INTRODUCTION

The role of epithelial cells for EBV primoinfection and persistence in healthy carriers is still unclear but it is clear that both B-lymphocytes and epithelial cells can be involved in EBV-associated oncogenesis. For example, Burkitt’s lymphomas and posttransplant lymphomas are derived from B-lymphocytes, while EBV-associated undifferentiated nasopharyngeal carcinomas (NPC) are derived from epithelial cells. Association of undifferentiated NPC with EBV is constant regardless of patient origin. In these tumors, all malignant epithelial cells contain the EBV-genome (Klein et al., 1974; Teng et al., 1986; Zur Hausen et al., 1970) and express viral proteins like EBNA1, LMP1, LMP2A, and BARF0 (Brooks et al., 1992; Busson et al., 1992; Fahraeus et al., 1988; Gilligan et al., 1991; Hitt et al., 1989; Young et al., 1988) as latent protein and BARF1 (Sbih-Lammali et al., 1996a), DNAase (Sbih-Lammali et al., 1996b), BZLF1 (Cochet et al., 1993), and EA-D (Luka et al., 1988) as early antigens. Gastric carcinoma is another epithelial malignancy which can be associated with EBV, but with less consistency; approximately 10% gastric carcinomas are EBV-positive (Osato and Imai, 1996). Again, in the EBV-positive subgroup of gastric carcinomas, all malignant cells contain the EBV genome and express viral latent proteins, except the LMP1, EBNA2, and EBNA3 groups (Yoshima et al., 1997).

There are several experimental models to investigate B-lymphocyte infection and transformation by EBV. In vitro EBV infection can growth-transform not only human B-lymphocytes but also primate B-lymphocytes. In addition, intravenous injection of purified virions of the B95-8 strain of EBV in tamarin induces a fast growing lymphoma containing the EBV genome (Zhang et al., 1992) and leading to animal death in about 3 weeks (Cleary et al., 1985). This indicates that EBV is oncogenic in primate B cells. In B cell lines transformed in vitro by EBV infection, about 11 viral genes (Kieff, 1996) are expressed and contribute to the growth-transformed phenotype. Six of them are known to be indispensable for this process (Kieff, 1996).

In contrast, it is still difficult to investigate EBV infection and transformation of epithelial cells (Gan et al., 1997; Sixbey et al., 1983). Several limiting factors should be considered. For a long time, many reports have drawn attention to the low rate of EBV penetration in epithelial cells due to the absence or very low expression of the CD21 molecule, which is the EBV membrane receptor on human B-lymphocytes (Birkenbach et al., 1992). To overcome this difficulty, the CD21 molecule has been artificially overexpressed or bypassed by various means, for example, using the IgA transcytosis pathway (Knox et al., 1996; Li et al., 1992; Gan et al., 1997). However, there are clearly additional factors distinct from the low rate of

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virus penetration which hamper EBV infection in epithelial cells. The epithelial cell environment seems to be often unfavorable both to an efficient replicative cycle and to the establishment of a stable mode of EBV latency. Recently EBV conversion of established epithelial cell lines has been achieved using a recombinant form of EBV (Imai et al., 1998). This report has two major innovative points. First, infection of epithelial cells was obtained using cocultivation with Akata producer cells with apparently no need for the CD21 receptor. Second, cells retaining the EBV genome in a latent state of infection were positively selected using the neomycin resistance gene carried by the recombinant virus. More recently an epithelial cell line from stomach material was obtained after infection of a recombinant lymphotropic EBV particle (Kawanishi et al., 1999). The established cell line did not express CD21 receptor.

Our aim was to obtain latent EBV infection of epithelial cells associated with growth transformation, in other words, an experimental situation where EBV-infected epithelial cells would be positively selected on the basis of their proliferative advantage. For this purpose, we chose monkey kidney primary epithelial cells as recipients and the NPC C17 tumor line as a source of natural EB virus (Busson et al., 1988). We here report that it was possible not only to infect these monkey primary cells with cell-free C17 virus preparation but also to obtain stably EBV-infected and growth-transformed cell clones. All transformed cells express EBNA1 and BARF1, in the absence of detectable LMP1 or lytic proteins. BARF1 has oncogenic activity in rodent fibroblasts (Wei and Ooka, 1989) as well as a human B cell line (Wei et al., 1994), but also an immortalizing activity in primary monkey epithelial cells (Wei et al., 1997). Moreover the BARF1 gene was specifically expressed in NPC biopsies (Decaussin et al., 2000). The BARF1 gene could therefore play an important role in epithelial oncogenesis. Our experimental model is useful in understanding the oncogenic mechanism induced by EBV.

RESULTS

Cell growth after infection by C17 tumor-derived EBV

Primary epithelial cells were subjected to infection by EBV secreted by NPC cells derived from the C17 xenograft. These cells were dispersed and maintained in culture in vitro for two passages. Release of EBV virions in culture medium was analyzed by PCR (amplification of BARF1 sequence, see the primers used in Table 1) or by electron microscopy analysis after sedimentation of the virus. One milliliter of concentrated virus derived from 250 ml of culture medium collected at passage 2 was used for infection (250-fold). For the detection of the EBV particle, DNA was extracted from 250-fold concentrated medium. PCR was carried out to amplify an entire BARF1 sequence with 1 μg of DNA extracted from virus, B95-8, and IB4. Louckes DNA was used as a negative control. A slight amplified fragment was obtained from virus preparation, while BARF1 negative Louckes DNA did not show any band (Fig. 1). The concentration of virus determined by negative coloration on electron microscopy was evaluated as about 1 × 10^5 particles/ml (data not shown).

Infected and control uninfected primary cells were seeded at a density of 0.6 × 10^6 cells in 10-cm petri dishes. The primary Patas monkey kidney epithelial cells are known to senesce after 10–16 passages in DMEM supplemented with 10% FCS and EGF (Wei et al., 1997). As expected, uninfected Patas stopped their growth between passages 12 and 16 (Fig. 2), while cells infected by C17 EBV kept growing. EBV-infected Patas cells were numbered at each passage. Major variations were ob-
served in their rate of proliferation. A sharp increase in cell proliferation was observed around passage 10, followed by a marked slowdown until passage 14. A new progressive increase in cell proliferation was noted from passages 14 to 27. Beyond this period, the rate of proliferation tended to stabilize at about three- to fourfold the growth rate of uninfected Patas cells (1.2 $\times$ $10^6$ cells for uninfected cells and 3.6–4.8 $\times$ $10^6$ for infected cells per dish per week of culture). Recently (after 80 passages), a new progressive increase in cell proliferation was again observed (Fig. 2).

At the 20th, 35th, and 50th passages, EBV-infected cells were examined for growth capacity at low cell density for two reasons: this property is one of the classical criteria used to assess oncogenic transformation, and practically this procedure is required to isolate monoclonal sublines. Infected cells taken at the above-mentioned passages were seeded in six-well plates at the rate of 250, 500, or 1000 cells per well, in 3 ml of culture medium (Wei et al., 1997). After 30 days, an average of 3, 10, and 15 colonies were recovered in wells seeded with 250, 500, and 1000 infected cells, respectively. In contrast, no colonies were observed when uninfected Patas cells (passage 2) were seeded at low density. Ten clearly separated colonies were picked up and designated subclones CD1 to CD10. In all subclones, cell morphology was quite different from the morphology of parental uninfected cells. While primary Patas cells grew as disordered large cells (Fig. 3A), cells from each subclone had two kinds of epithelial morphology either with compact or with regular form (Fig. 3: CD/C17, B; CD1, C; CD4, D).

The cell growth kinetics of the infected subclones was similar to that of the parental infected cells (CD/C17) except for the subclone CD1, which slightly reduced its growth at the 50th passage (Fig. 2). Both parental and subclone cells grow over 100 passages (corresponding to a period of 24 months).

**Identification of the viral genome in infected cells**

The above-reported results had shown that EBV infection resulted in growth transformation of the primary
epithelial cells. To determine whether the EBV genome was directly involved in this process, we first investigated the presence of the EBV genome in transformed cells. Three viral loci were investigated preferentially. These loci corresponded to the genes encoding EBNA1 (BKRF1), LMP1 (BNLF1), and BARF1. Indeed, EBNA1 is consistently expressed in all EBV-infected cells, LMP1 is an oncogene indispensable for the growth transformation of primary B cells, and BARF1 by itself has the ability to growth-transform primary epithelial cells. Viral genome fragments encoding EBNA1, LMP1, and BARF1 were detected by PCR using appropriate primer pairs (see Table I). The presence of this viral DNA fragment was monitored, each five passages, until the 60th passage of the parental infected cells (CD/C17) (Fig. 4). All three genes were detected at all passages, but the amounts of PCR products were quite variable.

Interestingly, we obtained a high intensity of amplified fragment at the 2nd, 15th, 20th, 25th, and 30th passages for all BARF1, LMP1, and EBNA1 genes. An optimum peak of cell growth obtained at the 5th passage (see Fig. 2) showed very low intensity, while an optimum intensity was observed at the 20th passage for all genes. The amplified band at the 20th passage had the highest intensity for three viral genes. The EBNA1 bands in Fig. 4B, lanes 5, 10, 35, and 45, which were difficult to visualize, come probably from the low quantity of EBV ge-
nome in these cultures (in particular at the 35th passage). It is quite possible that the loss of the EBV genome occurs during cell culture by a “hit end run” mechanism (Galloway and McDougall, 1980). The EBV-negative Ramos and uninfected Patas cells used here as negative controls did not give any significant response (Fig. 4, lanes Ramos and Patas).

The 10 clones derived from the infected Patas cells (CD1 to CD10) at the 59th passage were subjected to PCR analysis of the BARF1 and LMP1 genes. The parental CD/C17 cell line was processed in the same experiments (Fig. 5). Interestingly, all 10 clones were positive for each of the two genes. However, some subclones (CD3 and CD5) gave relatively low amounts of PCR products.

To confirm the presence of the EBV genome, Southern blot was carried out on CD/C17 and CD8 (which are positive to PCR) and P3HR-1 as well as IB4 (as positive controls) DNA. DNAs digested with BamHI were electrophoresed, transferred onto nylon filters, and hybridized with a radiolabeled BamHI A fragment (11.8 kb). As illustrated in Fig. 6, P3HR-1 TK− (containing 11 EBV genome copies) gave a strong signal, while IB4 DNA (containing 1 or 2 copies) showed a weak signal. Signals from CD/C17 and CD8 were slightly higher than that of IB4. It was very difficult to visualize a positive signal with other CD clones.

In order to know whether the EBV genome found in Patas subclones is the origin of C17 tumors, we amplified a small part of the LMP2 exon-1B region of CD/C17 and C17 original EBV genome using two primers: at positions 167020–167039 as sense and 169913–169933 as antisense for the EBV genome (Busson et al., 1995). Amplification for CD/C17 was somehow difficult due to a lower quantity of amplified fragment. Ten PCR reactions were therefore put together to obtain a large enough quantity of amplified fragment. The results confirmed that EBV from CD/C17 was of C17 origin, because we found two mutations specific to C17 EBV (in comparison with those of the B95-8 strain): at 169740 (T → A) and at 169764 (A → G) (data not shown) in the CD/C17 strain. This suggests that EBV present in CD subclones comes from C17 tumor.

A 382-bp nucleotide sequence contained in the BARF1 ORF was also PCR-amplified and sequenced from the C17 and B95 prototype EBV strain. No differences between the two EBV strains were found in this segment of the viral genomes. The same sequence was also amplified from the CD1 clone and from parental EBV-infected

![Figure 5](image5.png)

**FIG. 5.** Analysis of two EBV-encoded genes by PCR from DNA extracted from a parental transformed Patas cell line and Patas-derived subclones. PCR analysis was carried out on DNA extracted from immortalized Patas subclonal cells at the 59th passage. Amplified fragments are 382 bp (A) and 181 bp (B) for the BARF1 and LMP1 sequences, respectively. EBV-negative Ramos and primary uninfected Patas cells were used as negative controls. IB4, Akata, and C17 tumor cells were used as positive controls. Amplified DNAs were transferred onto Hybond filters. The filters were hybridized with the following probes: the C55 cDNA sequence for BARF1; and a CB281 cosmid for LMP1. CD/C17 is a parental transformed cell line.

![Figure 6](image6.png)

**FIG. 6.** Identification of BamHI-A fragment in CD clones by Southern blot. (A) CD8; (B) CD/C17; (C) IB4 (as a positive control); (D) P3HR-1-TK− (as a positive control); (E) Patas cells. The arrows indicate the size of the BamHI-A fragment.
Patas cells. Again, no differences were found with the C17 and B95 sequences (data not shown).

Expression of viral genes in growth-transformed epithelial cells

Expression of the BARF1 protein was analyzed in each EBV-carrying clone by immunoblot using an antipeptide rabbit polyclonal antibody (Wei et al., 1997). TPA-induced P3HR1 cells were used as a positive control. A characteristic 31- to 33-kDa band was detected in all clones, although with some variations in their intensity (Fig. 7A). Interestingly, a protein extract derived from the C17 xenografted tumor was also strongly positive for the BARF1 protein (Fig. 7A) (note that only 5 μg of the C17 protein extract were loaded, whereas 50 μg of protein was loaded for the clones). We also examined BARF1 protein expression in Patas-derived cell lines by immunofluorescence using a rabbit polyclonal antiserum purified by an affinity column. The results are presented in Fig. 8. Immunofluorescence was observed in the cytoplasm and nuclear periphery (Figs. 8B–8I), while no such immunofluorescence was detected in uninfected Patas cells (Fig. 8A).

The presence of lytic proteins were also examined in using a monoclonal antibody against p135kDa (OT13B) and BZLF1 for early proteins and a monoclonal antibody against gp350 for late protein. However, we never found the expression of p135kDa and gp350 proteins in the transformed cell lines, while a very weak response of BZLF1 was obtained with CD/C17 and CD1 subclones (data not shown).

EBNA1 protein expression was also analyzed in each clone (Fig. 7B). The size of the EBNA1 protein is variable depending on the EBV strain. The EBNA1 bands ob-
tained for our positive controls, the Raji and IB4 cell lines, were at the expected sizes, 65 and 75–78 kDa, respectively (Fig. 7, lanes 1 and 2). We found a much larger size (90–95 kDa) for the EBNA1 protein encoded by the C17 virus strain. This is consistent with observations made on earlier passages of this nude mouse tumor (P. Busson, personal data). The EBNA1 protein was detected in all clones, with a size similar to the size of the C17 EBNA1, around 95 kDa, which was revealed with monoclonal anti-EBNA1 and corresponds to that of the C17 nude tumor (Fig. 7B, lane 4). The level of EBNA1 expression varied among the subclones. As already observed for CD5 containing a very low EBV genome (Figs. 5A and 5B), the expression of EBNA1 was also low (Fig. 7B, lane 8).

To investigate EBNA1 expression at the single cell level, we analyzed its expression by immunofluorescence on fixed cells using a serum from a North African NPC patient (Fig. 9). Typical nuclear fluorescence was observed in CD/CD17 (Fig. 9D), CD1 (Figs. 9B and 9C), CD8 (Fig. 9E), and CD10 (Fig. 9F) clones, while the parental patas cells did not show any positive immunofluorescence (Fig. 9A). The level of EBNA1 expressing cells was almost 100% for CD/C17 and CD10, while the other clones expressed EBNA1 protein 90, 72, 64, 30, and 21% for CD9, CD1, CD8, CD5, and CD4, respectively.

LMP1 expression was also analyzed in the EBV-infected clones (Fig. 7C). It has been previously shown that LMP1 is not expressed in the C17 tumor (Clausse et al., 1997). As expected, LMP1 was undetectable in the C17 tumor extract but it was readily detected in the Akata and IB4 cell lines. None of the epithelial clones had LMP1 detectable by immunoblot. To achieve a greater sensitivity in our detection and to rule out the possibility of some antigenic polymorphism that would hamper recognition by the S12 monoclonal antibody, we searched LMP1 transcripts by RT–PCR using the primers indicated in Table I. But LMP1 transcripts (amplification of a 460-bp spliced sequence) were also undetectable in the C17 material and in all epithelial clones (Fig. 10), while Akata and Raji cell lines gave a positive response.

Expression of CD21 in Patas cells

In order to know whether EBV could have used the CD21 molecule to penetrate growth-transformed epithelial cells, we intended to investigate the expression of this receptor in Patas monkey cells. We first verified that primers designed to amplify a segment of the human CD21 gene were also able to amplify the homologous segment in Patas monkey cells (Fujisaku et al., 1989). This was the case, as shown in Fig. 11A. An expected 329-bp fragment was amplified in all DNA samples of human or monkey origin and these fragments hybridized...
with an entire human CD21 sequence used as a probe (Fig. 11B). In fact the nucleotide sequence analysis of amplified fragment was identical in monkey (Patas and B95-8) and human (Raji and Akata) B cells (data not shown). Thus in the next step, CD21 gene transcription was assessed by reverse PCR, using the same primer pair, in control human cell lines (Raji, Akata, and 293) and in epithelial monkey cells (noninfected primary cells, infected parental cells, and clone CD1). A high level of CD21 mRNA was detected in Raji and Akata cells and to a lesser extent in 293 cells. In contrast, no CD21 mRNA could be detected in Patas and Patas-derived cell lines.

Cytokeratin expression

To examine whether the immortalized Patas cell lines were of epithelial origin, immunofluorescence with two monoclonal antibodies specific for different kinds of cytokeratins was performed on Patas cells, EBV-infected parental CD/C17, and a subclone, CD1 (Fig. 8). With K8-60 antibody raised against both cytokeratins 10 and 11, primary Patas cells and immortalizing cell lines were positive (Figs. 8B, 8E, and 8H). With AE1/AE3 antibody raised against both acidic and basic simple cytokeratins, primary Patas cells and immortalizing Patas-derived cell lines were also positive (Figs. 8C, 8F, and 8I). As in the case of Patas cell line established by BARF1 transfection (Wei \textit{et al.}, 1997), both cytokeratins were expressed in cell lines immortalized with EBV. The detection of cytokeratins confirmed the epithelial origin of our immortalized clones.

Tumorigenicity assays in nude mice

We examined whether EBV-infected patas clones are tumorally transformed. The tumorigenicity of CD/C17, CD10, CD9, CD8, CD3, and CD1 as well as Patas (as a control cells) and HeLa cells (as a positive control) was initially assayed by injection of $10^6$ cells per animal in nude mice. Tumor development was followed up for 4 weeks. HeLa cell-injected mice developed tumors as early as 1 week after injection, and their tumor size continued to increase until 4 weeks, while none of the CD subclone-injected mice developed tumors (data not shown).

DISCUSSION

There is strong evidence that EBV is the causative agent of the process of growth transformation obtained in monkey primary epithelial cells. This is a first demonstration that primary epithelial cells were immortalized by a wild EBV. First, we did not observe, at any time, spontaneous transformation in noninfected Patas cells, which consistently senesced before passage 16. Second, all cell clones obtained by culture at low density retained the EBV genome and expressed some viral proteins. In addition, by immunofluorescence, it was possible to demonstrate that all cells of the transformed clones or at least the vast majority of these cells were EBNA1 positive. This suggests that the maintenance of the EBV genome is required for the persistence of the transformed phenotype. However, we observed a diminution of EBV genome content between the 20th and 40th passages. A very low amount of EBV genome was constantly found after the 40th passage. The subclones contained equal or inferior numbers of EBV genome copies per cell to those of IB4 cells (in which only one copy of the EBV genome was found). Similar contents of EBV genome were found in the subclones cultured for over 100 passages. The above observations suggest that almost all cells were infected by EBV at the 20th passage, but some cells probably lose the EBV genome after this period. In fact, when the expression of EBNA1 was examined in EBV-infected cells by immunofluorescence, three of the subclones expressed almost 80–100% EBNA1 protein, while the other subclones had EBNA1
expression between 20 and 70%. Possibly a "hit and run" mechanism could occur in the latter, as observed in HSV1 infection (Galloway and McDougall, 1980). Possibly, the EBV genome is able to modify the host genome specifically or may drive the primary cells to prolifirate, allowing other mutations resulting the maintenance of growth transformation after loss of EBV genome. This kind of phenomenon was recently observed in continuous growing Akata cells which lost the EBV genome (Shimizu et al., 1994).

Despite the fact that Patas cells were obviously infectable by EBV, they were completely lacking CD21 expression at the messenger level. Recently, it has been shown that several established human epithelial cell lines were infectable by EBV, although they did not express detectable levels of CD21. However, in this system, cocultivation of epithelial target cells with EBV-producing Akata cells was required to achieve infection of epithelial cells (Imai et al., 1998). In contrast, in our experimental system, EBV infection of epithelial cells was obtained and also in the absence of CD21 expression, but with cell-free virus. It will be important in future investigations to determine which membrane molecules or molecular complexes mediate EBV entry in Patas cells.

All the growth-transformed clones contained an EBNA1 protein with a size identical to that of C17 EBNA1. This was a confirmation in which the process of growth transformation resulted from the infection by an EBV particle derived from the C17 NPC xenograft. Our extended study showed that lymphocyte-derived EBV like B95-8 or Akata was not able to immortalize Patas cells. One may speculate that an EBV isolate derived from an NPC tumor has better molecular equipment to penetrate and stably transform epithelial cells.

It is probable that the products of several viral genes acted in synergy to establish the growth-transformed phenotype of EBV-infected Patas cells. Among them, the BARF1 gene is likely to have played a major role. Not only was the BARF1 protein consistently expressed in all cell clones but we have previously shown that the BARF1 gene by itself can transform Patas epithelial cells (Wei et al., 1997). However, transformed cells obtained by EBV infection or by BARF1 gene transfection had distinct characteristics. BARF1-transfected cells proliferated at a higher speed, reaching about $4 \times 10^5$ cells after 1 week of culture, starting from $0.6 \times 10^5$ per 10-cm plate, whereas EBV-infected Patas cells reached only $3-4 \times 10^5$ under the same experimental conditions (Wei et al., 1997). To understand this paradox one should keep in mind that in EBV-infected cells, BARF1 was expressed under its natural promoter instead of the retroviral LTR promoter. It is conceivable that in this context, BARF1 had different biological effects. Its transforming activity might be less marked and could require cooperation with the products of other viral genes.

LMP1 was completely undetectable in growth-transformed Patas cells, at both the protein (immunoblot) and messenger RNA levels (RT–PCR). This is not so surpris-
malignant transformation (Wei et al., 1998). One of our aims is to simplify the process of Patas cell EBV transformation to provide a novel transformation assay that could be used in routine investigations of EBV isolates from various sources (saliva and other body fluids, supernatants of primary cultures of malignant cells...). One way to make the assay shorter could be to seed infected cells at low density early after infection, for example, a few days postinfection. Such an experimental model will be useful in qualitatively and quantitatively assessing the functional consequences of strain polymorphism affecting viral genes which are not expressed in most cells of invasive tumors (for example, the ZEBRA gene).

**MATERIALS AND METHODS**

**Cells and tumor material**

Burkitt cell lines—EBV-carrying P3HR-1 and EBV-negative Louckes and BJAB cells—were maintained in RPMI 1640 containing 10% fetal bovine serum with antibiotics as previously described by Ooka et al. (1980). In order to induce the lytic cycle, P3HR-1 cells were treated with 50 ng/ml of 13-O-tetradecanoylphorbol ester (TPA) and 2 mM sodium butyrate (SB) (Ooka et al., 1983). The EBV-negative human epithelial HaCaT cell line (a gift from Dr. Fuesenig, Institut für Biochemie, Heiderberg, Germany) (Boukamp et al., 1988) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

We also used C15, C17, and C18 NPC tumor lines routinely propagated as xenografts in nude mice (Busson et al., 1988). C17-derived virus was obtained through a step of *in vitro* culture. A xenografted tumor collected at passage 15 was minced in fragments smaller than 1 mm³. Dispersed cells and fragments were maintained in culture in DMEM medium supplemented with EGF (0.01 μg/ml) in 10-cm petri dishes (Wei et al., 1997). After two passages *in vitro*, 250 ml of culture medium was collected and cleared from floating cells by a short centrifugation at 1000 g. The supernatant was first filtered at 0.8 μm and then with 0.45 μm to eliminate cellular debris. Cell-free virus was concentrated by a 2-h centrifugation at 10,200 g. The pellet was resuspended in 1 ml (250-fold concentration) of RPMI 1640 and used to infect primary kidney epithelial cells derived from the monkey *Erythrocebus patas* (Patas cells purchased from Rhône-Mérieux, Lyon, France).
Preparation of cell extracts for immunoblotting

Cell pellets were resuspended in RIPA buffer containing 20 mM Tris–HCl (pH 7.5), 5 mM ethylenediaminetetraacetic acid (EDTA), 650 mM NaCl, 1% Triton, 0.5% deoxycholate sodium, 1% SDS, 1 mM PMSF, and 1 μM protease inhibitor cocktail kit (ICN Biochemicals, CA). Lysates were frozen and thawed three times, sonicated twice for 30 s with a Branson B-15 sonicator, and then centrifuged at 105,000 g for 60 min in a Beckman 50 Ti rotor. After the quantitation of protein, the supernatant was frozen at −80°C until required.

Antibodies

The p33 BARF1 protein was detected with a rabbit polyclonal antiserum (named peptide III) prepared against a synthetic peptide (produced by Société Bioatlantic, Nantes, France) corresponding to a presumed epitope (NGGVMKEKD, amino acids 172 to 180). This BARF1 protein antiserum has been previously used for immunoblot and flow cytometry analysis at a 1:1000 dilution (Tanner et al., 1993). Nonspecific protein binding sites were blocked by overnight incubation of blotted filters in phosphate-buffered saline (PBS) with 0.1% Tween 20 and 5% dried skimmed milk or 5% commercialized blocking agent (Blot-QuickBlocker, Chemicon Int., CA). The filters were subsequently incubated overnight at 4°C with specific antibodies or control preimmune sera or mouse IgG at the same dilution. Filters were washed and incubated for 1–2 h at room temperature with peroxidase-labeled polyclonal anti-rabbit or monoclonal anti-mouse secondary antibodies. The antigen–antibody complexes were then visualized using an enhanced chemiluminescence system (ECL; Amersham) as instructed by the manufacturer.

Detection of BARF1, EBNA1, and LMP1 genes by polymerase chain reaction (PCR)

PCR analysis was carried out in order to identify the BARF1, EBNA1, and LMP1 genes in growth-transformed Patas cells. Experimental conditions were as previously described (Wei et al., 1997, for BARF1, Bathia et al., 1996, for EBNA1, and Books et al., 1992, for LMP1) with some modifications. EBV DNA was amplified in a final reaction mixture volume of 25 μl containing Taq polymerase buffer (1 mM Tris–HCl, pH 8.3; 50 mM KCl), 2.5 mM MgCl₂, 20 μM each deoxyribonucleoside triphosphate, 250 nM each EBV primer, and 1 μg of DNA sample. The lengths of the PCR products were 382, 330, and 181 bp for BARF1, EBNA1, and LMP1, respectively. After 10 min incubation at 95°C for denaturation of DNA, the mixture containing 1.2 unit of Taq polymerase (Perkin–Elmer) was added. Samples were subjected to 35 cycles. The denaturation step was 20 s at 94°C for all three amplified genes. The annealing step was 30 s at 60°C for BARF1, LMP1, and CD21, 30 s at 60°C for EBNA1. Elongation was done for 1 min at 75°C for BARF1, LMP1, and CD21 and at 72°C for EBNA1. After the last cycle, samples were held for 5 min at 72°C (BARF1, LMP1, and CD21) or 72°C (EBNA1) and then cooled to 4°C. Twenty-five microliters of the PCR mixture was analyzed on a 2% agarose gel, transferred, and hybridized as described (Sbih-Lammali et al., 1996). C56 cDNA and the BamHI-K and the EcoD-het genomic fragments were used as probes for the BARF1, EBNA1, and LMP1 genes products, respectively.

Immunoblotting

Thirty to fifty milligrams of proteins quantitated by a Bio–Rad protein assay (Bio–Rad Laboratories, Inc.) was diluted with 1 vol of gel sample buffer (0.2% bromophenol blue, 4% SDS, 200 mM DTT (dithiothreitol), 20% glycerol, 125 mM Tris–HCl, pH 6.8) and boiled for 5 min. Protein samples were separated on polyacrylamide gels and blotted onto nitrocellulose membrane. The transfer was carried out by electrotransferring, in a buffer containing 20 mM Tris–HCl, pH 7.4, EDTA, 50 mM NaCl, 150 mM; Sarkosyl, 1% and proteinase K, 20 mg/ml. The mixture was incubated first for 20 min at 55°C, followed by 1 h at 37°C, and then incubated twice with 1 vol of phenol and chloroform, before precipitation. For Southern blot analysis, 10 μg of cellular DNA was digested with the restriction enzyme BamHI and separated by electrophoresis in 1% agarose gels, followed by transfer to reinforced nitrocellulose filters (Schleicher & Schuell). The probe (11.8-kb BamHI-A fragment) was labeled with random primers and hybridization (1 × 10⁶ cpm/ml of hybridization solution) was carried out for 15 h at 65°C.
Detection of CD21 and LMP1 transcripts by reverse transcriptase–polymerase chain reaction (RT–PCR)

RNA was extracted as previously described (Wei et al., 1994). To eliminate contaminating DNA, the RNA preparation was treated twice with 2 units of Escherichia coli DNase-I/μg of RNA. After ethanol precipitation of RNA, 5 μg of total RNA was used for first strand cDNA synthesis in a 40-μl reaction volume using oligo(dT)15 as primer. Reverse transcription was done with Superscript reverse transcriptase according to the instructions of the manufacturer (GIBCO, BRL). Newly synthesized cDNA was aliquoted and stored at −20°C (Sbih-Lammali et al., 1996). PCR amplification was performed using 0.1 vol of the first strand reaction mixture, 1.2 U of Taq polymerase (Perkin–Elmer), 2.5 mM MgCl2 and 250 nM each primer on the CD21 RNA sequence, giving 329 bp of amplified fragment in a 25-μl reaction mixture containing 20 μM each deoxynucleoside triphosphate and a standard PCR buffer. For each sample, a control tube without reverse transcriptase was added (designated RT−). Amplifications were performed in a DNA thermal cycler (Bio–Rad) as follows: denaturation at 94°C for 20 s; annealing at 55°C for 30 s; and extension at 75°C for 1 min for 35 cycles. PCR mixtures were subsequently analyzed by electrophoresis through a 1% agarose gel followed by Southern transfer onto Hybond N+ nylon membranes (Amersham). Detection of amplified CD21 and LMP1 sequences was achieved by hybridization to a 32P-labeled pZipCR2 vector (containing 3.3-kb CR2 cDNA, a kind gift of Dr. F. Wang (Harvard Medical School) for CD21 and EcoDhet for LMP1, using a random primer DNA labeling kit (Stratagene). The filters were hybridized as previously described (Wei et al., 1997).

Immunofluorescence

Standard immunofluorescence was performed for detection of intracellular cytokeratins (Henle and Henle, 1986). The EBNA1 protein was identified at the single cell level using the anticomplement immunofluorescence (ACIF) reaction (Reedman and Klein, 1973). Epithelial cells were grown on plastic chamber slides resistant to acetone treatment, washed with PBS, fixed with cold acetone/methanol (v/v) for 15 min, and dried. Fixed cells were incubated for 45 min at 37°C with a 1:80 dilution of TU 088, a serum from a Tunisian NPC patient, containing a high titer of antibodies to EBNA1 and low titers of antibodies to early (EA) and late (VCA) EBV antigens (EBNA1, 1:1280; EA, 1:<10; and VCA, 1:40/80). Slides were further incubated 30 min with purified C3 (EBNA-1-negative human serum) at a 1:10 dilution. After three washes with PBS, they were treated with FITC-anti-C3 antibodies (Sigma) at a 1:10 dilution for 30 min and washed three times with PBS and water.

DNA sequencing

The amplified 329 bp of CD21 and 382 bp of BARF1 PCR products were sequenced by the ACT gene (Paris, France).

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REFERENCES


