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Induction by sodium butyrate of cytomegalovirus replication in human endothelial cells

Brief Report

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Summary. Cultured endothelial cells are shown to be induced in regard to permissiveness to human cytomegalovirus by temporary treatment postinfection with sodium butyrate (1–2 mM). Drug-treated cells are demonstrated to exhibit expression of immediate early and early viral antigens, synthesis of viral DNA and viral structural glycoprotein B. Progeny virus could be visualized by electron microscopy.

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Cytomegalovirus (HCMV) is known to exhibit not only species specificity but also marked cell type restriction [14, 24]. For efficient propagation in vitro human fibroblast cultures are generally used [12]. However, a number of reports have documented in vitro replication also in cultured epithelial cells [24]. Thus in addition to fibroblastic phenotype other parameters, such as ploidy and extent of differentiation also seem to determine cellular susceptibility to HCMV. With regard to cultured endothelial cells reported attempts to propagate HCMV have been unsuccessful [6]. On the other hand, HCMV has been identified in several instances in endothelial cells in vivo [5].

In order to examine their in vitro susceptibility endothelial cell cultures were prepared from human umbilical vein and cultured as described previously [11]. Studies were performed on primary as well as cultures between the first and forth passage. The cultures were devoid of conspicuous contamination by fibroblasts as judged by their microscopic morphology.

Our initial experiments revealed that cultures of human endothelial cells are indeed nonpermissive for HCMV (strain Towne [7]) at a low multiplicity of infection (moi 1). Even at high moi of 10 a maximum of 2 to 5 percent of the

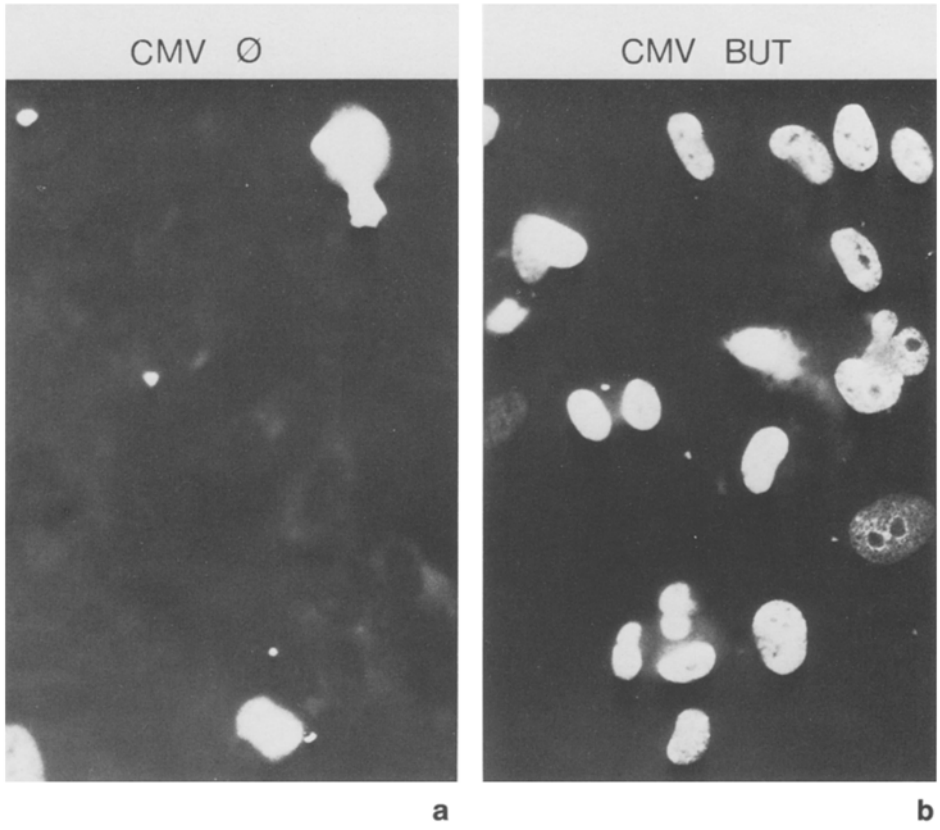

Fig. 1. HCMV-specific early nuclear antigen in **a** untreated and **b** butyrate-treated (1 mM) human endothelial cells visualized 72 hours p.i. by indirect immunofluorescence using commercial monoclonal antibody against a viral 52 kd early polypeptide (Dupont, Bad Nauheim, Federal Republic of Germany) and FITC-labelled rabbit anti-mouse-IgG (Dakopatts, Hamburg, Federal Republic of Germany). Cells were seeded on gelatine-coated cover slips in Leighton tubes and grown to subconfluency with medium 199 plus 10 percent human serum which was replaced by medium containing fetal calf serum 24 hours prior to adsorption of HCMV (moi 10) for 1 hour at 37 °C. Subsequently cultures were maintained in culture medium without or with butyrate until 72 hours p.i. when they were fixed in cold acetone and processed for immunofluorescence [19]. Fluorescence microscopy was performed with the UV-epi-illumination- and camera equipment of a Zeiss microscope at a magnification of 1 : 400

cells exhibited viral antigen as examined 72 hours postinfection (p.i.) by indirect immunofluorescence [19] with a monoclonal antibody (mab) recognizing a virus-specific early nuclear polypeptide of 52 kilodaltons (kd; Fig. 1a) [8]. When sodium butyrate, at concentrations between 1–2 mM, was present in the culture medium p.i., infected endothelial monolayers developed cytopathic changes which were comparable to the typical cytopathic effect induced by HCMV in fibroblast cells [1]. Indirect immunofluorescence at 72 hours p.i. showed that butyrate induced early viral antigen in the nuclei of the majority of the cells (60–90 percent; Fig. 1b). Essentially the identical result was obtained when

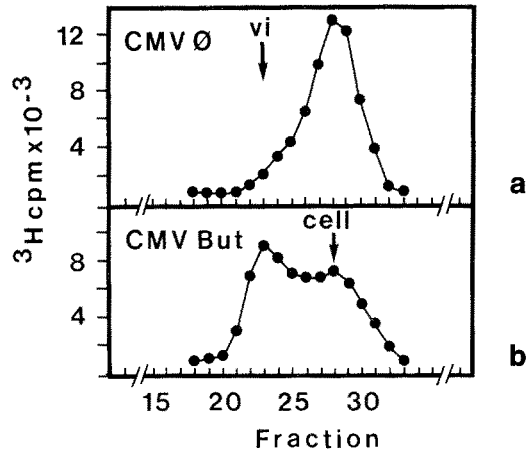

Fig. 2. Radioactivity profiles of neutral CsCl gradients run with DNA (3 μg per gradient) from **a** untreated and **b** butyrate-treated (see text) HCMV-infected subconfluent endothelial cells grown in gelatine-coated 25 cm^2 plastic flasks and pulse labelled from 72–96 hours p.i. with ^{3}H-thymidine (10 μCi/ml; specific radioactivity 26 Ci/mmol) prior to extraction of DNA and isopycnic centrifugation in neutral CsCl [12]. *vi* Position of viral DNA = 1.716 g/ml; *cell* ^{14}C-labelled DNA from human fibroblasts which was included in the gradients as an internal marker. Centrifugation conditions and determination of acid-insoluble radioactivity in individual fractions were performed as described previously [12]

immunofluorescence was performed with mab p63-27 (generously provided by Dr. W. Britt, Birmingham, Alabama, U.S.A.) which recognizes nuclear immediate early (IE) antigen of 68 kd. This observation supports the view that HCMV is normally blocked in most of the cells prior to IE antigen expression. Induction of IE and early antigen expression was also observed following only temporary presence of the drug: Removal of the drug at 12 hours p.i. did not impair the extent of antigen development at 72 hours p.i. On the other hand, pretreatment of the cultures prior to infection did not promote increased antigen expression.

In order to examine whether induction by the drug also concerned viral structural components, infected endothelial cultures (0.8–1 × 10^6 cells per subconfluent monolayer in 25 cm^2 gelatine-coated plastic flasks; moi 10) without and with temporary butyrate treatment (1–12 hours p.i.) were pulse labelled with ^{3}H-thymidine from 48–72 hours p.i. prior to extraction of DNA and isopycnic centrifugation in CsCl [12]. The radioactivity profiles in Fig. 2 document that viral DNA synthesis was several fold induced by the drug. Continuous presence of butyrate p.i. and during the labelling period clearly reduced incorporation into viral DNA.

Glycoprotein B (gB) of HCMV has been shown to be a component of mature virions [3]. Drug-induced permissiveness should result in enhanced expression of structural viral proteins and thus of gB. To address this projection subconfluent HCMV-infected endothelial cultures (see above; moi of 10 of cell-free HCMV) drug-treated for 12 hours p.i. were harvested at (72 to) 96 hours p.i.

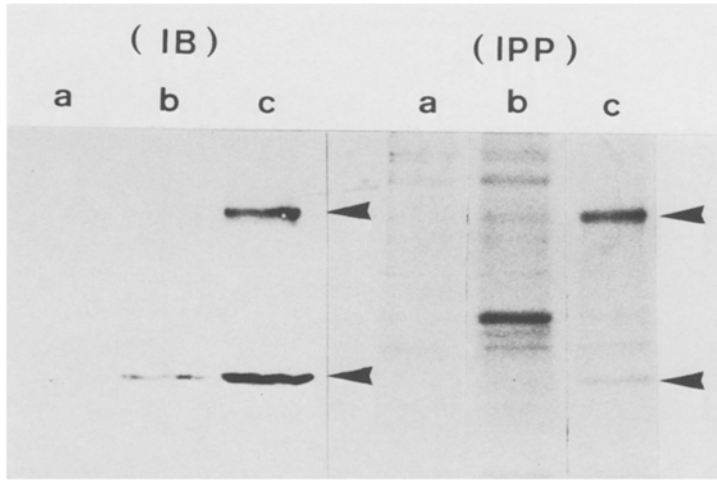

Fig. 3. Immunoblotting (*IB*) and immunoprecipitation (*IPP*) with HCMV-gB-specific mab 27-156 of extracts from noninfected (*a*) as well as untreated (*b*) and butyrate-treated (*c*) (see text) HCMV-infected subconfluent endothelial cells grown in 25 cm^2 plastic flasks and pulse labelled with ^{35}S-methionine (20 µCi/ml; specific radioactivity 3,000 Ci/mmol) from 93–96 hours p.i. prior to preparation of total cellular extracts [12]. Equivalent amounts of the different extracts were used for immunoblotting and immunoprecipitation (corresponding to 50 µg of protein in each sample). Immunoprecipitation was performed with protein A-sepharose CL4B beads (Sigma, Dreisenhofen, Federal Republic of Germany) coated with rabbit anti-mouse immunoglobulin (Dakopatts, Hamburg, Federal Republic of Germany) according to Kari et al. [13] followed by electrophoretic separation of the precipitates [12] and fluorography [2]. ► Positions of the gB precursor of 130 kd and gB (58 kd)

and subjected to electrophoretic separation and immunoblotting by standard techniques described previously [12, 18] using monoclonal HCMV-gB-specific antibody (mab 27-156 generously donated by W. Britt, Birmingham, Ala., U.S.A.). Mock-infected and HCMV-infected parallel cultures not exposed to butyrate served as controls. Using equivalent amounts of cellular extracts from controls and butyrate-treated cultures for this analysis, considerably more gB was stained in blots of extracts from drug-induced cells (Fig. 3, IB). The notion that synthesis of gB was indeed induced by butyrate was further examined by immunoprecipitation [13] of gB with mab 27-156 from extracts of an identical set of cultures pulse labelled with ^{35}S-methionine for 3 hours at 4 days p.i. (Fig. 3, IPP). Subsequent fluorography [2] of the electrophoretically separated precipitates [12] indeed revealed that butyrate significantly enhanced incorporation of methionine into the precursor of gB of 140 kd [3] whereas comparatively little radioactivity accumulated in product gB of 58 kd verifying that cleavage of the precursor is a relatively slow process [3] (Fig. 3, IPP). Furthermore, precipitation of a labelled polypeptide of about 68 kd was consistently observed with extracts from untreated infected cells. It is not clear at present whether this product represents an aberrantly processed form of gB or a co-precipitated host protein. Again, when butyrate-treatment p.i. was continued

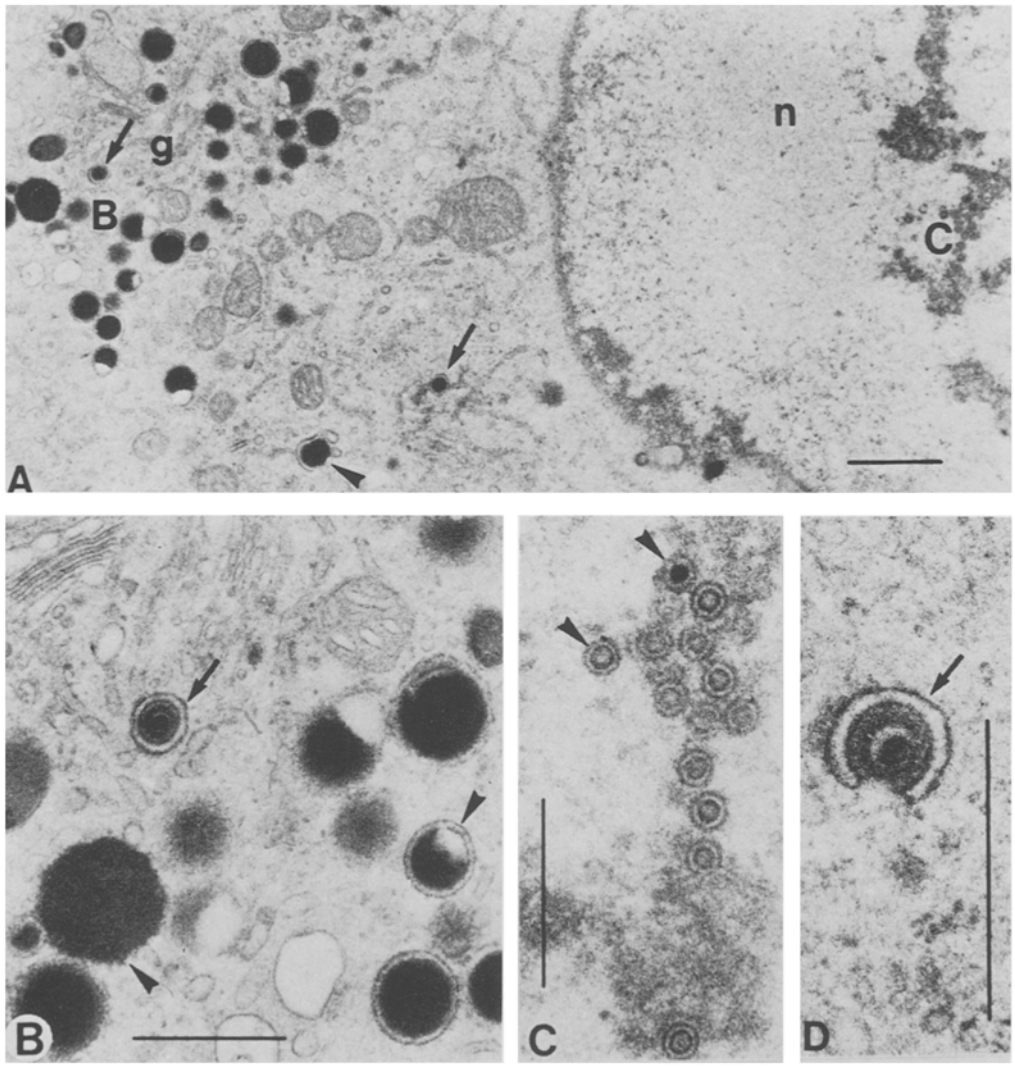

Fig. 4. Electron micrographs of representative HCMV-infected endothelial cell from human umbilical vein exposed to sodium butyrate for 12 hours p.i. At 96 hours p.i. endothelial monolayers were fixed with 2 percent glutaraldehyde in phosphate buffered salt solution and processed for transmission electron microscopy [4]. **A** Overview of parts of the nucleus (*n*) and the Golgi membranes (*g*). Note budding dense body. Areas indicated in **A** were reproduced at higher magnification. **C** The nucleus contains numerous nucleocapsids with lucid as well as dense cores (►). **A**, **B**, and **C** Arrows point to virions surrounded by two unit membranes. **D** The virion (taken from a different infected cell) is in the stage of budding into a smooth surfaced membrane sac. **B** Dense bodies with and without a double membrane (►). Bars in **A** 1 μm, in **B**–**D** 0.5 μm

until harvest of the cells at (72 to) 96 hours, induction of gB was not observed (see above).

For direct visualization of virus formation cultures were temporarily exposed to butyrate as described above; at 96 hours p.i. the monolayers were fixed with glutaraldehyde and processed for transmission electron microscopy [4]. Most

of the cells exhibited nuclei with naked viral nucleocapsids, with lucid as well as dense cores (Fig. 4C). The cytoplasm of these cells also contained virions with dense cores and a double membrane (Fig. 4B). In addition, numerous dense bodies were observed which were mostly enveloped (Fig. 4A, B). Occasionally virions as well as dense bodies were seen in the stage of budding into the smooth-surfaced membranes of the Golgi-area (Fig. 4D). As contamination of the cultures by fibroblasts was not apparent (see above) this observation clearly demonstrates productive infection of endothelial cells by HCMV.

Induction by butyrate of production of infectious viral progeny was further verified by comparative titration by end point dilution [12] of culture medium taken at various times p.i. from untreated and temporarily drug-treated (see above) infected endothelial cultures. As compared with infected fibroblasts which yielded approximately 10^5–10^6 infectious units (IU)/ml under comparable conditions only low titers were obtained in endothelial cells. HCMV titers of 10^2 IU/ml at 24 hours p.i. were followed by an increase at 72 hours p.i. when the titers of drug-treated cells exceeded those of the control on the average by one order of magnitude, e.g. 10^4 IU/ml versus 10^3 IU/ml.

The effects of butyrate have been investigated in a variety of non-infected as well as virus-infected cell systems [9, 10, 19, 20]. In noninfected cells this fatty acid possibly interferes with histone acetylation and thus with DNA replication [22]; as a consequence most of the cells accumulate in G_1-phase [9]. At the same time drug-treatment may promote terminal cellular differentiation [17]. It is not clear whether these cellular reactions are related to the effects of butyrate on virus-infected cell systems. For Epstein-Barr-virus (EBV) infected cells it has been shown that viral DNA synthesis is induced by the drug in producer-, not, however, in nonproducer cells [20]. Distinct cellular control mechanisms with different sensitivities to butyrate were suggested to function in producer and nonproducer cells, respectively. In permissive HCMV-infected fibroblasts butyrate appears to selectively inhibit host cell glycoprotein synthesis [19].

With regard to our observations on HCMV-infected endothelial cells adsorption and penetration of infecting virus were apparently not affected by butyrate: Cellular pretreatment by butyrate and/or its presence during the adsorption period prior to temporary exposure of the cultures after infection as described above, did not result in additional effects on subsequent development of viral IE or early antigen (data not shown). As a working hypothesis we assume that in untreated cells HCMV replication is hindered prior to expression of IE antigens similarly to the situation in EBV producer lymphoblastoid cells [20]. It might further be valid for the cell system described here that butyrate, by elimination of inhibitory host cell functions, allows progression to synthesis of IE and early products. A consecutive second mechanism involved in the butyrate effect is possibly concerned with the release by drug-removal of the infected cell population from G_1-phase into S-phase, a cellular stage known to facilitate production of HCMV progeny [16, 21].

Finally it merits comment that endothelial cells exhibit polarized growth behaviour [15]. Induction of permissiveness in these cells thus opens the novel opportunity to study aspects of regulation of polarized transport [23] of HCMV-particles as well as HCMV gene products. Further analysis of this new system might not only be valuable as an experimental model for human virus-host cell interaction in this field but might also add to our understanding of the complex HCMV biology.

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