

# An Epstein–Barr virus early protein induces cell fusion

(nasopharyngeal carcinoma/biological properties of Epstein–Barr virus)

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**ABSTRACT** Superinfection of Raji cells with Epstein–Barr virus (EBV) leads to syncytium formation. Studies using metabolic inhibitors and amino acid analogues suggest that the fusion-inducing factor belongs to the early group of virus-specified proteins. Induction of early EBV protein synthesis in Raji cells by using various chemicals also leads to syncytium formation, indicating that the fusion process is not caused by a virion membrane protein introduced into the cells upon infection. The relevance of these findings to the association of EBV with carcinoma of the nasopharynx is discussed.

Previous studies (1) indicated that superinfection of Raji cells with Epstein–Barr virus (EBV) derived from P3HR1 cells leads to the formation of polykaryocytes. Moreover, it was demonstrated that superinfected Raji cells could fuse to cells lacking receptors for the virus. Virus treated with neutralizing anti-serum or inactivated with UV failed to induce cell fusion, indicating that viable virus is required for this process. Furthermore treatment of infected cells with sodium azide, which prevents virus penetration, or cycloheximide, which prevents all protein synthesis, precluded the formation of polykaryocytes. These data suggested that a viral protein synthesized after superinfection was responsible for the fusion potential of EBV. However, these experiments did not exclude the possibility that a virus membrane protein, which could have been integrated into the cellular membrane during penetration of the virus, caused fusion and that the various treatments we used prevented the necessary processing of this protein (e.g., proteolytic cleavage) that would enable it to induce cell fusion. In the series of experiments described in this communication we show that the synthesis of viral proteins in superinfected cells is essential for fusion and that the viral genes, inducible in Raji cells by treatment with chemicals such as iododeoxyuridine (2) or phorbol ester (3), code for the necessary functions that lead to syncytia formation.

## MATERIALS AND METHODS

**Tissue Culture and Virus Preparation.** Raji cells were grown in plastic tissue culture flasks in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal calf serum. Cultures were split 1:4 when the cell density reached  $10^6$  cells per ml. P3HR1 cells, which release infectious EBV, were grown as 200-ml cultures in 32-oz (950-ml) glass prescription bottles in RPMI 1640 medium. After subculture the cells were allowed to stand at 34°C for 10 days, the culture supernatant was clarified by centrifugation at  $4000 \times g$  for 15 min, and the virus was sedimented by centrifugation at  $27,000 \times g$  for 2 hr at 4°C. The virus pellet was resuspended in tissue culture medium and stored over liquid nitrogen.

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**Preparation of Lymphoblastoid Cell Monolayers.** Plastic plates (Flow Laboratories) 35 mm in diameter were incubated with 2.5% (vol/vol) glutaraldehyde at room temperature for 2 hr and then thoroughly washed with sterile phosphate-buffered saline; 1 ml of diluted anti-lymphocyte globulin (ALG) (16  $\mu\text{g}/\text{ml}$ ) was added and the plates were incubated overnight at 4°C. The unadsorbed ALG was removed with a pipette and the plates were then rinsed with phosphate-buffered saline several times to remove remaining unadsorbed ALG. Cells were added to the coated plates and after 1–2 hr at 37°C the nonadherent cells were removed by rinsing the plates with tissue culture medium. After refeeding the cultures were ready for use.

**Fluorescent Antibody Staining of Fixed Cells.** Smears of suspension cultures or monolayers of adherent cells were dried under a stream of cold air and then fixed for 10 min at –20°C in acetone when glass surfaces were used. For plastic surfaces we used petroleum benzene (bp 50–75°C) (4). The smears were stained either directly by using serum conjugated with fluorescein isothiocyanate or indirectly by using unconjugated serum followed by fluorescein isothiocyanate-conjugated anti-human IgG. Stained cells were embedded in semipermanent mounting medium (5) and examined in a Zeiss epi-illuminated fluorescence microscope.

**Induction of Early Antigen (EA) Synthesis in Raji Cells.** Raji cells were synchronized by using a double thymidine block and EA synthesis was induced according to the procedures described (2) by adding iododeoxyuridine (20  $\mu\text{g}/\text{ml}$ ), hypoxanthine (14  $\mu\text{g}/\text{ml}$ ), and aminopterin (1  $\mu\text{g}/\text{ml}$ ) 1 hr after the onset of S phase. Other inducing agents—sodium butyrate at 3 mM (6), phorbol ester (3), or the methylation inhibitor cycloleucine at 2 mM—were added directly after the release of the thymidine block. In experiments in which induced cells were treated with inhibitors or amino acid analogues the drugs were added at the same time as the EA-inducing reagents.

## RESULTS

Superinfection of Raji cells with P3HR1 EBV leads to a lytic cycle of viral expression. In a number of studies we (7–9) and others (10) have described an ordered synthesis of virally induced proteins within superinfected Raji cells. Furthermore, we were able to divide the replicative cycle of EBV into several phases by the use of a number of metabolic inhibitors, these data are summarized in Fig. 1. Fig. 2 shows the appearance of Raji cells superinfected and immobilized in the presence of phosphonoacetic acid (*Lower Left*) and canavanine in the presence of arginine (*Lower Right*). Reference to Fig. 1 shows that in the presence of these inhibitors a limited expression of the spectrum of EBV-induced proteins occurs and these cells are capable of undergoing fusion. In a previous study we have shown (1) that addition of inactivated virus to Raji cells failed to induce fusion.

Abbreviations: EBV, Epstein–Barr virus; ALG, anti-lymphocyte globulin; EA, early antigen.

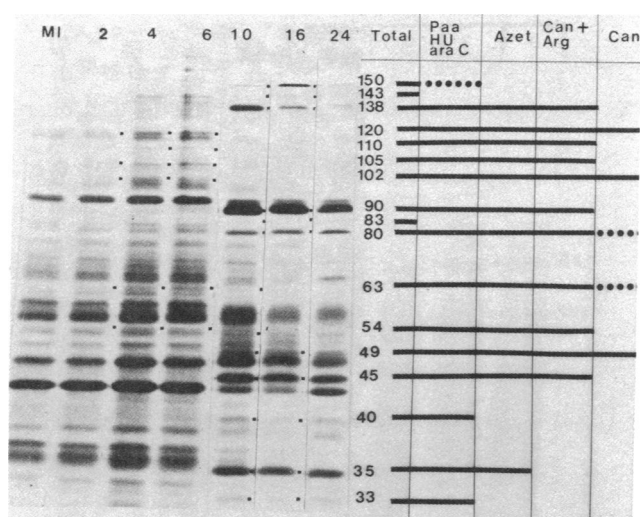


FIG. 1. Synthesis of proteins in EBV-superinfected Raji cells. Raji cells were superinfected with EBV derived from P3HR1 cultures and pulse labeled with [ $^{35}$ S]methionine at various times after infection (the starting times, in hr, for the 30-min pulses are given above the tracks; MI is the profile of proteins observed in uninfected Raji cells). The approximate molecular masses are given in kilodaltons at the side of the gel. The infected labeled cells were disrupted in sample buffer [50 mM Tris-HCl (pH 7.0)/2% sodium dodecyl sulfate/5% (vol/vol) 2-mercaptoethanol/30% sucrose] and heated to 100°C for 5 min. The samples were resolved on 10% polyacrylamide gels crosslinked with 0.26% diallyltartardiamide, using a stacking gel containing 3% acrylamide, 0.08% diallyltartardiamide, and a discontinuous buffer system. The resolved stained gels were dried and autoradiograms were prepared, using LKB 2208 Ultrofilm. In a series of similar experiments various metabolic inhibitors were added at the time of infection and the cells were labeled at 12–16 hr after infection. Solid lines on the right indicate protein synthesis in the presence of the inhibitor named in the column heading. PAA, phosphonoacetic acid at 200  $\mu$ g/ml; HU, hydroxyurea at 4 mg/ml; ara C, cytosine arabinonucleoside at 50  $\mu$ g/ml; Azet, azetidine at 500  $\mu$ g/ml; Can + Arg, canavanine at 500  $\mu$ g/ml in normal arginine-containing medium; and Can, canavanine at 500  $\mu$ g/ml in arginine-free medium. The column headed "Total" indicates the spectrum of proteins seen in uninhibited infected Raji cells. The 150-kilodalton protein is synthesized in cultures treated with phosphonoacetic acid or hydroxyurea but not in cultures treated with cytosine arabinonucleoside; the 80- and 63-kilodalton proteins can be identified in extracts of canavanine-treated cells only after immunoprecipitation because they are relatively poorly synthesized under these conditions.

Because addition of inhibitors to uninfected cultures did not result in the formation of polykaryocytes, it can be concluded that at least a partial expression of EBV is required for fusion to occur. Although it cannot be said that the fusion-inducing protein is one of those visualized in the gel profiles presented in Fig. 1 (because the protein might comigrate with a host protein or be present in minor amounts and not be visualized), it may be concluded that the protein has the same properties as the proteins seen in the early profiles and in the inhibitor-treated samples.

Fluorescent antibody staining of the cultures revealed that the cells were synthesizing EA but not viral capsid antigen. It has been shown that chemicals (2, 3, 6) and other agents (11, 12) can be used to induce EA synthesis in Raji cells, and it was necessary to test whether the proteins responsible for fusion are synthesized in induced or only in superinfected Raji cells. Previous experiments (11) have shown that Raji cells immobilized in confluent monolayers can no longer be induced to synthesize EA. Because confluency is necessary for the observation of cell fusion we overcame this problem by synchronizing the Raji cells

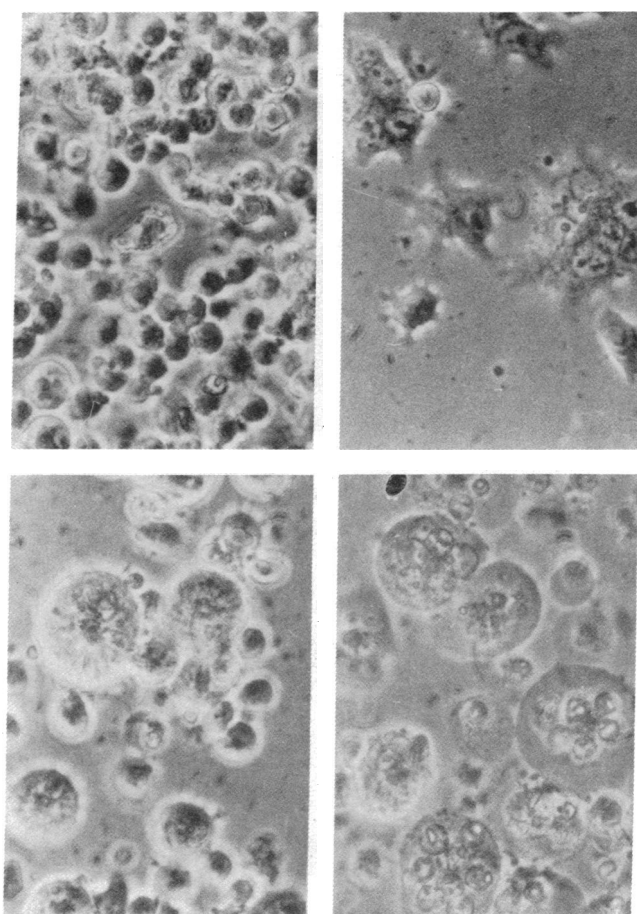


FIG. 2. Formation of polykaryocytes in cultures of immobilized superinfected Raji cells. Raji cells were superinfected or mock infected. To the culture shown in *Lower Left*, phosphonoacetic acid was added at the time of infection; canavanine was added to the culture shown *Lower Right* at infection. After the virus had absorbed to the cells, the cells were applied to the ALG-coated plates and allowed to form monolayers. Uninfected cells do not form polykaryocytes (*Upper Left*), whereas after 4 hr the superinfected cultures contained fused cells (*Upper Right*). The treatment with either phosphonoacetic acid or canavanine (*Lower Left* and *Right*) did not prevent the formation of polykaryocytes. ( $\times 650$ .)

in suspension; these free-floating cells were then induced to synthesize EA by the addition of various inducing agents, including iododeoxyuridine (2), phorbol ester (3), sodium butyrate (6), or the methylation inhibitor cycloleucine. After the Raji cells had entered into a cycle of EA synthesis they were applied to ALG-coated plates and allowed to form partially confluent monolayers. In many places the cells had settled out as islands and thus the fusion process could occur if the required proteins were present. Twelve hours after the cells were immobilized they were prepared for fluorescent staining or examined and photographed with a phase-contrast photomicroscope. Under conditions in which the cells synthesize EA the cells were capable of undergoing fusion (representative cultures are shown in Fig. 3 *Upper Right* and *Lower Left* and *Right*). Immobilized, synchronized Raji cells at high density (when they do not synthesize EA in the absence of inducers) did not lead to polykaryocyte formation (Fig. 3 *Upper Left*). Treatment of the induced cells with the various inhibitors described in Fig. 1 (we did not test cytosine arabinonucleoside or hydroxyurea) did not prevent the formation of polykaryocytes, once again suggesting that a subset of very early proteins may be involved in the fusion process.

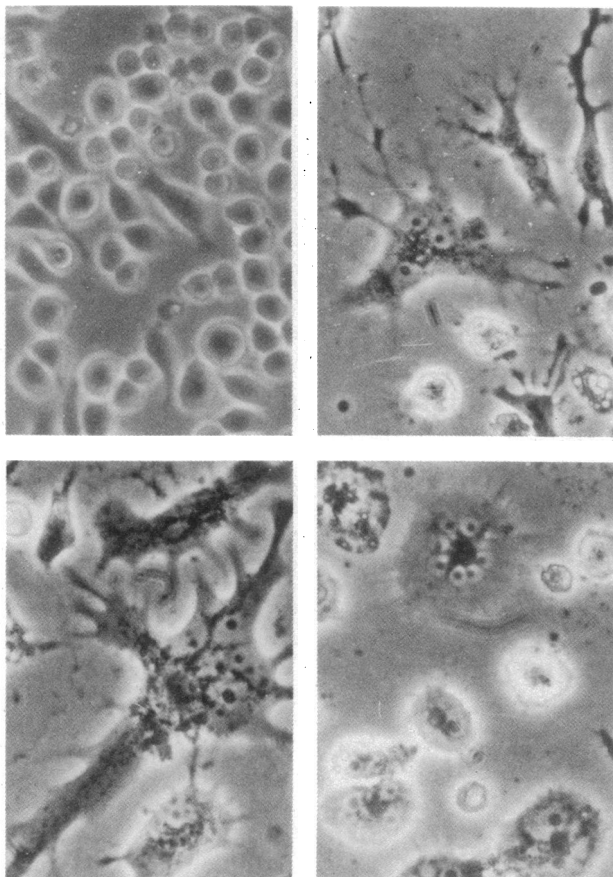


FIG. 3. Formation of polykaryocytes in cultures of induced immobilized Raji cells. Raji cells were induced to synthesize EA as described in the text, the cells were applied to ALG-coated Petri plates and allowed to attach, and 12 hr later the cultures were photographed in phase contrast. (Upper Left) uninduced cells; (Upper Right) cycloheximide present; (Lower Left) sodium butyrate present; (Lower Right) 12-*O*-tetradecanoylphorbol 13-acetate present. ( $\times 650$ .)

Control experiments with the EBV-negative cell line BJA showed that the added chemicals did not cause fusion by themselves. The morphology of immobilized cells seems to be dependent upon the length of time for which the cells have been attached to the surface. A few hours after immobilization the cells are rounded and appear as normal lymphoblasts (Fig. 2 Upper Left); at later times the cells spread out and present an epithelioid or fibroblastoid morphology (Fig. 3 Upper Left). The infected or induced cells are also capable of adopting a flattened morphology even under conditions when fusion takes place. Certain metabolic inhibitors (e.g., canavanine; Fig. 2 Lower Right) or inducers (e.g., phorbol ester; Fig. 3 Lower Right) seem to prevent the adoption of a flattened morphology; this is likely to be due to interference with normal cellular processes in addition to the effects which these agents have on viral antigen synthesis. The reasons for the adoption of this flattened appearance and the formation of the fine processes (a good example can be seen in Fig. 3 Upper Right) are not clear, but this morphology could be due to the interaction of the cell membrane with the antibodies used to immobilize the cells.

Previous studies by us (9) have suggested that treatment of uninfected Raji cells with cycloheximide for several hours followed by its removal leads to a very limited expression of the endogenous EBV genome. For this reason we decided to see if such a treatment would lead to polykaryocyte formation. As shown in Fig. 4, treatment of Raji cells with cycloheximide for

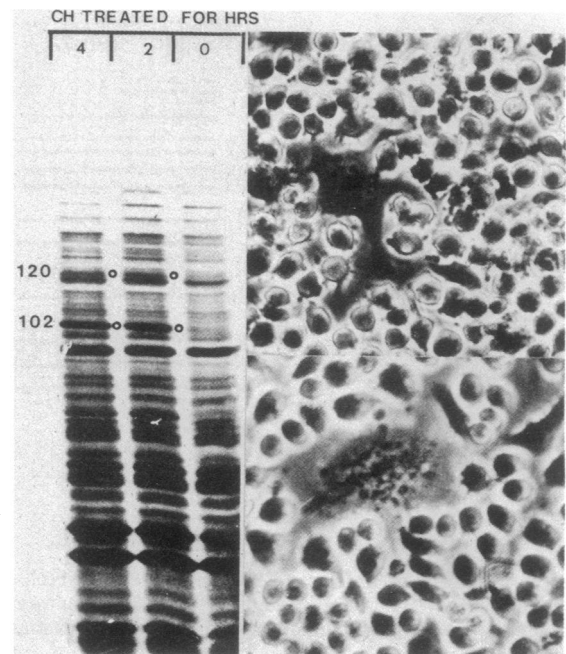


FIG. 4. Induction of polykaryocyte formation in Raji cells by cycloheximide (CH) treatment. Raji cells were treated with cycloheximide for 0, 2, or 4 hr, the drug was then washed from the cells, and the cells were labeled for 30 min with [ $^{35}$ S]methionine. Cell extracts were prepared and analyzed as described in the legend to Fig. 1. The two cycloheximide-inducible proteins (which are also induced by superinfection; see Fig. 1) are marked with open circles. Adjacent to the cell profiles are phase-contrast photomicrographs of cultures of immobilized Raji cells that were exposed to cycloheximide for 3 hr; after this exposure the drug was washed away and the cultures were incubated for 2 hr (Upper) or 18 hr (Lower). Note the formation of polykaryocytes and the increase in size after longer incubation. ( $\times 650$ .)

2–4 hr followed by its removal leads to the synthesis of two new proteins with molecular masses of 120,000 and 102,000 daltons, respectively. After immobilization of such cultures local polykaryocyte formation can be observed. The number and size of the polykaryocytes, in comparison to those seen in Figs. 2 and 3, is small and may indicate that occasionally cells express additional proteins which induce fusion. The amounts of the two cycloheximide-inducible proteins seen on the gels suggest that most of the cells in the treated cultures must be synthesizing them. If they were responsible for the fusion, then polykaryocyte formation should be observed more often in the treated cultures. Staining of these cultures with antisera against EA failed to reveal the presence of this antigen within large numbers of cells; however, a few weakly positive cells were seen. Further studies using immunoprecipitation should be carried out to investigate this point.

## DISCUSSION

The data presented indicate that the EBV-mediated cell fusion occurs from within and is not due to a virion membrane protein as is the case with the paramyxovirus group. Further studies will be necessary to identify the protein responsible for the fusion process, although the results of the experiments with inhibitors narrow the number of possible candidates to a group of proteins synthesized very early in the viral replicative cycle. This observation is of importance for processes *in vivo*, where antibodies in the serum would probably attack and destroy EBV-carrying lymphocytes before they entered into the later phases of the viral replicative cycle.

EBV genomes have been found in epithelial cells of nasopharyngeal carcinoma by several unrelated techniques: *in situ* hybridization (13, 14), cell separation (15), passage of cells through nude mice (16), and identification of EBV-specified nuclear antigen in touch preparations of tumor tissue (13, 17). These observations present an enigma because attempts to infect human nontumorous cells other than B lymphocytes *in vitro* have failed (18). Also, negative results from binding studies using radiolabeled EBV (unpublished data) do not support the presence of EBV receptors on human untransformed nasopharyngeal epithelial cells. One report (19) indicated that explants of normal human nasopharyngeal tissue could be stimulated into rapid outgrowth by addition of EBV and could be cultured for periods of up to 1 year; however, no EBV "footprints" could be found in the infected cells. It is of interest to note that the infected explants, in contrast to the uninfected ones, contained multinucleate giant cells and cells with abnormal nuclei that could have arisen from a fusion process. Our data describing EBV-induced cell fusion provide a possible explanation for the presence of EBV in EBV receptor-negative cells.

It cannot be ruled out that lymphocytes present in the specimens were the primary targets for EBV and after entering a lytic cycle they transferred their viral genomes to the epithelial cells via a partial or complete fusion process. The exceptionally close contact between the immobilized cells within the monolayers prepared for these studies allowed us to detect the fusion-inducing ability of Raji cells expressing early viral functions. One might speculate that similar events could take place *in vivo*. The exceptionally close contact between lymphocytes and epithelial cells found in the Waldeyer's ring of the throat (20) may provide the necessary conditions of intimate cell-to-cell contact for cell fusion. A lymphocyte carrying EBV and expressing the fusion-inducing proteins in close contact with an epithelial cell could fuse to it and so transfer its load of EBV genomes along with other cellular components. The infectability of epithelial cells from tumors (21) may reflect an alteration in the biological properties of these cells; it is conceivable that among the materials transferred from the lymphocytes during a fusion event may be not only EBV genomes but also something conferring the ability to carry the EBV receptors on the cell surface. Under normal conditions one would expect that fusion is not a frequent event because the induction of EBV in lymphocytes seems to be a rare event [a consequence of this is that antibodies to EA disappear rather rapidly after acute disease (22)], thus one would expect that EBV-containing epithelial cells would be present in very small numbers and would finally, after years of latency, give rise to clinically manifested malignant growth.

The geographical distribution of EBV-associated nasopharyngeal carcinoma could reflect the distribution of various strains of EBV differing in their ability to induce cell fusion. Alternatively, it may reflect the presence of agents within high-risk areas with the potential to induce the synthesis of early EBV proteins in latently infected lymphocytes and so increase the probability of fusion events. Indeed, recent studies (23) demonstrate that the high incidence area of nasopharyngeal carcinoma in southern China follows almost exactly the geographical distribution of *Croton tiglium* Linnaeus, a plant containing phorbol esters, which is used in traditional Chinese herbal remedies. The apparent correlation between the presence of phorbol esters in the environment and the occurrence of nasopharyngeal carcinoma might at first seem rather puzzling because these substances lead to expression of viral antigens and hence cell death. Synthesis of early membrane antigens may be

enough for the immune system to eliminate these lymphocytes. However, if latently infected lymphocytes were induced to synthesize immediate early EBV proteins that were nonantigenic (9), they could then fuse to the epithelial cells in Waldeyer's ring, transferring their EBV genomes.

Previous studies (24) have shown that the inducibility of EBV is highly dependent upon the type of cell within which it resides and, furthermore (25), it was shown that a human epithelioid cell line (D 98 variant of HeLa) contained a factor that repressed the expression of EBV in superinfected nonproducer cells (F 256).

Thus EBV genomes from induced lymphoid cells may be transferred by fusion to epithelial cells devoid of EBV receptors, where cell specific factors could repress the further expression of the viral genomes, allowing the manifestation of the immortalizing potential of EBV.

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