BUTYRATE DOES NOT INDUCE SINGLE STRAND BREAKS IN FRIEND ERYTHROLEUKEMIA CELLS OR IN 3T6 MOUSE FIBROBLASTS

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1. Introduction

Sodium butyrate has several effects on animal cells. It induces terminal differentiation in some cell systems [1–4], inhibits the synthesis of differentiation-specific proteins in others [5] and generally causes a block in DNA replication and cell division [6–9]. Cells grown in the presence of butyrate accumulate acetylated forms of histones (particularly of histones H3 and H4) due to an inhibition of histone deacetylase [10–13]. In addition various other modifications have been observed in chromosomal proteins of butyrate-treated cells [14,15].

In [16], another striking effect of butyrate on Friend erythroleukemic cells was reported. Like dimethylsulfoxide (DMSO), hydroxyurea and other agents which induce terminal differentiation in these cells, butyrate was claimed to cause breaks in DNA. This property was discussed as possibly being an early step in the control of differentiation of erythroleukemia cells [16]. Determination of single strand breaks in DNA was carried out by 2 methods, namely by alkaline sucrose gradient centrifugation and by centrifugation of folded genomes in neutral sucrose gradients.

We have been using butyrate as an agent to study the requirements for induction of viral and cellular DNA replication in SV 40 and polyomavirus-infected, as well as in serum-stimulated, mouse cells ([9], unpublished). We found that while sodium butyrate blocked cells in the G1 phase of the cell cycle and inhibited the initiation of cellular and viral DNA replication, these effects were readily reversible at least if the butyrate treatment did not exceed 24 h. As it is clear that the presumptive production by butyrate of single strand breaks in DNA would have a profound influence on the interpretation of the results obtained with butyrate-treated cells, we have carefully re-examined the question of whether the fatty acid causes breakage in DNA. We have used the sensitive method of alkaline elution [17] to monitor single strand breaks in DNA. Using a concentration of butyrate (7 mM) which leads to a complete but reversible block of DNA replication and cell division in 3T6 mouse fibroblasts, we were unable to detect any degradation of DNA in cells treated for various times (up to 24 h when all cells accumulate in the G1 phase of the cell cycle). We then repeated these experiments with Friend erythroleukemia cells and again could not obtain evidence for breaks in DNA of cells treated for 24 h with 2 mM butyrate or with 3% DMSO, conditions which cause the induction of terminal differentiation in these cells [16].

2. Methods

2.1. *Cell culture*

3T6 mouse fibroblasts were grown on plastic Petri dishes at 37°C in Dulbecco's modification of Eagle's medium containing 5% calf serum. Friend erythroleukemia cells (kindly donated by Dr P. Swetly, Ernst Boehringer Institute, Vienna) were grown in suspension culture using the same medium but 10% fetal calf serum. Cells were labeled for 24 h with [^{3}H]thymidine (1 μCi/ml medium). Sodium butyrate or DMSO was then added to the final concentration specified in the figure legends. Incubation was continued at 37°C and cells were used at various times (maximally 24 h) thereafter. Treatment of cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was done by adding the mutagen from a stock solution (0.05 M in dimethylsulfoxide) to the final concentration required. MNNG treatment was for 30 min at 37°C and the cells were then used immediately. The concentration of DMSO added with MNNG did not exceed 0.5%.

2.2. *Analysis of single strand breaks by alkaline elution*

Labeled and treated cells were collected (by cen-

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trifugation in case of Friend cells, by trypsinization and centrifugation in case of 3T6 cells) and washed with phosphate-buffered saline. Cells were then suspended in phosphate-buffered saline and cell number and radioactivity determined in aliquots. At the time of harvest cultures usually contained ~5 X 10^6 cells on a 10 cm dish (3T6 cells) or in 10 ml suspension (Friend cells). Between 5 X 10^5 and 10^6 cells (containing 5 X 10^5–10^6 cpm) were applied onto polyvinylchloride filters (Millipore BSWP 02500, 25 mm diam., 2 μm pore size), lysed with 2 ml lysis mixture (2% SDS, 0.1 M glycine, 0.02 M EDTA, pH 10) and DNA was eluted (at 2.5 ml/h) as in [17]. Eluted DNA was collected in 13 fractions and radioactivity determined in 2 ml aliquots. DNA remaining on the filter was also measured. The sum of the DNA recovered in eluted fractions and that on the filter agreed within 10% with the amount expected from the measurement of an aliquot of the cells applied to the filter.

3. Results

Fig.1 shows the elution profile of DNA from 3T6 fibroblasts treated for 24 h with 7 mM sodium butyrate. There was no difference between the DNA of treated or untreated cells. The same result was obtained when butyrate treatment was for 2,4 or 6 h (not shown). The butyrate concentration used had been found to lead to an optimal, reversible block of 3T6 cells in the G1 phase of the cell cycle after ~20–24 h treatment. As a positive control we analysed DNA from cells exposed to low concentrations (0.05 mmol/l)

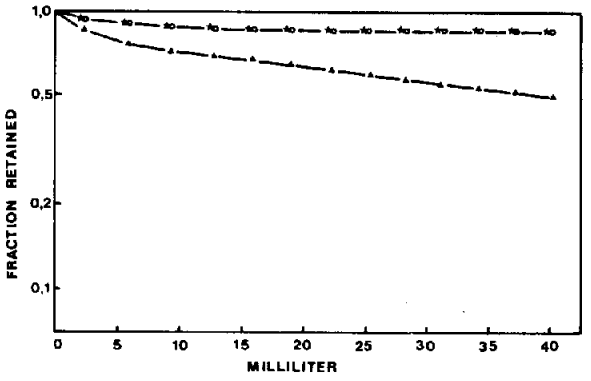

Fig.1. Alkaline elution profiles of DNA from 3T6 mouse fibroblasts: (★) control cells; (○) cells incubated for 24 h with 7 mM butyrate; (▲) cells treated for 30 min with 0.05 mM MNNG. The ordinate shows the fraction of DNA retained on the filter after elution with the volumes indicated in the abscissa.

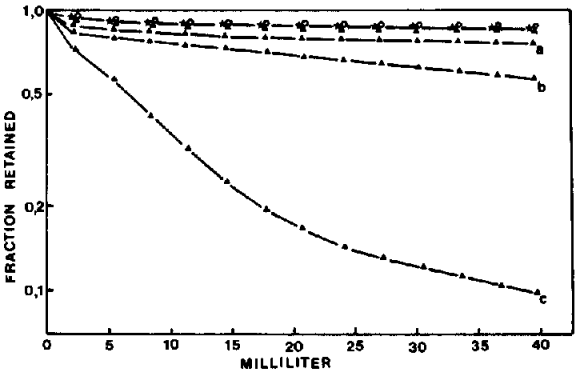

Fig.2. Alkaline elution profiles of DNA from Friend erythroleukemic cells: (★) control cells; (○) cells incubated for 24 h with 2 mM butyrate; (△) cells incubated for 24 h in presence of 3% DMSO. The points for the 3 curves superimpose. (▲) Friend cells incubated for 30 min with MNNG of following final concentration: (a) 0.012 mM; (b) 0.025 mM; (c) 0.05 mM.

of MMNG. As shown in [18], this mutagen induces the production of single strand breaks in mouse fibroblasts which can be detected by alkaline elution as well as by alkaline sucrose gradient centrifugation. A comparison of the 2 methods revealed that alkaline elution is the more sensitive one [18]. We thus conclude that the absence of detectable breakage in DNA by butyrate is significant.

As it was reported that butyrate induces breaks in the DNA of Friend erythroleukemic cells [16], we extended our study to these cells. The results are shown in fig.2 and again give no indication for fragmentation of DNA. DNA from control cells and from cells incubated for 24 h with 2 mmol/l of butyrate (which is optimal for induction of terminal differentiation [2,16]), gave exactly the same elution profile. The same holds true for cells incubated for 24 h with DMSO, another inducer of differentiation, used at 3%. Although 3% DMSO was reported to cause single strand breaks [16], in our experiments there was no indication for this. As a positive control we again used an incubation of cells with the mutagen MNNG. Friend cells turned out to be even more sensitive to this substance than 3T6 fibroblasts. Breaks could be detected with certainty by 30 min treatment of Friend cells (5 X 10^5 cell/ml) with MNNG at 0.01 mmol/l final conc. (1.5 μg/ml). We concluded that neither butyrate nor DMSO cause breaks in DNA of Friend cells at concentrations which allow these cells to proceed through terminal differentiation. As expected, synthesis of hemoglobin did occur in our treated cultures.

4. Discussion

The question whether agents which induce terminal differentiation cause the production of single strand breaks in DNA is of general importance. Therefore, the report [16] that sodium butyrate, DMSO and other agents which trigger differentiation in Friend erythroleukemia cells produce such lesions in DNA was of interest. The mechanism by which these various substances induce differentiation is unknown and it is not unlikely that the cellular site of action is a different one for each agent. Butyrate exerts a variety of effects on animal cells which seem to depend on the cell type (section1). Of particular interest was the observation that the fatty acid need not necessarily induce terminal differentiation characterized by the synthesis of differentiation-specific proteins, rather in at least one case butyrate was shown to do just the opposite. In chicken oviduct explants the substance was found to inhibit the hormone-dependent synthesis of ovalbumin and transferrin mRNAs [5]. This together with many other effects of butyrate on eukaryotic cells makes it unlikely that it acts by a common mechanism, e.g., involving DNA strand breaks. We have therefore re-examined the effect of butyrate on DNA of 2 lines of mouse cells (3T6 and Friend) using a very sensitive method to detect single strand breaks, namely alkaline elution. We could not obtain evidence for the production of DNA breaks by the fatty acid. The main difference between our experiments and those in [16] lies in the methods employed to measure single strand scissions. We consider alkaline elution to be more sensitive than alkaline sucrose gradient centrifugation. The latter method, in particular as applied in [16], including long preincubation of DNA in alkaline solution prior to centrifugation, (which results in random breakage and thereby shortening of long DNA molecules) is not without criticism [19]. The second method used in [16], i.e., the sedimentation of folded genomes, may be the most sensitive, but not specific method to detect DNA breaks. Changes in the sedimentation properties of folded genomes are likely to occur by alterations in chromatin other than single strand scissions, such as modifications in chromosomal proteins. As butyrate and possibly also DMSO cause such alterations, these could explain the observed effects on folded genomes.

Although our results make it unlikely that DNA strand breaks play a role in the induction by butyrate or DMSO of terminal differentiation in Friend erythroleukemic cells, we do not want to imply that this is a general rule. There may well be differentiation-specific events in some cells which do depend on the production of breaks in DNA. Any differentiation process requiring genome rearrangements would depend on a transient opening of the polynucleotide chain albeit at rather specific sites. It could well be that such processes can be initiated by substances which induce enzymes involved in this process [20]. Sodium butyrate, however, does not seem to be such a substance.

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