Effects of n-Butyrate on Epstein-Barr Virus-carrying Lymphoma Lines

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SUMMARY

n-Butyrate has been shown to induce Epstein-Barr virus (EBV) antigen synthesis in certain EBV-carrying lymphoma lines (Luka et al., 1979). We have studied the effect of n-butyrate on two EBV-positive Burkitt lymphoma lines by immunofluorescence and electron microscopy. In the producer line P3HR-1, the drug induced not only early antigen (EA) and virus capsid antigen (VCA) synthesis, as shown before, but also increased the number of cells containing virus particles. The transition from VCA expression to the formation of virus particles was much more effective in treated cells than in EBV antigen-producing cells of the same line. The productive cycle was associated with the development of characteristic morphological changes. In the non-producer Raji cells, n-butyrate induced EA in only a minor fraction of the cells. There were, however, clear signs of differentiation in the direction of plasma cells. Two days after the addition of n-butyrate 80% of the Raji cells could be classified as plasmablasts. After 72 h, 20% of the cells appeared as typical plasma cells.

INTRODUCTION

Human lymphoma lines that carry Epstein-Barr virus (EBV) genomes can be subdivided into virus producers and non-producers. Both types of cells express the EBV-determined nuclear antigen (EBNA) (Reedman & Klein, 1973). In producer lines a small proportion of the cells enter the virus cycle with early antigen (EA) synthesis, followed by virus DNA replication, virus capsid antigen (VCA) production and the release of virus particles (for review, see Ernberg & Klein, 1979). Non-producer cells can be induced to enter the virus cycle by halogenated pyrimidines (Gerber 1972; Hampar et al., 1974), anti-IgM (Tovey et al., 1978), corticosteroids (Magrath et al., 1979), mitomycin C (Ernberg & Moar, 1981) and certain tumour-promoting agents (zur Hausen et al., 1979).

Recently, it was shown that n-butyrate is a powerful inducer of the virus cycle in the two prototype producer lines, P3HR-1 and B95-8, and that it can also induce EA in the non-producer Raji line (Luka et al., 1979). The massive induction has facilitated the analysis of EBV-specific protein synthesis during the virus growth cycle (Kallin et al., 1979). The effects of n-butyrate on cellular and EBV-specific DNA synthesis in the P3HR-1 and the Raji line, have been recently described (Saemundsen et al., 1980).

In spite of the effective induction of the cycle by n-butyrate, increased production of biologically active virus was only occasionally seen (B. Kallin, personal communication). We have, therefore, examined the effect of n-butyrate on virus production in induced P3HR-1 cells by electron microscopy, in parallel with immunofluorescence microscopy. We have also examined the effects of n-butyrate on the non-producer Raji line.
METHODS

Cells. Two EBV-positive Burkitt lymphoma lines were used: the producer line P3HR-1 (Hinuma & Grace, 1967) and the non-producer Raji line (Pulvertaft, 1965). They were cultivated in RPMI 1640 medium containing 10% foetal calf serum, penicillin and streptomycin.

Induction. The cells were induced by n-butyrate as described by Luka et al. (1979). Cells were grown to a high density, pelleted by centrifugation and resuspended in fresh medium at 5 × 10^5 cells/ml. n-Butyrate (Merck) was added to the final concentrations, as indicated. Samples were collected for immunofluorescence and electron microscopy at 24 h intervals, up to 72 h.

Immunofluorescence microscopy. Smears of cells were air-dried and fixed in acetone at -20 °C for 5 min. EA and VCA were detected by direct immunofluorescence (Klein et al., 1972) using the F1-Esther conjugate (1:40) and Fi-Buya (1:20) respectively.

Electron microscopy. Electron microscopic examination was performed as described elsewhere (Anisimová et al., 1977). Briefly, the cells were pelleted and washed. The pellets were fixed in 3% glutaraldehyde (Serva, Heidelberg, F.R.G.) in phosphate buffer (pH 7.2) and post-fixed with 1% osmium tetroxide (IMC) in the same buffer. The preparations were embedded in Vestopal W (Serva). Ultrathin sections were contrasted with uranyl acetate and lead citrate. For observation and photography, a Philips EM 300 electron microscope was used. The percentage of cells containing EBV particles or EBV-associated morphological changes was determined from 500 to 1000 profiles. Cells exhibiting morphological changes were classified, according to the overall morphology, as either altered or disintegrated. Altered cells showed marked changes in concentration and distribution of chromatin, fragmentation and reduplication of the nuclear membrane, mitochondrial changes and formation of tubular structures. In some cells the full range of these changes was present, while others expressed only some of them. The cell membrane remained intact. In disintegrated cells, the continuity of the cell and the nuclear membrane were disrupted, and the cell contents leaked out, forming debris.

RESULTS

Morphological changes in P3HR-1 cells

Untreated P3HR-1 cells showed a typical lymphoblastoid morphology. Cells in mitosis and a small amount of degenerated cells were seen in the preparations. Approx. 0.1% of the cells in control cultures contained virus particles at any given time. This was significantly less than the proportion of VCA-positive cells. Cells containing virus particles showed characteristic morphological changes: margination of chromatin, accumulation of small dense granules in the nucleoplasm and alterations of the nuclear membrane, indicated by fragmentation and multilayered reduplication followed by disruption or lysis (Fig. 1). Mitochondria were larger, cristae and matrix were replaced by beaded electron-dense material, and virus particles were present in either altered or disintegrated cells in the form of empty capsids or nucleocapsids containing nucleoids of various morphologies (Fig. 1).

n-Butyrate-treated cells showed a marked increase in the number of cells with virus-associated changes, even by 24 to 48 h after initiation of the treatment. These changes resembled those seen in untreated cells but were more pronounced. This concerned mainly the nuclear membrane reduplication and mitochondrial changes (Fig. 2a). In addition, the cytoplasm showed frequent formation of hollow tubular structures, distributed individually or accumulated as bands of regularly arranged tubules (Fig. 2a, b). Cell disintegration was usually not observed before 48 h. Interestingly, some of the altered cells contained virus
Fig. 1. Electron micrograph of a thin section through the nucleus of a degenerating untreated P3HR-1 cell, spontaneously producing virus. The nucleoplasm exhibits characteristic EBV-induced cytopathic changes: margination of chromatin (ch), reduplication of nuclear membrane (arrows) and the accumulation of dense granules (g). Typical immature EBV particles can be seen in nuclear substance (v).

particles, while others were virus-free. Seventy-two h after induction, the morphological character of the cell population changed; the number of altered cells decreased and most cells had disintegrated.

Formation of virus particles after butyrate treatment

n-Butyrate treatment significantly increased the number of cells containing virus particles. They were found in altered cells or, particularly at later time intervals, in disintegrated cells. The morphology of the virus particles was similar in n-butyrate-treated and untreated cells. Immature particles, empty or with a central ring-shaped or dense nucleoid were most frequent. Enveloped particles were only rarely detected. They were most abundant at 72 h post-induction, together with capsids containing a dense nucleoid. The envelopes were derived from the inner layer of the nuclear membrane or from the membranes of vacuoles. Budding through the cellular membrane was not observed.

Table 1 shows the relationship between the expression of virus antigens and virus particles, and the concentration of n-butyrate. At 48 to 72 h the average percentage of cells expressing EA and VCA increased at 3 mM- and 5 mM-butyrate approx. 30- and 12-fold respectively. On the other hand, the percentage of particle-positive cells increased more than 100-fold. The influence of the lowest n-butyrate concentration was less marked. The increase in the number of particle-positive cells followed the increase in the frequency of VCA-positive cells. At 48 to 72 h post-induction, the ratio between VCA-positive and particle-positive cells was about 1.5, suggesting that virus particles were not formed in all VCA-positive cells. Interestingly, the mean value of this ratio was about 20 in control cultures, suggesting that virus assembly is
more efficient in treated cells. The number of virus particles per section area was relatively low, however, with 2 to 10 particles in morphologically altered cells and 6 to 30 particles in cellular debris.
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Fig. 3. Electron micrograph of an untreated Raji cell. Typical features of lymphoblasts may be detected: large irregular nucleus (N), cytoplasm containing few mitochondria (m) and low content of rough endoplasmic reticulum (arrows).

Table 1. Effect of various concentrations of n-butyrate on the expression of virus antigens and the number of cells containing virus particles in P3HR-1 cells*

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>1 mM</th>
<th>3 mM</th>
<th>5 mM</th>
<th>Control</th>
<th>1 mM</th>
<th>3 mM</th>
<th>5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EA+</td>
<td>VCA+</td>
<td>No. of cells containing virus particles (%)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>0.8</td>
<td>1.0</td>
<td>0.1</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>1.9</td>
<td>2.4</td>
<td>7.1</td>
<td>0.2</td>
<td>0.2</td>
<td>0.7</td>
<td>2.2</td>
<td>1.7</td>
</tr>
<tr>
<td>48</td>
<td>1.7</td>
<td>4.8</td>
<td>23.1</td>
<td>0.8</td>
<td>8.5</td>
<td>12.9</td>
<td>14.9</td>
<td></td>
</tr>
<tr>
<td>72</td>
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<td>54.5</td>
<td>54.5</td>
<td>16.9</td>
<td>28.2</td>
<td>11.8</td>
<td>12.7</td>
<td>19.5</td>
</tr>
</tbody>
</table>

* Cells were treated with n-butyrate at the final concentrations indicated, as described in Methods.
† Controls represent non-treated cell cultures grown in parallel.

Effects of n-butyrate on Raji cells

Untreated Raji cells showed the typical appearance of large, poorly differentiated lymphoblasts (Nilsson, 1978) containing rounded or lobular nuclei with 1 or 2 nucleoli. The chromatin was diffusely distributed within the nucleoplasm. The cytoplasm contained a well-developed Golgi apparatus and a weakly pronounced endoplasmic reticulum. Many ribosomes or polyribosomes were dispersed throughout the cytoplasm (Fig. 3). Cells in mitosis were frequently seen.

Induction of virus antigens in Raji cells with 3 mM-n-butyrate, was relatively ineffective and limited to the expression of EA only (Luka et al., 1979; Saemundsen et al., 1980). However, electron microscopic examination showed that a considerable proportion of the cells was induced to differentiate into plasma cells. The first signs of differentiation were observed 24 h
Fig. 4. Raji cells 48 h post-addition of n-butyrate. The cytoplasm contains extensive regular stacks of rough cisternae of the endoplasmic reticulum (arrows), well-developed mitochondria (m) and lipid vacuoles (V). A portion of the nucleus (N) is visible in the upper right-hand corner.

Fig. 5. Fragment of a Raji cell, 72 h post-induction possessing the markers of a typical plasma cell. The enormous accumulation of rough endoplasmic reticulum, with parallel arrangement of its compartments, can be seen (arrows). In the centre a lipid vacuole is visible (V).
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post-induction. Approx. 20% of the cells formed irregularly distributed new compartments of endoplasmic reticulum. The membranes of the newly synthesized endoplasmic reticulum contained relatively small numbers of ribosomes. Mitotic cells contained an increased amount of rough endoplasmic reticulum. At 48 to 72 h post-induction, a relatively large amount of rough endoplasmic reticulum was found, mainly localized in ring-like structures around the nucleus and, as a consequence, the mitochondria formed groups pressed into zones (Fig. 4), where the endoplasmic reticulum was missing. The number of mitochondria increased, lipid vacuoles appeared in the cytoplasm (Fig. 4) and the nuclei were localized either centrally or eccentrically. In some cells the chromatin was margined and clumped near the nuclear periphery. Approx. 80% of the cells contained variable amounts of rough endoplasmic reticulum, significantly more than was seen at 24 h. This type of cell may be classified as a plasmablast (Nilsson, 1978). At this stage cell division was markedly inhibited.

By 72 h post-induction, about 20% of the cells showed the morphological markers of a typical plasma cell (Nilsson, 1978). The nuclei were localized eccentrically, nuclear chromatin was margined and clumped near the nuclear membrane. A substantial amount of rough endoplasmic reticulum was present throughout the cytoplasm (Fig. 5). Endoplasmic sacs were mainly flat. Dilated sacs were rarely seen and contained varying amounts of light or dark amorphous material. We found no signs of excretion of cytoplasm and did not observe cells exhibiting EBV-associated changes at any time interval post-induction.

DISCUSSION

We have found that n-butyrate can induce not only EA and VCA synthesis in P3HR-1 cells (Luka et al., 1979), but also the formation of virus particles, associated with the development of specific morphological changes in the cells. The association of these changes with EBV production has been confirmed in numerous studies (for review, see Epstein & Achong, 1979).

There were only slight differences between morphological alterations found in virus-containing cells of n-butyrate-treated cultures and the few positive cells of untreated cultures. However, in n-butyrate-treated cells, a large amount of tubular structures were seen in the cytoplasm. The nature and the possible significance of these structures for EBV replication is not clear. These microtubules resemble those found in P3HR-1 cells induced to produce EA, VCA and EBV particles by incubation at elevated temperatures (E. Anisimová et al., unpublished results) and in cells activated with BrdUrd (Trumper et al., 1976). It is interesting to note that similar structures have also been found in the nucleus of herpes simplex virus type 2 (but not type 1)-infected cells (Murphy et al., 1967; Mori et al., 1973; Young et al., 1977).

The ratio between VCA-positive cells and particle-positive cells was close to 1.5 in treated cells as compared to 10 to 30 in untreated cells. This indicates that virus assembly was much more efficient in the induced cells. However, enveloped (infective) virions occurred relatively rarely. Naked capsids were mainly observed, either empty or containing nucleoids in various stages of maturation.

In Raji cells, where n-butyrate induced the expression of EA in only a very low proportion of cells, EBV-associated morphological changes and virus particles were never observed. On the other hand, we detected signs of differentiation, in the direction of plasma cells, in a significant proportion of the cell population. Similar results have been obtained after induction of chronic lymphocytic leukaemia cells lines with the phorbol ester TPA (Tötterman et al., 1980). It should be pointed out that differentiation-inducing effects of n-butyrate have been reported in other systems. It can induce erythroid differentiation in immature erythroleukaemic cells (Leder & Leder, 1975) and haemoglobin synthesis in human erythroleukaemic cells (Andersson et al., 1979). n-Butyrate has also been known to affect
various morphological and biochemical properties of cultured cells (for review, see Prasad & Sinha, 1976).

Butyrate induced no differentiation-related change in P3HR-1 cells. The difference might be related to the cytopathogenicity of the activated P3HR-1 virus, capable of turning off host macromolecular synthesis (Gergely et al., 1971; Nonoyama & Pagano, 1972; Steinitz et al., 1978). Alternatively, P3HR-1 might represent a clone of cells in a different stage of differentiation than that of Raji. The primary effect of n-butyrate would then be to inhibit cellular mechanism(s) that control the expression and replication of the EBV genome (Saemundsen et al., 1980) leading to a switch on of the virus growth cycle. It is interesting, in light of these findings, that many phorbol esters that effectively induce EA and VCA in P3HR-1 cells, but only EA in Raji cells (zur Hausen et al., 1979), have been shown to have differentiation-inducing capacities (Miao et al., 1978; Huberman & Callaham, 1979; Lotem & Sachs, 1979; Rovera et al., 1979; Nagasawa & Mak, 1980; Tötterman et al., 1980).

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