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Establishment of Epstein-Barr Virus-positive Human Gastric Epithelial Cell Lines

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We have established two Epstein-Barr virus (EBV)-positive cell lines, GT38 and GT39, derived from human gastric tissues of two patients bearing gastric carcinoma. Both cell lines were positive for cytokeratin, an epithelial marker, but not for lymphocyte-related markers. Unlike GT39 cell line, GT38 cells lacked the property of contact inhibition. EBV genome was detected in both cell lines. The cell lines were positive for latent membrane protein 1, and EBV-determined nuclear antigen 1 (EBNA1). EBNA2 was also detected in GT38. These cell lines should be useful for studying the interaction of EBV with gastric epithelial cells and its role in gastric carcinogenesis.

Key words: Epstein-Barr virus — Gastric carcinoma — Cell line — LMP1 — EBNA1

Epstein-Barr virus (EBV) has been associated with a variety of malignancies in lymphoid tissues, nasopharyngeal epithelium, testis, salivary gland, and lung.[1–5] Recently it has been reported that EBV is associated with some gastric carcinomas.[6–14] In most EBV-associated gastric carcinomas, EBV genome is present in a monoclonal episomal form,[15–19] suggesting that EBV infects a cell prior to the neoplastic transformation and may be involved in the gastric carcinogenesis. EBV encodes a number of latent infection proteins. Latent membrane protein 1 (LMP1) is considered to be one of the oncogenic proteins, because the transfection of *LMP1* gene into rodent fibroblasts made the cells tumorigenic in a nude mouse.[20] Expression of LMP1 also inhibits the differentiation of human epithelial cell lines, and promotes their proliferation.[21, 22] In spite of a number of reports on the association of EBV with gastric carcinomas, studies on the role of EBV in neoplastic transformation of gastric epithelial cells have been hampered by the lack of a suitable *in vitro* system. We have been trying to establish EBV-positive cell lines from gastric tissues in order to study the interaction of EBV with gastric epithelial cells, and its involvement in the carcinogenesis. Here we report the establishment of two latently EBV-infected epithelial cell lines derived from human gastric tissues.

MATERIALS AND METHODS

Culture media Eagle's minimum essential medium (MEM) and Keratinocyte-SFM were purchased from Gibco BRL (Rockville, MD). Fetal calf serum (FCS) was obtained from Flow Lab. (Costa Mesa, CA).

Antibodies Fluorescein isothiocyanate (FITC)-, horseradish peroxidase (HRP)-, or alkaline phosphatase-labeled goat anti-mouse antibodies were from Zeimed (South San Francisco, CA). Mouse anti-LMP1 antibodies were kindly provided by Dr. K. Hirai, or were purchased from Dako Japan (Kyoto). Mouse antibody to EBV-determined nuclear antigen 1 (EBNA1) was obtained from Chemicon (Temecula, CA), and mouse anti-EBNA2 and anti-human cytokeratin (45–56.5 kDa: clone MNF116) were from Dako. FITC-labeled mouse anti-CD3, and anti-CD19 were from PharMingen (San Diego, CA).

Gastric tissues for culture For culture we used stomachs, excised by operation from 35 patients with gastric cancers (26 males, 9 females). We established cell lines in two cases. They were from non-cancerous portions of the stomachs of two male patients (59, and 69 years old). Antibody titers to EBV antigens (viral capsid antigen, LMP) were elevated in both of the patients at the time of diagnosis. The tissues were cut into small pieces (less than 1 mm^3), which were put into plastic dishes and cultured at 37°C in a mixture of MEM and Keratinocyte-SFM (1:1), supplemented with 10% heat-inactivated FCS. The medium was changed once a week. We observed that cells started to grow rapidly after 6 months of culture. Then the medium was replaced with MEM containing 10% FCS.

After establishing the two cell lines, we characterized the cells and examined the presence and expression of EBV genome.

Fluorescence flow cytometry analysis The expression of CD3 and CD19 (surface markers for T- and B-lymphocytes) was analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA). The attached cells in dishes were washed in phosphate-buffered saline (PBS), and harvested by scraping. They were incubated with mouse monoclonal FITC-anti-CD3, or FITC-anti-CD19 on ice for 20 min, then washed with PBS three times, and the fluorescence was measured. The background fluorescence was determined using mouse FITC-IgG as a negative control.

Immunocytochemistry The cells were cultured on glass coverslips for 3–5 days, fixed with 2% paraformaldehyde/0.1 *M* phosphate buffer (pH 7.4) for 30 min at room temperature, washed with PBS, and permeabilized by incuba-

tion for 10 min with 0.1% Triton X-100/PBS. They were then incubated overnight at room temperature with anti-LMP1, anti-EBNA1, or anti-EBNA2 followed by FITC- or HRP-labeled goat anti-mouse IgG. We also used B95-8 cells as positive controls, and Kato cells (EBV-negative human gastric cell line)[23] as negative controls with the same immunocytochemical procedures.

Immunoblotting The cells were washed once with homogenization buffer (10 m*M* Tris-HCl, pH 7.4, 0.25 *M* sucrose, 2 m*M* $MgCl_2$) and then harvested by scraping in a minimal volume of homogenization buffer (1 ml). Cells were cooled to 4°C and passed 20 times through a homogenizer. Unbroken cells and nuclei were removed by centrifugation (1000*g*) for 5 min and postnuclear supernatants were mixed with an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer (0.25 *M* Tris-HCl, pH 6.8, 2% SDS, 30% glycerol, 10% β-mercaptoethanol), and then boiled for 5 min. SDS-polyacrylamide gel (SDS-PAGE) was conducted in 10–20% linear gradient gels, which were then blotted onto polyvinylidene-difluoride membrane filters. The membranes were blocked in 1% nonfat milk/0.05% Tween 20/PBS, and stained with mouse anti-LMP1 or with rabbit anti-cytokeratin for 1 h at 37°C. They were washed with PBS, then incubated with alkaline phosphatase-conjugated goat anti-mouse Ig, and bands were visualized by using a combination of nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. B95-8 and Raji cells were used as EBV-positive or cytokeratin-negative controls.

***In situ* hybridization** The cells cultured on glass coverslips for 3–5 days were fixed with acetone at 4°C for 10 min. The presence of EBV genome in the cells was examined by using an *in situ* hybridization kit for detection of the EBV genome (Enzo, Farmingdale, NY). The kit contained biotin-labeled DNA probes specific to *Bam*HI-W fragments of the EBV genome.

RESULTS

We established two cell lines named GT38 and GT39, from the gastric tissues of two male patients. GT38 cells were isolated from a patient (69 years old) with EBV-positive advanced gastric carcinoma (subserous; moderately differentiated tubular adenocarcinoma). GT39 cells were established from the other patient (59 years old) with early gastric carcinoma (submucosal; well differentiated tubular adenocarcinoma). For at least 3 months after the tissue blocks were placed in primary culture, there were no distinct colonies of cells growing in the dishes. After the sixth month, a few rapidly growing colonies appeared. Some of these colonies were selected and seeded for subsequent cultures. Two cell lines, derived from the two different patients, have been cultured *in vitro* for more than 30 months and are considered as permanent cell lines.

Cell growth and morphology The two cell lines grew well as monolayers and both were epithelioid in morphology. GT38 piled up and formed foci in confluent cultures, indicating the loss of cell contact inhibition (Fig. 1a), in contrast to GT39, which showed no piling up of cells (Fig. 1b). The number of GT38 cells increased 8-fold over 3 days and had not reached saturation by the fifth day after the passage (Fig. 2). The doubling time was approximately 24 h for GT38, and 22 h for GT39 cells.

Characterization of cell lines To determine the cell type of GT38 and GT39, we investigated by flow cytometry the expression of CD19 and CD3 as markers for B- and, T-lymphocytes, respectively, and we also examined the presence of cytokeratin, a marker for epithelial cells, by

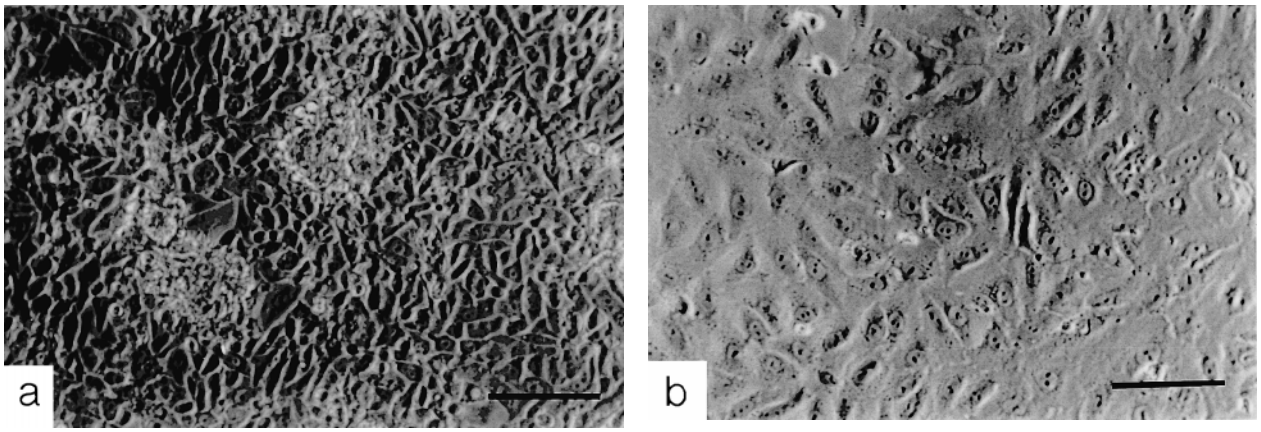

Fig. 1. GT38 and GT39 cell lines in confluent cultures as seen by phase contrast microscopy. a, GT38 showing piled-up foci; b, GT39 with flattened morphology (bars=100 μm).

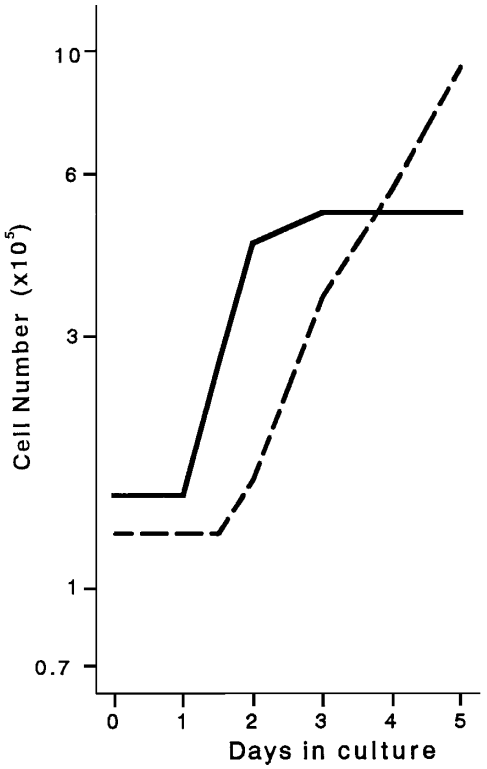

Fig. 2. Growth curves of GT38 and GT39 cells, at passages 69 and 70 respectively. Cells were seeded in Nunclon culture tubes (100×14 mm) and harvested using 0.1% trypsin at 24-h intervals. The cell number in each tube was counted and expressed as the mean of two tubes for each point. ---- GT38, —— GT39.

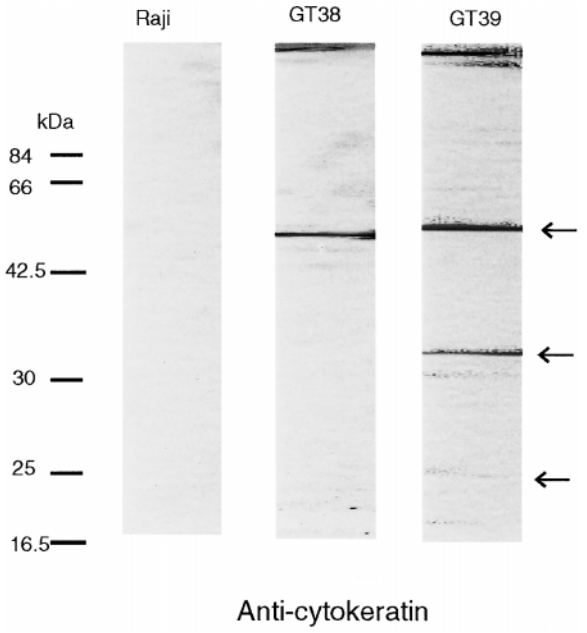

Fig. 4. Immunoblots of cytokeratin. Postnuclear supernatants of GT38, GT39, and Raji cells (negative control) were subjected to SDS-PAGE, transferred to polyvinylidene-difluoride membranes, and reacted with anti-cytokeratin followed by alkaline phosphatase-labeled secondary antibody. Cytokeratin was detected as multiple bands (arrows).

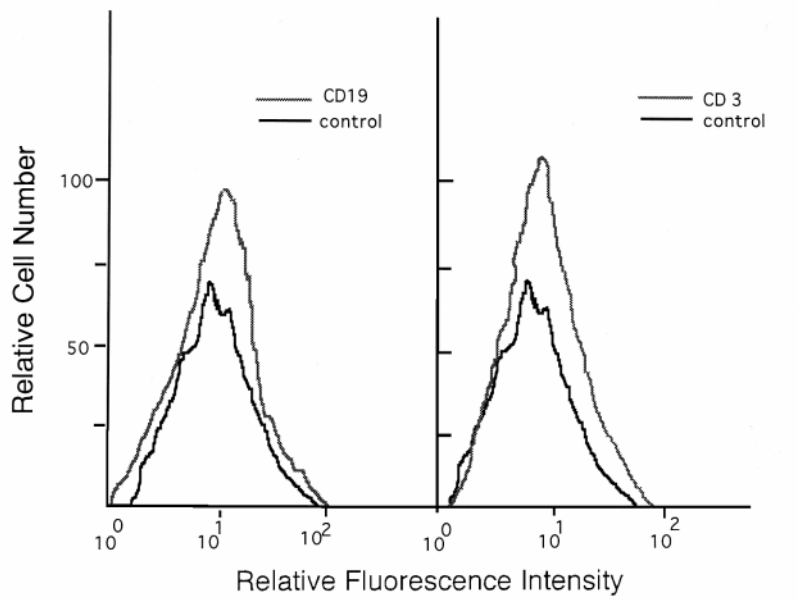

Fig. 3. Flow-cytometric analysis of CD19 and CD3 expression on GT38 cell line. The cells were negative for these surface markers.

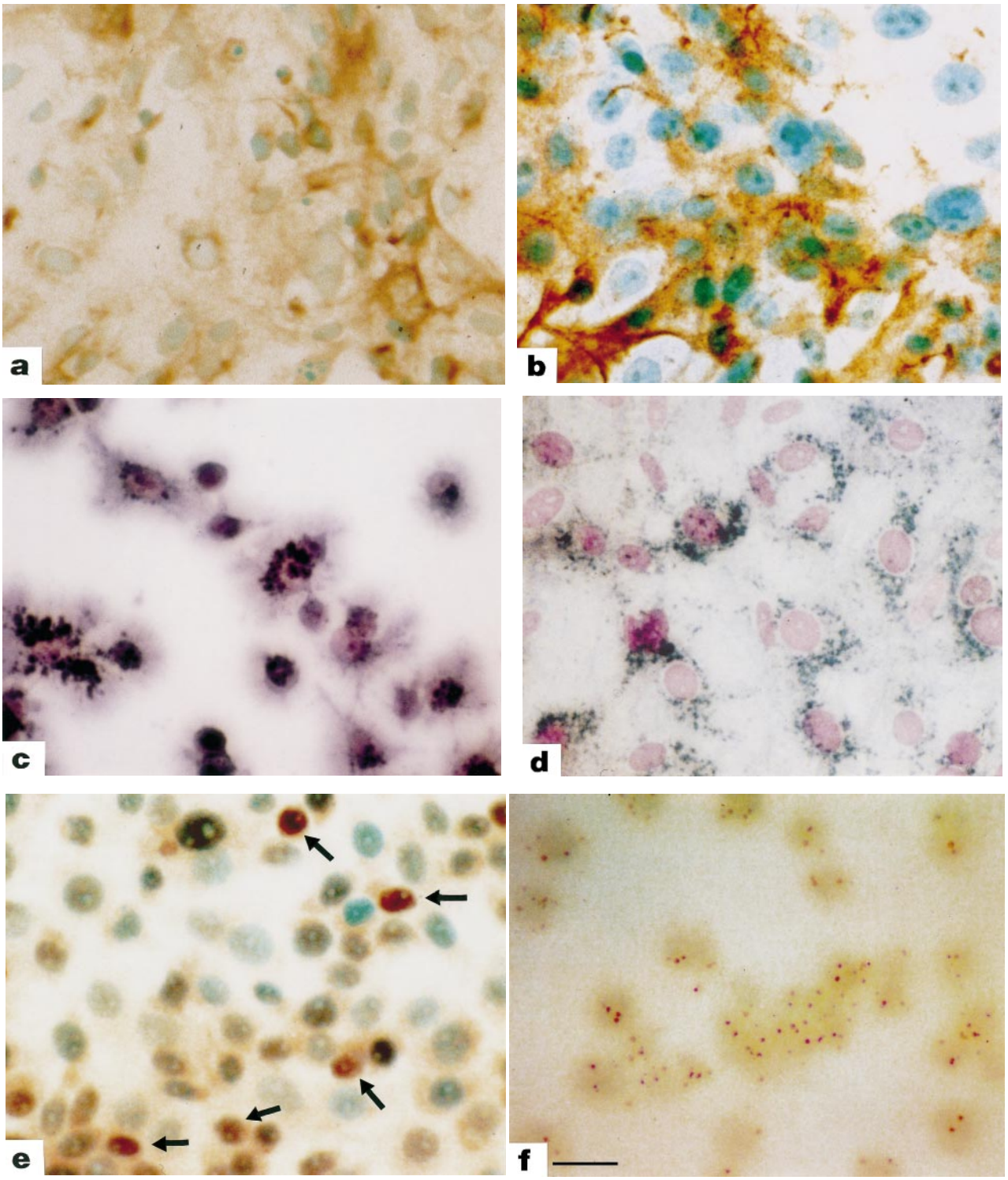

Fig. 5. Immunohistochemistry (a–e), and *in situ* hybridization (f) of GT38 (a, c, e, f) and GT39 (b, d) cells. Reaction products of HRP were used to visualize target molecules. a, b, cytokeratin (brown) is seen in the cytoplasm (counterstained with methyl green); c, d, LMP1 (dark blue) localized on the cell surface and in the cytoplasm (counterstained with nuclear fast red). e, EBNA2 (brown)-positive nuclei are shown by arrows; f, probes specific to *Bam*HI-W fragments of EBV genome are localized in the nuclei or cytoplasm, appearing as brown dots (bar=40 μm).

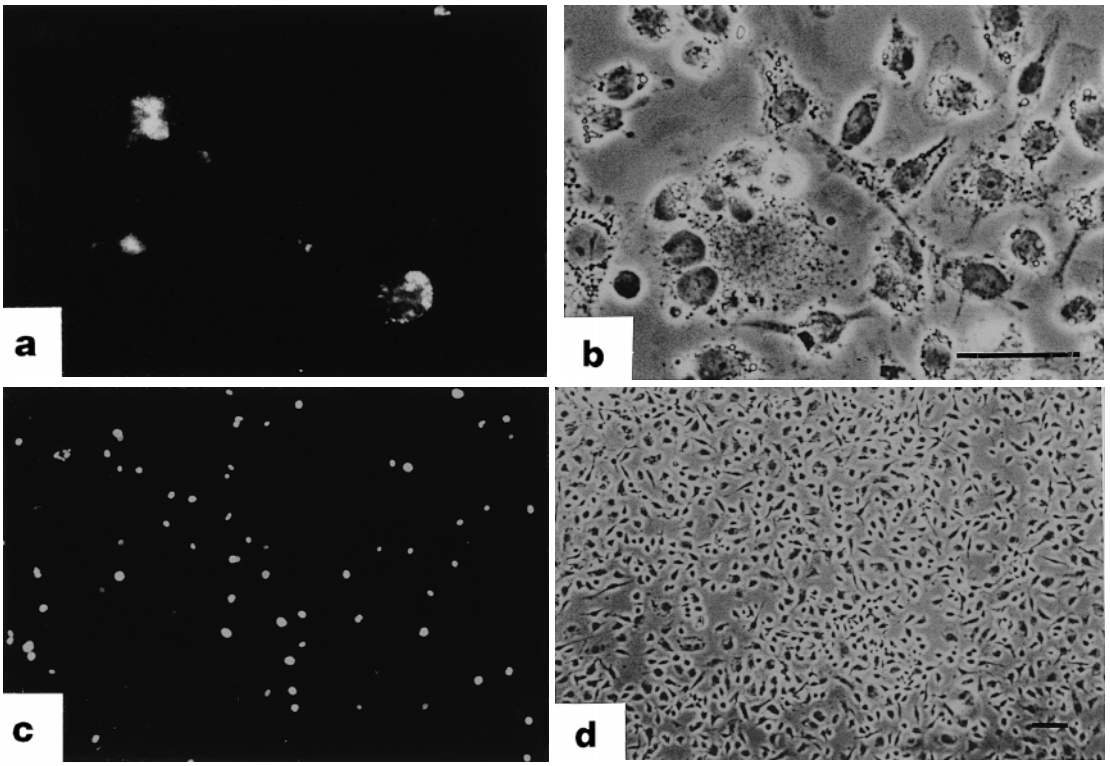

Fig. 6. Visualization of latent infection proteins of EBV by immunofluorescence microscopy. LMP1 (a) and EBNA1 (b) in GT38 cells, and the corresponding phase contrast micrographs (b, d) (bars=40 μm).

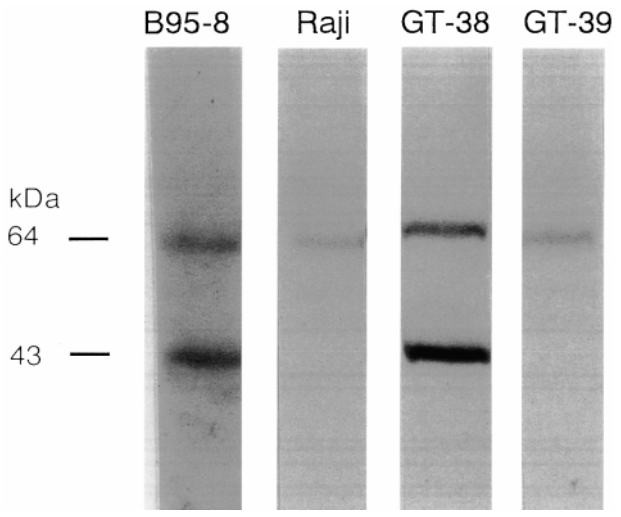

Fig. 7. Immunoblots of LMP1. Postnuclear supernatants of GT38, GT39, B95-8, and Raji cells were reacted with anti-LMP1 by using the same procedure as described in the legend to Fig. 4. LMP1 was detected as two bands of 64 and 43 kDa.

immunocytochemistry and by immunoblotting. While the two lymphocyte markers were not detected (Fig. 3), both cell lines were positive for cytokeratin (Figs. 4, 5a and 5b), suggesting they were of epithelial origin.

The presence of EBV genome and its expression EBV genome was detected in virtually all cells of GT38 and GT39 as shown by *in situ* hybridization using a DNA probe specific to the *Bam*HI-W fragment of EBV genome (Fig. 5f). It appeared as several small spots in the nucleus or cytoplasm. Immunocytochemistry revealed that subpopulations of both cell lines expressed LMP1 (Fig. 5, c and d), and EBNA1; moreover GT38 was EBNA2-positive. LMP1 and EBNA1 were positive in approximately 10% of GT38 cells (Fig. 6), and EBNA2 in 2% (Fig. 5e). LMP1 showed patchy distribution on the cell surface, and was localized in the cytoplasm as well. The expression of LMP1 was confirmed in both GT38 and GT39 cells by immunoblotting (Fig. 7). GT38 showed two different LMP1 bands, of 43 kDa and 64 kDa. Only the 64 kDa band was observed in GT39 cells. B95-8 showed two (43 kDa, 64 kDa) bands which correspond to those of the GT38, whereas only one band, 64 kDa, was detected in Raji cells.

In spite of the presence of EBV genome and its expression, viral particles have not so far been found by electronmicroscopy in either G38 or G39 cells.

DISCUSSION

We have established two EBV-positive epithelial cell lines (GT38, GT39) from human gastric cells. This is the first report of the EBV-associated human gastric cell lines, although some human gastric cell lines have already been described.[23, 24)]

We can not determine whether the GT38 cell line was established from a normal gastric epithelial cell or from a neoplastic cell *in situ*. Since GT38 cells were isolated from a patient bearing an advanced gastric carcinoma accompanied with EBV, it is likely that neoplastic cells may have invaded the non-cancerous portion of the stomach and that the GT38 cell line was derived from a neoplastic gastric epithelial cell. This idea is supported by the observation that GT38, but not GT39 cells lacked the property of contact inhibition in confluent cultures. Further, GT38 cells develop into a tumor when transplanted into a nude mouse (unpublished data).

Both cell lines carried the EBV genome and expressed LMP1 and EBNA1. However, the expression pattern of EBV-latent infection proteins in these cell lines does not fit in with those of most gastric carcinomas *in situ* reported so far. LMP is not expressed in EBV-associated gastric carcinomas,[15, 16, 25, 26)] except in a few cases.[6, 17, 27)] Our two cell lines revealed a patchy distribution of LMP1, as seen in other types of cell lines,[28, 29)] although the expression of LMP1 was relatively low in GT39 cells. It has been reported that the expression of LMP is tightly regulated by cellular factor(s),[30)] and that LMP is not expressed in EBV-infected epithelial cell lines, except after addition of differentiating agents.[31)] The difference in LMP expression between gastric carcinomas and gastric cell lines reported here may reflect a difference in the cellular differentiation or in the physiological state. GT38 cells, unlike GT39 cells, revealed two different LMP1 bands on immunoblotting. LMP1 has a short half-life in some cell lines.[32, 33)] The accumulation of LMP1 in the cytoplasm as observed by immunocytochemistry presumably reflects the rapid internalization and turnover of LMP1 in GT38 cells. The lower-molecular-weight bands of LMP1 in GT38 and in B95-8 may be due to a truncated form of the protein expressed in a lytic cycle of EBV infection, as has been found in other cells.[32)]

EBV genome and its expression have been detected in a number of gastric carcinomas, of which at least some presumably developed from a gastric epithelial cell which had been infected by EBV and had undergone neoplastic transformation.[15)] However, the precise role of EBV in gastric carcinogenesis is poorly understood. The two cell lines established here could provide a useful model for analyzing the molecular mechanisms of neoplastic transformation by EBV in gastric epithelial cells.

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