pZip 3</td>
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<td>18</td>
<td>60</td>
<td>90</td>
<td>8</td>
<td>8</td>
<td>13</td>
<td>87</td>
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<tr>
<td>LouZARI-1-3</td>
<td>76</td>
<td>23</td>
<td>68</td>
<td>251</td>
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<td>10</td>
<td>14</td>
<td>94</td>
</tr>
<tr>
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<td>60</td>
<td>64</td>
<td>242</td>
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<td>11</td>
<td>37</td>
<td>85</td>
</tr>
<tr>
<td>LouZ55-4</td>
<td>79</td>
<td>22</td>
<td>63</td>
<td>260</td>
<td>7</td>
<td>11</td>
<td>20</td>
<td>85</td>
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<tr>
<td>LouZ55-5</td>
<td>76</td>
<td>122</td>
<td>64</td>
<td>373</td>
<td>8</td>
<td>10</td>
<td>13</td>
<td>85</td>
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</tbody>
</table>

Fig. 3. Flow cytofluorometry analysis of the BARF1 transfectants. BARF1 transfectants or vector-transfected control cells were stained with anti-CD21, anti-CD23, and anti-CD71 (transferrin receptor) monoclonal antibodies and fluorescein isothiocyanate-conjugated goat anti-mouse Fab fragments. A profile of fluorescence intensity by cell sorter analysis is shown. Filled tracing, profile of pZip vector-transfected control cells. A, anti-CD21; B, anti-CD23; C, anti-CD71. Fluorescence intensity is denoted on a logarithmic scale.
Table 2 Tumorigenicity of either BL-derived or in vitro established LCLs and Louckes cell clones

<table>
<thead>
<tr>
<th>Clones</th>
<th>Days after injection</th>
<th>Frequency*</th>
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<tr>
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<td>7</td>
<td>9</td>
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<tr>
<td>LCL-1</td>
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<tr>
<td>LCL-2</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>LCL-3</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>BJAB-B95</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>BJAB-1</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>BJAB-2</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>Louckes</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>Lou-pZip-1</td>
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<td></td>
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<tr>
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<td>Lou-pZip-5</td>
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<td>LouZAR1-1</td>
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<td>LouZ55-4</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>LouZ55-5</td>
<td>0/6</td>
<td></td>
</tr>
</tbody>
</table>

* Tumor formation after s.c. injection of 10⁷ cells into newborn rats treated with the anti-thymocyte serum during the first 2 weeks.

** Cord blood B-lymphocyte immortalized by B95–8 strain.

† The average size of all tumors (in cm) on that day.

‡ BJAB cell line converted by B95–8 virus strain.

§ The animals were killed to isolate the tumor tissue sample.

A subclone issued from original BJAB cell line.

BAJ cells frozen in 1980.

Louckes cell clone containing pZip vector alone.

Louckes cell clone containing 1.1-kilobase genomic BARF1 sequence.

Louckes cell clone containing 0.74-kilobase cDNA sequence.

Cultured for 2 months after isolation under selection in 3 mg/ml neomycin.

Cultured for 6 months without neomycin.

microscopy. All biopsies tested showed a diffuse lymphoma with many small cells (Fig. 4). To examine whether the tumor biopsies contain the BARF1 gene, DNA (10 μg) extracted from biopsies and digested with EcoRI restriction enzyme was analyzed by Southern blot techniques using a 1.1-kilobase Smal fragment as a probe. If the recombinant plasmids are not integrated in cellular DNA, a positive signal should be 8.8 kilobases for the cDNA sequence and 9.2 kilobases for the genomic sequence. The results presented in Fig. 5 signal differences of integrated sites in cellular DNA. This integration presumably occurs through the LTR sequence (5.4 and 5.8 kilobases of LTR to LTR size with cDNA or genomic sequence, respectively).

The above experiments were carried out with clones from transfected Louckes cells at an early passage (2 months after isolation under neomycin selection). However, when we tested the tumorigenicity of the same clones cultured over 6 months without neomycin (LouZAR1–3, LouZ55–4, and LouZ55–5: Fig. 5, a–c), no tumor formation was observed in most cases (Table 2). These late passages of Louckes cell clones did not express BARF1 mRNA, and only low levels of c-myc mRNA expression were detected (data not shown). However, the analysis of total cellular DNA by Southern blots showed that these long-cultured Louckes cells retained the recombinant plasmids (data not shown). This indicates that human lymphoid cells can inhibit the expression of a foreign gene by a mechanism not yet identified. In contrast, BARF1-transfected rodent fibroblast BALB/c3T3 cells still expressed mRNA after long periods in culture, although at somewhat of a reduced level (13). Similar tumor development in newborn rats was observed with two of three polyclonal lymphoblastoid cell lines, LCL-1, LCL-2, and LCL-3, which were established in vitro from human cord blood B-lymphocytes immortalized in vitro by B95–8 EBV strain. The tumorigenicity assays revealed that both LCL-2 and LCL-3 express about 1–2% EA- and about 4–8% VCA-induced tumors in newborn rats on as early as the 7th day. The tumors reached a maximal size on days 14–16 postinjection and then regressed by 27–30 days. By contrast, EA-VCA-negative LCL-1 did not develop any tumors (Table 2). LCL-2 and LCL-3 showed significant c-myc transcription, whereas the nontumorigenic line, LCL-1, showed no significant expression of c-myc (data not shown).

DISCUSSION

There are no ideal human lymphoid cell lines for studying the oncogenic roles of EBV genes. Two known EBV-negative, immortalized B-cell lines, BJAB and Louckes, are candidate lines which are suitable for transfection experiments, although the former (BJAB) in our hands proved highly fragile and clones of it were actually found to be tumorigenic. This was not the case with the Louckes cell line. As shown in this report, Louckes cell lines could be used for tumorigenicity experiments since they are transfectable and are not tumorigenic in newborn rats even in the presence of the pZip vector. Therefore, the introduction of the BARF1 gene into target human B-lymphoid Louckes cells was carried out. Injection of resulting cell clones in newborn rats caused the induction of tumors within 2–3 weeks, but not in all cases. Two of the cell clones used contained the BARF1 genomic sequence, and two clones contained the cDNA sequence. The frequency of tumor formation varied among the clones. Similar low frequencies of tumor induction have been observed with EBV-positive LCLs transfected with the c-myc gene (23). The cell populations used for tumor induction were not clonal, and this might account for frequency variation, as well as for differences in the features of tumor induction (some clones such as Lou-ZAR1–3 induce tumors at an early time, while others such as Lou-Z55–4 induce...
tumors at a later time). The tumor biopsies resembled diffused lymphomas and contain BARF1 sequences. The size of the tumors was smaller than those obtained from rodent fibroblast BALB/c3T3 cells which express the BARF1 gene, and the Lou-BARF1 tumors regressed after 3–4 weeks postinjection in newborn rats. On the other hand, the oncogenic activity of BARF1 in rodent fibroblasts resulted in an aggressive and evolutive tumor (13). The observed tumor induction in response to BARF1-carrying Louckes cells seems to be significant in that we have never observed induction from either parental cells or parental cells bearing the pZip vector up to 8 weeks postinjection. Furthermore, Louckes cells in which the expression of the BARF1 gene was lost in culture did not induce any tumors in newborn rats. The tumor regression pattern observed in the case of LCL-2 and LCL-3 resembles that observed for LCL immortalized by the B95–8 virus (24), yet LCL-1 was not tumorigenic, probably because of the lack of expression of lytic antigens. Nevertheless, in our case we must also consider whether tumor regression after the third week may have been due to the lack of anti-thymocyte serum since rats were subjected to immune suppressive treatment only for the first 2 weeks after cell injection. A recovered immune system might prevent the progression to highly aggressive tumors. An alternative explanation is that both LCL- and BARF1-expressing Louckes cell lines might lack some factors from cellular and/or viral origin necessary for inducing evolutive tumors. We recently confirmed the tumorigenicity of BARF1-expressing Louckes cell clones in severe combined immunodeficiency disease mice, indicating their tumorigenicity in two different animal models.

Increased expression of cell surface proteins, particularly CD21, CD23, and the transferrin receptor, was observed in BARF1 transfectants at levels similar to those obtained with LMP1-transfected Louckes cells (9, 10) which express high levels of LFA1, LFA3, CD23, ICAM-1, and transferrin receptor molecules (25). No induction of LFA1 and ICAM-1 molecules was observed in the BARF1 transfectants, except for LFA3 which showed a slight increase in expression compared to controls. Our recent results with a rabbit polyclonal antibody against p33 showed that this viral protein seems to be localized also in the cell membrane.4 The BARF1-encoded p33 protein could be interacting with other cell membrane proteins like LMP1 and may be implicated in EBV-infected B-cell growth regulation (25, 26).

Since no tumor induction was observed with cells that had lost the BARF1 gene, its expression seems to be responsible for the observed tumorigenicity. The presence of high transcription of the c-myc gene in tumorigenic BARF1-expressing cell clones implies that activated expression of c-myc is involved in the tumor induction process. Recent work (23) indeed demonstrated that overexpression of the c-myc gene in EBV-positive LCL led to induction of tumors in nude mice that were B-cell lymphoma-like tumors, like tumors induced with Louckes cells. The BL-derived B-cell lines were reported to show generally some higher c-myc expression due to the chromosome translocation. The BL-derived control Louckes cell transcribed the c-myc gene at a lower level, similarly to that of LCL-1. However, this expression increased in BARF1-transfected Louckes cell lines. The mechanism of c-myc gene activation by the BARF1 gene is not yet known.

The role of BARF1-encoded protein in tumor induction remains unknown. Our recent works revealed possible transcription of this gene in lymphomas from patients with acquired immune deficiency syndrome, in biopsies from patients with North African nasopharyngeal carcinoma,5 and in a tumor biopsy induced in tamarins by EBV infection (27). Computer analysis showed a possible homology between the respective amino acid sequences of BARF1 and ICAM-1 genes. The overall homologies were 23.5% of identically aligned residues and 6.8% of similarly aligned residues over 221 amino acid sequences of BARF1; in some regions this homology reached up to 50%. A possible transmembrane domain in the BARF1 protein was found in the region where the ICAM-1 molecule also had a transmembrane sequence (28), as well as a possible peptide signal sequence. Homology of BARF1 with the immunoglobulin gene was reported earlier (29). An adhesion molecule, ICAM-1, can be activated by LMP (25). We did not see activation of the ICAM-1 molecule in BARF1-expressing Louckes clones by FACS analysis. A possible amino acid sequence homology between BARF1 and ICAM-1 raises the question of whether the BARF1 gene product can

Fig. 4. Histological analysis of the induced tumors. Frozen tumors from clone LouZ55-5 were cut, stained with m-cresol purple, and photographed under a microscope. Magnification: A, ×40; B, ×100.

Fig. 5. Identification of BARF1 sequence in tumors by Southern blot. a, clone LouZAR1-3; b, clone LouZ55-4; c, clone LouZ55-5; d, positive control from tumor induced by BZ55-27, BALB/c3T3 clone bearing BARF1 sequence (13); e, negative control from normal rat tissue. Arrows, DNA marker in kilobases.

4 J. Tanner, M. X. Wei, A. Ahmad, C. Alfieri, P. Tailor, T. Ooka, and J. Menezes. Epstein-Barr virus protein BARFI expressed in transfected cells serves as a target for antibody-dependent cellular cytotoxicity, manuscript submitted.

5 M. X. Wei, G. Decaussin, and T. Ooka, Unpublished data.
be a ligand for the LFA1 receptor (30, 31). The existence of a putative transmembrane domain and a putative peptide signal in the BARFI-encoded protein might suggest possible secretion of this protein into the culture medium. Further studies using specific antibodies against the protein encoded by the BARFI gene will be necessary to identify its biological function(s).

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