Sodium butyrate selectively inhibits host cell glycoprotein synthesis in human fibroblasts infected with cytomegalovirus

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Host cell as well as viral DNA synthesis in human fibroblasts infected with human cytomegalovirus was found to be largely resistant even to high concentrations of sodium butyrate. Likewise, production of viral progeny was reduced by 1-2 orders of magnitude but not abolished. On the other hand, the drug allowed (modified) glycosylation only of viral polypeptides whereas that of host proteins was suppressed. Immunofluorescence studies on living cells suggested that butyrate may interfere with processing and intracellular transport of virus-specific surface membrane antigens.

Unlike other members of the herpesvirus group, human cytomegalovirus (HCMV) has been shown by various experimental approaches to exhibit a pronounced host cell dependency. Thus, in contrast to that of herpes simplex virus, HCMV growth appears to be restricted in UV- or actinomycin-treated human fibroblasts (8,17). Furthermore, evidence has been presented in this context, from two laboratories using synchronized cells, for a particular S-phase dependency of HCMV multiplication (9,19). This latter finding is in line with the earlier observation of a characteristic increase in host-specific S-phase enzyme activities during the early phase of the HCMV infectious cycle (13). HCMV may in fact induce an S-phase like cellular state for provision of host enzymes essential for viral replication since arrested cultures acquire, early after infection, a glycosphingolipid pattern which is typical of S-phase fibroblasts (25).

Butyrate is known to effectively arrest cell cultures in the G1-phase of the cell cycle thus preventing cellular progression to DNA replication and cell division (12). This mechanism of action was intended to be taken advantage of here to study HCMV-host cell interaction. The experiments described show that the effect of butyrate on cellular as well as virus-specific macromolecular synthesis in permissive fibroblasts is complex. A clear inhibitory action was evident only with respect to host-specific glycoprotein synthesis. Production of infectious viral progeny was reduced but not prevented in inhibitor-treated cultures.
Materials and Methods

Cells and virus

Human foreskin fibroblasts (HFF; generously donated by Dr. B. Fleckenstein, Erlangen) were used between the 10th and 20th passage for all experiments as well as for propagation of HCMV (strain Towne; 7). Minimal essential medium (MEM, Gibco) with Earle's salt solution supplemented with 200 units of penicillin and 50 μg of refobacin/ml and 10% fetal calf serum served for cell cultivation. For experimental use HFF were grown in plastic flasks (Nunc; 75 cm² or 175 cm², corresponding to 5 x 10⁶ and 2 x 10⁷ cells) or on glass cover slips (2 x 10⁵ cells) and partially arrested by serum starvation (5, 28). For experimental infection a multiplicity of infection (MOI) of about 3 was used, which induced viral antigen in approx. 90% of the cells. Virus titers were determined by the endpoint dilution method combined with indirect immunofluorescence for viral antigen (6, 28).

Labeling, extraction and analysis of DNA

For pulse labeling of DNA, ³H-thymidine (25 Ci/mmol) was included into the culture medium (5 μCi/ml) for various time intervals postinfection prior to extraction by established methods (13, 23). The DNA content of the samples was estimated by the procedure of Giles and Myers (11). Separation of viral and cellular DNA was achieved by isopycnic centrifugation in neutral CsCl where HFF DNA bands at a density of 1.696 g/ml and HCMV DNA at 1.717 g/ml (31). Trichloroacetic acid (TCA) precipitable radioactivity of DNA was determined after transfer of aliquots to glass fiber filters (GF/C; Schleicher & Schuell, Dassel, F.R.G.) which were washed successively with 10%, 5% TCA, ethanol and ether (26) followed by counting in a toluene-based scintillation cocktail (Quickszint, Roth, Karlsruhe, F.R.G.).

Polyacrylamide gel electrophoresis (PAGE) and fluorography

Electrophoretic separation of polypeptides was performed in polyacrylamide slab gels in the presence of sodium dodecylsulfate (SDS) according to the method of Laemmli (16) as described by Gallwitz et al. (10). During radiolabeling with the tritiated sugars, ³H-galactose (spec.act. 28 Ci/mmol) and ³H-glucosamine hydrochloride (spec.act. 40 Ci/mmol) prior to PAGE cell cultures were kept in MEM in which glucose was replaced by 10 mM pyruvate (24, 30). Cell extracts and subcellular fractions for PAGE were prepared following the method of Yamada et al. (37) as published previously (36). For the estimation of protein content in the samples the method of Lowry et al. (18) was used. TCA-precipitable radioactivity of protein was determined as described above for DNA. Fluorography (2) was carried out employing Rotifluoroszint D (Roth, Karlsruhe, F.R.G.) for impregnation and Kodak SB2 films for detection of radioactivity.

Immunoblotting

For immunoblotting (34), transfer from gel slabs after PAGE to nitrocellulose sheets (BA 85, Schleicher & Schuell, Dassel, F.R.G.) was achieved by electrotransfer at 30 V and 200 mA overnight at 4°C in a chamber constructed in our laboratory (27). The transfer buffer
consisted of 20 mM Tris/HCl, pH 8.3, 150 mM glycine and 20% methanol. Indirect immunostaining of transferred viral polypeptides was performed with anti-HCMV hyperimmune serum (Biotest) at a dilution of 1:50 as the first antibody, biotinylated anti-human IgG (sheep) as the second antibody followed by incubations with streptavidin-biotinylated horseradish peroxidase complex and 4-chloro-l-naphthol as the substrate exactly according to the instructions of the producer (Amersham, U.K.).

Immunofluorescent staining of surface membrane antigens

The double staining procedure used comprised the following steps: Infected cover slip cultures were washed twice in phosphate buffered saline and incubated with F(ab')\textsubscript{2} fragments (32) of anti-HCMV IgG (Biotest) at a dilution of 1:20 at 4°C for 90 min prior to fixation with cold acetone, incubation at 37°C for 60 min with a monoclonal antibody directed to an 'early' antigen of HCMV (DiagVitro, Munich, F.R.G.) followed by staining at 37°C for 60 min with a mixture of rhodamine-labeled goat anti-human IgG and FITC-labeled rabbit anti-mouse IgG (Miles-Scientific). Control incubations with the fluorescent IgGs did not result in nonspecific binding. Fluorescence microscopy was performed with the UV-epi-illumination equipment of a Leitz microscope.

Results

Effect of butyrate on infected cell DNA synthesis and production of viral progeny

To determine the effective drug concentration for HFF cells a set of serum-starved parallel cultures (5 x 10\textsuperscript{6} cells each) were induced for DNA synthesis by serum addition or by infection with HCMV (MOI of approx. 3) in the presence of various concentrations of butyrate. Pulse labeling with \textsuperscript{3}H-thymidine (5 \textmu Ci/ml) was performed from 36-48 h postinduction, the interval of the expected DNA replication response (24, 36).

Under these conditions the effect of serum on precursor incorporation was found to be completely abolished at concentrations above 3 mM butyrate (Table 1, Expt. 1). For HCMV-infected cultures, on the other hand, induction of thymidine uptake appeared to be resistant at 3 mM and still partially resistant at the highest drug concentration used (Table 1, Expt. 2). Pretreatment of the cultures with 10 mM butyrate prior to infection did not affect this relative resistance of HCMV-induced DNA synthesis (Table 1, Expt. 3). Most of the label from untreated as well as drug-treated infected cultures banded in the position of heavy viral DNA (31) in isopycnic neutral CsCl gradients (not shown). Likewise, viral multiplication in the presence of high concentrations of butyrate was reduced by 1-2 orders of magnitude but not abolished (Fig. 1).

To examine the drug effect on virus-mediated enhancement of host-specific DNA synthesis (31), infected cultures kept under phosphonoacetic acid (PAA; 100 \textmu g/ml), which allows expression only of 'early' viral genes (14), were exposed to butyrate and pulse
Table 1. Inhibition by sodium butyrate of induction of DNA replication by HCMV or serum

<table>
<thead>
<tr>
<th>Induction by Drug concentration Precursor incorp* (%) of control</th>
<th>before infection</th>
<th>post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2% FCS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15% FCS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>15% FCS</td>
<td>-</td>
<td>1 mM</td>
</tr>
<tr>
<td>15% FCS</td>
<td>-</td>
<td>3 mM</td>
</tr>
<tr>
<td>15% FCS</td>
<td>-</td>
<td>10 mM</td>
</tr>
<tr>
<td>Expt. 2**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2% FCS</td>
<td>-</td>
<td>10 mM</td>
</tr>
<tr>
<td>HCMV</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCMV</td>
<td>-</td>
<td>1 mM</td>
</tr>
<tr>
<td>HCMV</td>
<td>-</td>
<td>3 mM</td>
</tr>
<tr>
<td>HCMV</td>
<td>-</td>
<td>10 mM</td>
</tr>
<tr>
<td>Expt. 3**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCMV</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCMV</td>
<td>10 mM</td>
<td>-</td>
</tr>
<tr>
<td>HCMV</td>
<td>10 mM</td>
<td>3 mM</td>
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<tr>
<td>HCMV</td>
<td>10 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>Expt. 4***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2% FCS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCMV</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HCMV</td>
<td>-</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

*100% corresponds to 4700 c.p.m./μg DNA in Expt. 1, 2500 c.p.m./μg DNA in Expt. 2, 44 600 c.p.m./μg DNA in Expt. 3 and 2600 c.p.m./μg DNA in Expt. 4. Labeling with 3H-thymidine (5 μCi/ml) was performed from 48-60 h except for Expt. 3, where it was from 60-72 h postinduction.

**Induction by HCMV was performed in the presence of 0.2% FCS.

***Induction by HCMV was performed in the presence of 0.2% FCS and 100 μg phosphonoacetic acid/ml.

In this experimental setup butyrate did indeed not prevent induction of host DNA synthesis in infected cultures (Table 1, Expt. 4).

Effect of butyrate on protein and glycoprotein synthesis in HCMV-infected cultures

For the analysis of protein and glycoprotein synthesis in the presence of butyrate, untreated or inhibitor-treated cultures (5 x 10^6 cells) were pulse labeled either with a mixture of 3H-leucine/3H-lysine or 3H-glucosamine/3H-galactose (2.5 μCi/ml of each) from 48-72 h.
postinfection prior to separation of polypeptides by SDS-PAGE and visualization of radioactivity by fluorography. $^3$H-amino acid incorporation by inhibitor-treated infected cultures was reduced by about 40%. However, synthesis of most of the virus-induced polypeptides remained relatively unaffected (Fig. 2, lanes b and c). Apparent effects of the inhibitor were (i) reduction of label particularly of histone H4 (Fig. 3), and (ii) enhancement of label over that in the untreated infected control in a polypeptide of about 100 kd (Fig. 2, lane c).

Inhibition of incorporation by butyrate-treated infected cultures into TCA-precipitable material, again by 40-50%, was also observed using tritiated sugars as the precursors. The subsequent further analysis revealed distinct drug-mediated alterations: of the numerous virus- and host-specific glycoproteins (21,22,27,32,33) induced by HCMV, butyrate allowed labeling of only three glycoproteins in the molecular size range of 140, 100 and 80 kd (Fig. 4Ac). These products correlated to virus-induced bands in the untreated infected sample although the intensity of label in corresponding positions differed (Fig. 4Ab). Parallel immunoblotting of the identical samples with anti-HCMV hyper-immune serum showed specific binding in the respective molecular weight range (Fig. 4B). Reduction of sugar uptake in the presence of butyrate appeared thus to reflect selective suppression of host glycopolypeptide synthesis.

**Expression of surface antigens in the presence of butyrate**

The observed influence of the inhibitor on the labeling of virus-specific glycoproteins led us to examine the expression of viral surface membrane antigens by living cell immunofluorescence. To recognize infected single cells a double staining procedure was used (see Materials and Methods). Figs. 5a and c show that in both inhibitor-treated and untreated control cultures, infected cells exhibit typical
Fig. 2. (Above, left) Fluorogram of SDS-PAGE (7-20% gradient slab gel) with comparable amounts of cell extracts from uninfected (lane a) and HCMV-infected untreated (lane b) as well as drug-treated (lane c) HFF pulse labeled with $^3$H-leucine/$^3$H-lysine (2.5 μCi/ml of each) from 48-72 h postinfection. The arrows in lane b indicate positions of virus-induced polypeptides (see Results), arrow in lane c that of an induced polypeptide of 100 kilodaltons. The numbers give molecular weights in kilodaltons (kd) for the following marker proteins: RNA polymerase, beta-chains 160 kd, alpha-chain 39 kd, bovine serum albumin 67 kd and phosphorylase B 97 kd.

HCMV-induced surface fluorescence in contrast to uninfected cells of the same culture. Nonspecific binding due to Fc-receptors was excluded (see Materials and Methods). However, butyrate treatment obviously decreased the surface membrane reactivity. On the other hand, there was no evident reduction of nuclear antigen in the same cells in comparison to untreated infected cells (Fig. 5b and d).

Discussion

HCMV has been shown to behave like the established oncogenic DNA viruses, SV40, polyoma and adenovirus (3,4,15,35), with regard to its potential to trigger a characteristic induction of host DNA
BUTYRATE EFFECT ON HCMV-INFECTED FIBROBLASTS

Fig. 4. (A) Fluorogram of SDS-PAGE with comparable amounts of cytoplasmic extracts of uninfected (lane a) and HCMV-infected untreated (lane b) as well as drug-treated (lane c) HFF pulse labelled with \(^3\)H-glucosamine/\(^3\)H-galactose (2.5 \(\mu\)Ci/ml of each) from 48-72 h postinfection and (B) immunoblotting with anti-HCMV hyperimmune serum (see Materials and Methods) of a parallel set of the identical samples. The arrows indicate corresponding positions of glycopolypeptides in (A) and blotted polypeptides recognized by the antiserum in (B). h labels the positions of polypeptides of presumable host cell specificity. The marker proteins (kd) are the same as those given in Fig. 1 except for alpha-2-macroglobulin with 170 kd.

replication concomitant with viral DNA synthesis. In the latter experimental systems butyrate has been used for elucidation of certain aspects of virus-host cell dependence. The results obtained were variable showing either (i) complete abolishment of host- as well as viral DNA synthesis in the case of polyoma virus (35); (ii) no reduction of host DNA synthesis and some impairment for viral DNA replication in the case of SV40 (3); or (iii), as for adenovirus, the drug effect in permissive differed from that in semipermissive cells where both host and viral DNA synthesis were prevented (4,15).
Fig. 5. Indirect double immunofluorescence on untreated (a,b) and drug-treated (c,d) HCMV-infected HFF. For the detection of virus-specific surface membrane antigens, living cells were reacted with anti-HCMV IgG (a,c) at 4°C prior to fixation with acetone. For subsequent demonstration of viral nuclear antigen a monoclonal antibody (b,d) was used (see Materials and Methods). Microscope magnification: 300 x.

Our studies on DNA synthesis in cytomegalovirus-infected permissive cells show resistance towards the inhibitor at concentrations which are highly effective in noninfected cells induced by serum addition. Only very high drug concentrations reduce viral but not host DNA synthesis. This latter observation implies that mechanisms different from those mediated by serum are involved in cellular induction by HCMV. The drug effect on DNA synthesis in HCMV-infected cultures compares best to that in SV40-infected cells (3). This holds true also for the production of viral progeny in the presence of butyrate (3). With respect to the latter system it has been suggested that host DNA synthesized in inhibitor-treated infected cells might be fragmented (3). This point remains to be examined for the experimental system described here.

With regard to our initial intention to dissociate host- from virus-specific synthesis by the use of butyrate, clear results were obtained during analysis of glycoprotein synthesis by carbohydrate labeling: while host-specific glycoproteins induced in untreated infected cultures (27) are largely suppressed by butyrate, glycosylation
of virus-specific polypeptides continues, resulting, however, in a different pattern of radiolabel. Considering the recent report on the polymorphism of HCMV glycoproteins due to co- and posttranslational modification (20), all of these glycopolypeptides belong immunologically to the A-class and are candidates for surface membrane antigens (27). Our results may indicate that butyrate interferes with the processing and consequently with the proper insertion of surface membrane antigens. This would explain the decrease in immunoreactivity observed during the living cell fluorescence studies. This action of the drug might prove to be a suitable experimental tool, as in the Epstein-Barr-virus system (32,33), for the study of the maturation of HCMV-specific glycoproteins.

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References