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Na-butyrate increases the production and α2,6-sialylation of recombinant interferon-γ expressed by α2,6-sialyltransferase engineered CHO cells

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Abstract

A non-human like glycosylation pattern in human recombinant glycoproteins expressed by animal cells may compromise their use as therapeutic drugs. In order to correct the CHO glycosylation machinery, a CHO cell line producing recombinant human interferon-γ (IFN) was transformed to replace the endogenous pseudogene with a functional copy of the enzyme α2,6-sialyltransferase (α2,6-ST). Both the parental and the modified CHO cell line were propagated in serum-free batch culture with or without 1 mM sodium butyrate. Although Na-butyrate inhibited cell growth, IFN concentration was increased twofold. The IFN sialylation status was determined using linkage specific sialidases and HPLC. Under non-induced conditions, IFN expressed by α2,6-engineered cells contained 68% of the total sialic acids in the α2,6-conformation and the overall molar ratio of sialic acids to IFN was 2.3. Sodium butyrate addition increased twofold the molar ratio of total sialic acids to IFN and 82% of total sialic acids on IFN were in the α2,6-conformation. In contrast, no effect of the sodium butyrate was noticed on the sialylation of the IFN secreted by the α2,6-ST deficient parental cell line. This study deals for the first time with the effect of Na-butyrate on CHO cells engineered to produce human like sialylation.

Abbreviations: α2,3-ST, α2,3-sialyltransferase; α2,6-ST, α2,6-sialyltransferase; dhfr, dihydrofolate reductase; IFN, human interferon-γ; LDH, lactate dehydrogenase.

Introduction

Mammalian cells are widely used for the production of recombinant drugs because of their ability to perform extensive post-translational modifications (Hodgson, 1993). However, they exhibit low production yields in comparison with procaryotic cells. Nevertheless, various strategies can be considered to overcome this drawback. Thus, we have developed new vectors (Monaco et al., 1994), serum-free media (Jenkins et al., 1994), bioreactor technologies for high cell density (Goergen et al., 1994) and culture control strategies (Lourenço et al., 1996), in order to optimise the production of recombinant proteins by animal cell cultures.

An alternative strategy is the use of stimulating agents such as dexamethasone, tetramethylurea, dimethylsulfoxide or sodium butyrate reported as enhancers of protein biosynthesis (Saito et al., 1992). We have already successfully used sodium butyrate to increase the production of proteins such as gamma-glutamyl transferase produced by CHO cells in bioreactor (Oster et al., 1993) and IgG produced by an OKT3 hybridoma (Chevalot et al., 1995). Other authors also report the over-expression of various proteins upon sodium butyrate addition: tPA (Arts et al., 1995), factor VIII (Palermo et al., 1991), von Wille-

brand factor, EPO (Dorner et al., 1989), FSH (Gebert and Gray, 1995) and monoclonal antibodies (Oh et al., 1993).

The gross structure of recombinant proteins produced following Na-butyrate addition appears not to be affected (Dorner et al., 1989; Palermo et al., 1991; Chevalot et al., 1995). Nevertheless, some conflicting results demonstrated that Na-butyrate addition may affect the cell's glycosylation machinery and more precisely the sialylation. The α2,6-sialyltransferase mRNA synthesis was reduced by 90% in hepatocytes in response to a 5 mM Na-butyrate concentration (Shah et al., 1992), and 3–5 mM Na-butyrate caused 80% inhibition in colonic cells (Li et al., 1995). Conversely, some authors pointed out a twofold increase in the specific activity of α2,3-sialyltransferase in CHO cells producing FSH as a result of 1.5 mM Na-butyrate addition (Chotigeat et al., 1994; Gebert and Gray, 1995). As far as we know, no paper has reported the effect of Na-butyrate on sialylation in cells engineered for the production of an exogenous sialyltransferase.

Although the CHO-derived glycan structures are similar to those present on natural human proteins, they are not identical (Jenkins et al., 1996). In CHO cells, sialic acids are exclusively added to terminal galactose via α2,3-linkages because the gene coding for the α2,6-sialyltransferase has become inactivated (Lee et al., 1989). Since it is known that the sialic acid linkage of α2,6 versus α2,3 are crucial to the biological activity and the half-life of proteins (Flesher et al., 1995; Kelm, 1995), we have recently expressed the cDNA coding for the rat α2,6-ST into the CHO 320 producing interferon-γ cell line (Monaco et al., 1996). The α2,6-ST was efficiently expressed in batch culture, and resulted in the increase of both the quantity of sialic acids linked in the α2,6-conformation and the total amount of IFN sialylation. IFN is a homodimeric cytokine exhibiting immunomodulatory and antiviral activities (Young and Hardy, 1995). Its mature polypeptide chain is 143 amino acids long and bears two N-glycosylation sites (Rinderknecht et al., 1984). The glycosylation status of IFN is critical for its dimerization and secretion (Sareneva et al., 1994), resistance to proteases (Sareneva et al., 1995), antigenicity (Meager and Leist, 1986) and stability in bloodstream (Sareneva et al., 1993). The IFN cDNA has been expressed in *E. coli* (Gray et al., 1982), insect cells (James et al., 1995), CHO cells (Hayter et al., 1991) and transgenic mice (James et al., 1995). When synthesized in CHO cells, the carbohydrate chains of the IFN were found to be predominantly bi- and triantennary complex structures with different degrees of sialylation (Gu et al., 1997; Hooker et al., 1995).

Here we report on experiments performed in controlled bioreactor cultures to study and compare the effect of Na-butyrate on IFN production and sialylation in both parental and α2,6-ST engineered CHO cells.

Materials and methods

Cell lines

The producing IFN CHO cell line (CHO 320) was constructed from the CHO-K1 (dhfr$^-$) cell line by cotransfection with the cDNAs for IFN and dhfr. It was supplied by the Wellcome Foundation Laboratories (now part of the Glaxo-Wellcome Research Group) and adapted to grow in suspension in a RPMI-1640 based serum-free medium (Hayter et al., 1991). We transfected the cDNA coding for the rat α2,6-ST (donated by Dr. J. Paulson, Cytel Corporation, San Diego, U.S.A.) into the CHO 320 cell line by using an original *in vitro* amplification expression method (Monaco et al., 1994). The resultant cell line (CHO C5) performs IFN α2,6-sialylation (Monaco et al., 1996). This cell line has been first adapted to grow in suspension in serum-containing medium (Lamotte et al., 1997), and subsequently was adapted to serum-free medium.

Culture media

Cultures were performed with a serum-free medium based on RPMI-1640 supplemented with 5 mg ml^{-1} BSA-fraction V (Bayer, Puteaux, France), 5 μg ml^{-1} human transferrin, 5 μg ml^{-1} bovine insulin, 1 mM sodium pyruvate, 0.1 mM alanine, 1 μm putrescine, 3 μm $FeSO_4$, 3 μm $ZnSO_4$, 10 nM Na_2SeO_3, 10 nM $CuSO_4$ and 0.1 μm methotrexate (all purchased from Sigma). Sodium butyrate was added to cultures from a stock solution (500 mM in PBS – Sigma B2503).

Culture conditions

Cultures (100 ml working volume) were initiated in 500 ml polycarbonate Erlenmeyer flasks (Corning) and scaled up to a 4 l bioreactor (Inceltech-SGI, France, 3 l working volume). Shake-flask cultures were carried out on an orbital agitation table (3 cm orbital diameter, 100 rpm) in a 37 °C incubator. Headspace of flasks were purged with 5% CO_2 in air in order to maintain pH at 7.2–7.4. Cell lines propagated

in shake-flasks were harvested in mid-exponential growth phase to seed the bioreactor at 1.5×10^5 cells ml^{-1}. The same number of cell propagation were performed for all experiments. In the bioreactor, the dissolved oxygen tension in the medium was controlled at 50% air saturation by oxygen sparging, the pH was maintained at 7.35 by CO_2 and NaOH (0.2 N) additions and the stirrer speed was fixed at 50 rpm.

Cell counting and metabolites assays

Twice a day, aliquots were collected from the bioreactor. Samples were centrifuged and supernatants stored at –20la °C for glucose, Na-butyrate and IFN measurements or at 4 °C for LDH assay. Viable cells were determined by trypan blue exclusion combined with a haemocytometer counting. Glucose and LDH were enzymatically determined using commercial assay kits (Sigma). IFN concentrations were measured using a sandwich ELISA procedure described elsewhere (Hayter et al., 1991). Sodium butyrate concentration was analysed by HPLC equipped with a Polypore-H column (Brownlee Laboratories) and a UV detector (210 nm, Waters). Elution was performed by H_2SO_4 (0.04 N) at 65 °C with a flow rate of 1.5 ml/min.

Kinetic data analysis

Specific rates of cell growth (μ) and IFN production (qIFN) were calculated from total (Xt) and viable cell densities (Xv), and IFN concentrations (IFN) according to the following equations:

$$\mu = (\mathrm{dXt/dt})(1/\mathrm{Xv}),\ \mathrm{qIFN} = (1/\mathrm{Xv})(\mathrm{dIFN/dt})\,.$$

Prior to derivation, experimental data were smoothed and interpolated with a software program based on the moving average method. Values of the specific rates were not plotted for the early hours of culture because of their poor accuracy. Total dead cells were evaluated by LDH release according to the method developed in our laboratory (Wagner et al., 1992). Dead lysed cells were calculated as the difference between total dead cells obtained from LDH assays and trypan blue stained cells (Chevalot et al., 1994). Intracellular LDH content of butyrate-treated viable cells, measured after induction of cell lysis in a Tween-20 solution (0.2% v/v in PBS solution), was found to be $14\ \mathrm{IU} \times 10^{-4}$ cells.

IFN sialylation

Prior to sialylation determination, IFN was purified from CHO cell supernatants using immuno-affinity chromatography (James et al., 1995). The monoclonal anti-IFN antibody covalently linked to sepharose beads recognises exclusively protein epitopes and not carbohydrates structures (Hooker et al., 1997). Purification procedure was therefore not influenced by potential changes in the IFN sialylation status.

The rpHPLC resolution and detection of fluorophore-labelled sialic acids were first reported by Reuter and Schauer (1994). We adapted it to discriminate between α2,3- and α2,6-linked sialic acids by use of conformation-sensitive sialidase enzymes (Monaco et al., 1996). Briefly, purified IFN was subjected to (a) total release of all sialic acids by incubation with *A. ureafaciens* sialidase (Oxford Glycosystems) or (b) selective release of only α2,3-linked sialic acids using Newcastle disease virus sialidase (Oxford Glycosystems). Sialic acids were recovered by micro-ultrafiltration, labelled with the fluorophore 1,2–diamino-4,5-methyl-dioxybenzene (Sigma), resolved using rpHPLC (Waters Ltd, using a C18 column in isocratic mode), and detected at 448 nm. This method also allowed the determination of the molar ratio of total sialic acids to IFN monomer. The average coefficient of variation was 6% for rpHPLC-based assays.

Results

Kinetics of cell growth and IFN production

Effect of different Na-butyrate concentrations

Since the literature reports a wide range of stimulating Na-butyrate concentrations, varying between 0.1 to 10 mM, we first tested the influence of four Na-butyrate concentrations (0, 1, 5 and 10 mM) using CHO 320 and CHO C5 cells in shake-flask cultures. After 36 h of culture, small volumes of a concentrated Na-butyrate solution (500 mM) were added in culture flasks in order to obtain the desired concentrations. Figure 1 shows the viable cell density and the IFN concentration throughout the cultures.

The influence of the sodium butyrate on cell density and IFN concentration was similar for both cell lines. In comparison with the control cultures (performed without Na-butyrate addition), 1 mM Na-butyrate resulted in a 40–50% decrease in the maximal

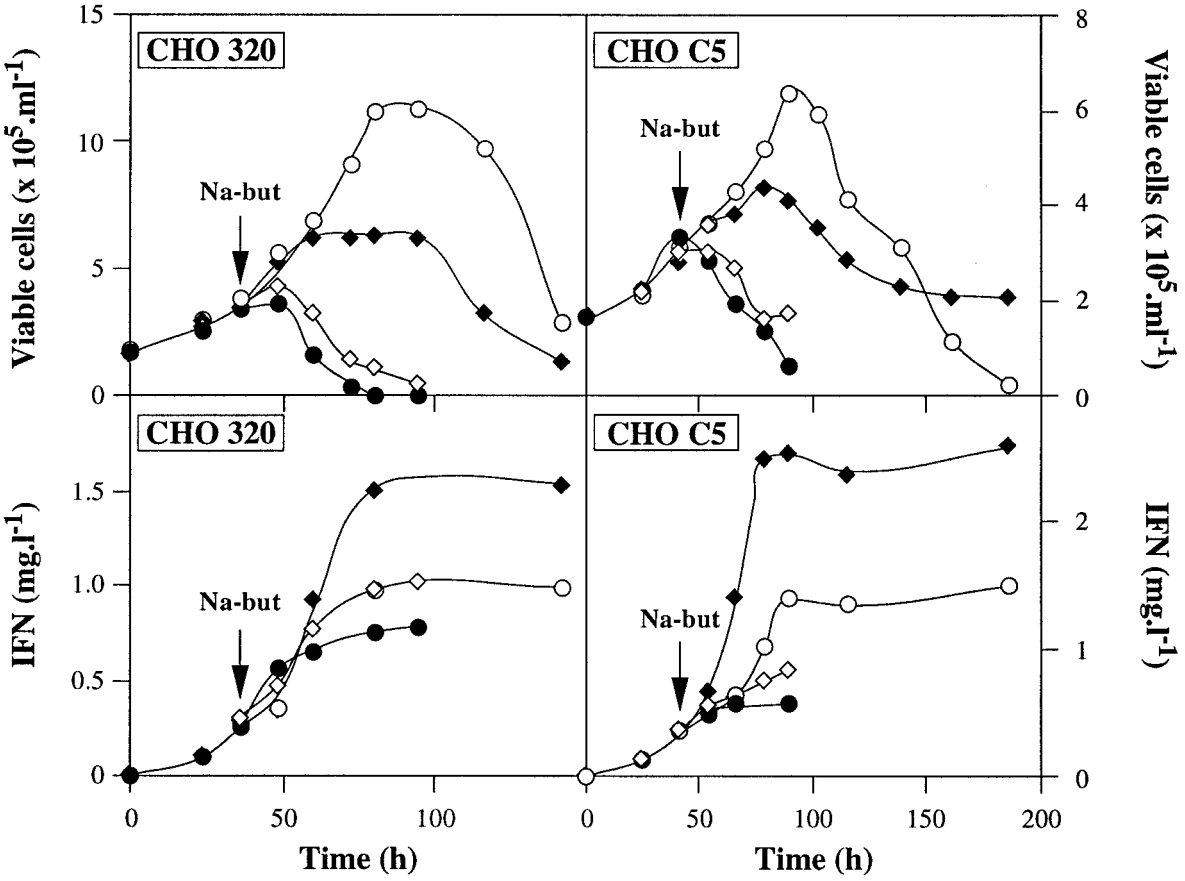

Figure 1. Viable cell density and IFN concentration over time during shake-flask cultures of CHO 320 and C5 cells with and without Na-butyrate addition (○: 0 mM, ♦: 1 mM, ◇: 5 mM, ●: 10 mM).

cell density, and 5 and 10 mM Na-butyrate led immediately to the arrest of the cell growth. In contrast, 1 mM Na-butyrate concentration increased by twofold the maximal IFN concentration in comparison with standard batch cultures. IFN production was poorly maintained with 5 and 10 mM Na-butyrate addition after the arrest of the growth and these experiments were discontinued after 100 h. Based on these results, a Na-butyrate concentration of 1 mM was chosen for further cultures.

Culture kinetics in the bioreactor

Batch cultures in the bioreactor were performed with and without Na-butyrate addition, in order to estimate Na-butyrate effects on cell kinetics and IFN sialylation. Experiments were performed on the control (320) and α2,6-ST engineered (C5) CHO cell lines (Figure 2). Chevalot et al. (1995) have demonstrated the optimal time for Na-butyrate addition is when the specific growth rate is highest. Thus, Na-butyrate was added when the specific cell growth rate was close to its maximal value (24–36 h).

In control cultures, the maximal cell density (8×10^5 cell ml^{-1}) was reached after 85–100 h with a maximal specific growth rate of 0.025 and 0.022 h^{-1} for CHO 320 and CHO C5 cells respectively. For both cell lines, the production of the IFN occurred exclusively during the growth phase. The specific rates of IFN production and cell growth varied in the same way during cultures of CHO 320 and C5 suggesting that IFN production and cell growth were closely related. However, as previously reported (Lamotte et al., 1997), the maximal IFN concentration was higher in CHO C5 cells (1.4 mg l^{-1}) than in CHO 320 cells (0.8 mg l^{-1}). This discrepancy was probably not due to the introduction of the α2,6-ST cDNA into the CHO 320 cell line but was the consequence of selecting high producing cells (CHO C5) at the early stages of transfection.

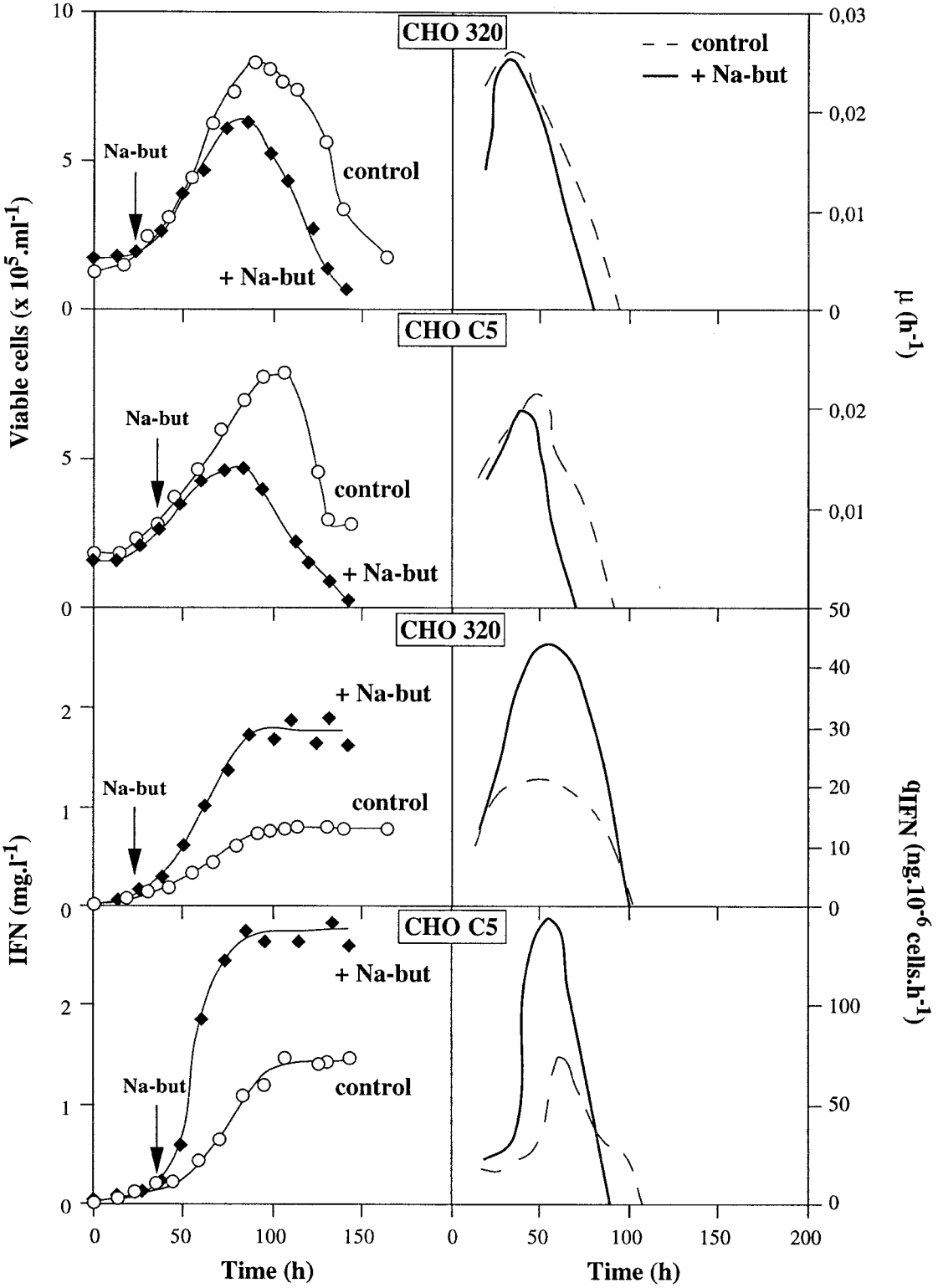

Figure 2. Viable cell density, IFN concentration, specific growth rate and specific IFN production over time during batch cultures of CHO 320 and C5 performed with Na-butyrate [♦: IFN and viable cells; (——): μ and qIFN] and without Na-butyrate [○: IFN and viable cells; (— —): μ and qIFN].

As expected, 1 mM Na-butyrate concentration increased the final IFN concentration by 90–130% and restricted the maximal viable cell density. The specific growth rate was suppressed more in the CHO C5 line than in the parental line although CHO C5 was directly derived from CHO 320 (maximal viable cell concentrations were 6×10^5 cell $ml^{-1}$for CHO 320 and $<5 \times 10^5$ cell $ml^{-1}$for CHO C5). The clonal variation is most likely explanation to that phenomenom. Na-butyrate exposure resulted in a major increase in the maximal specific IFN production rate reaching 45 and 150 $ng \times 10^{-6}$ cells h^{-1} for CHO 320 and CHO C5 respectively. However, the specific rates of IFN production and cell growth were closely correlated, as in non-induced cultures. HPLC measurements showed that the Na-butyrate concentrations were maintained at constant level (1 mM) throughout the cultures, suggesting that the molecule was neither used by cells in significant amounts nor degraded (data not shown). This indicates stimulation of cell physiology by Na-butyrate itself, and not one of its metabolites.

Figure 3 shows the time variation of the measured LDH and the calculated viable, dead non-lysed and lysed cells during the culture of the CHO C5 line with and without Na-butyrate. In both cases the amount of dead lysed cells was negligible ($0–0.3 \times 10^5$ cells ml^{-1}) during the growth phase. However, during the cellular decline phase cell lysis was found as a significant phenomenon with a level of 2.1×10^5 lysed cells ml^{-1} at the end of the culture performed with Na-butyrate. Moreover, cell debris were observed by optical microscopy in that decline phase confirming that cytoskeleton was damaged.

Previous work on CHO 320 cells has shown that glucose and glutamine depletion coincided with the onset of the CHO 320 stationary phase in batch cultures (Hayter et al., 1991). In butyrate-treated cultures reported here, cell death coincided with the glucose depletion, but the medium at that stage was not deficient in glutamine or other amino-acids (data not shown).

IFN sialylation

In order to characterise the IFN sialylation, IFN was immuno-precipitated from supernatants at the end of the growth phase of the batch cultures (80–100 h of culture) performed with and without Na-butyrate addition. The percentages of α2,6-linked sialic acids and the molar ratio of total sialic acids to IFN molecules were determined (Table 1). In control conditions, transfection of the rat α2,6-ST cDNA into CHO 320 cells to produce CHO C5 resulted in 68% of total sialic acids linked in the α2,6-conformation and nearly doubled the overall IFN sialylation, in comparison with the IFN from the parental cell line.

In parallel, the effect of adding Na-butyrate was determined for the two cell lines. As expected, because CHO 320 lacks α2,6-ST the conformation of the sialylation was not modified by Na-butyrate addition, and 100% of sialic acids were still in the α2,3-conformation. Conversely, 82% of sialic acids were in the α2,6-conformation on IFN secreted by CHO C5 line (α2,6-ST transfected) with Na-butyrate compared to 68% without Na-butyrate. Furthermore, the overall degree of IFN sialylation was twofold higher for CHO C5 cells subjected to Na-butyrate, whereas it was not enhanced by Na-butyrate exposure in the CHO 320 cell line. Therefore, the increase in the overall degree of sialylation on IFN produced by CHO C5 cells with Na-butyrate results predominantly from an increase in the molar ratio of total α2,6-linked sialic acids (+130%) rather than α2,3-linked sialic acids (+15%).

Discussion

We have shown that a 1 mM sodium butyrate addition significantly increased both the IFN volumetric production and the specific IFN production per cell. The molecular mechanisms involved in that phenomenon are still largely unknown. Nevertheless, previous work has shown that Na-butyrate treatment results in the over-expression of mRNAs coding for a variety of proteins (Smith et al., 1996; Oster et al., 1993). Others have observed that Na-butyrate decreases phosphorylation (Saito et al., 1991) and enhances hyperacetylation of histones (Arts et al., 1995), the latter playing an important role in the enhancement of the protein synthesis (Yamamoto et al., 1996). According to Oh et al. (1993), hyperacetylation may loosen the chromatin structure and allow increased access to RNA polymerase for mRNA synthesis. Furthermore, Na-butyrate addition can increase the production of some chaperone-like proteins (GRP 78 and 94) in the endoplasmic reticulum which can modulate the transport of other proteins within the endoplasmic reticulum and their passage to the Golgi (Dorner et al., 1989).

The effects of the Na-butyrate on cell proliferation are widely described. It is evident that Na-butyrate inhibits propagation of cells or viruses (Rocchi et

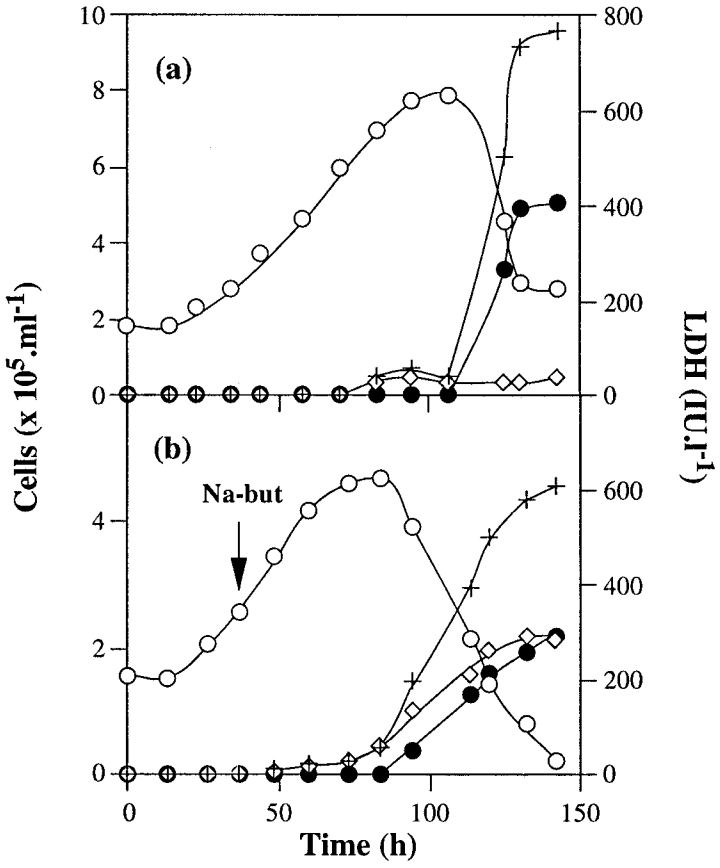

Figure 3. Viable (○), dead non-lysed (●) and lysed (◇) cells and extracellular LDH levels (+) over time during batch culture of CHO C5 cultured without (a) and with 1 mM Na-butyrate (b).

Table 1. Concentration and properties of IFN derived from CHO 320 and CHO C5 cells. Mean values of 3 measurements are shown. The average coefficient of variation was 6% for rpHPLC-based assays (rows 3–5)

	Cell line			
	CHO 320 (α2,6-ST$^-$)		CHO C5 (α2,6-ST$^+$)	
	Control	1 mM butyrate	Control	1 mM butyrate
Maximum viable cell density ($\times 10^5$ ml^{-1})	8.3	6.2	8	4.6
Maximum IFN concentration (mg l^{-1})	0.8	1.7	1.4	2.7
Molar ratio of sialic acid to IFN monomer	1.3	1.1	2.3	4.5
			(1.6 in α2,6)	(3.7 in α2,6)
			(0.7 in α2,3)	(0.8 in α2,3)
% α2,6-linked sialic acids	0	0	68	82
% α2,3-linked sialic acids	100	100	32	18

al., 1992; Shadan et al., 1994). As shown in our experiments performed in shake-flask cultures, the magnitude of the growth inhibition is dependent on the Na-butyrate concentration. 1 mM Na-butyrate suppressed the cell growth whereas the 5 and 10 mM concentration also compromised the cell viability. In low concentrations Na-butyrate may reduce the cell cycle transition because histone phosphorylation is critical for cell cycle progression (Yamada et al., 1992), but in higher concentrations Na-butyrate may irreversibly degrade the chromatine structure (Fillipovitch et al., 1994; Smith, 1986) and cause cytoskeleton damage (Kruh, 1982). A significant amount of lysed cells were found during the decline phase with 1 mM Na-butyrate which could have modified the cell structure and made cells more sensitive to shear stress in bioreactors. When using sodium butyrate to optimise the production of recombinant proteins by mammalian cells a concentration must be chosen that does not compromise cell viability (e.g. 1 mM).

Our results also highlight the effects of Na-butyrate in improving the overall sialylation of the recombinant IFN produced by CHO cells transfected with rat α2,6-ST cDNA (CHO C5) whereas the effect of the Na-butyrate was not apparent in control cells (CHO 320). IFN molecules from butyrate-treated CHO C5 cells should therefore probably have better pharmacokinetic properties because increased terminal sialic acids prevent the clearance of the protein from bloodstream via the asialoglycoprotein receptor (Cole et al., 1993; Lodish, 1991; Dickramer, 1991).

The improvement of the overall IFN sialylation by Na-butyrate in CHO C5 cells was almost exclusively due to the additional linkage of sialic acids in the α2,6-conformation, while the contribution of α2,3-sialic acids in the overall sialylation stayed roughly constant (Table 1). This result is in good agreement with the fact that IFN sialylation was not modified in the α2,6-ST deficient CHO 320 cells.

The reasons of the increase of α2,6-linked sialic acids on IFN produced by butyrate-treated CHO C5 are not yet elucidated. However, three non-exclusive hypotheses can be put forward to explain this phenomenom. First, the two sialyltransferases, α2,6-ST and α2,3-ST, should compete for CMP-sialic acid substrates and acceptor glycan chains. This could suggest that the activity of α2,6-ST was increased by Na-butyrate addition relative to the α2,3-ST. Second, it can not be excluded that the recombinant protein itself may influence the percentage of α2,6- and α2,3-linked sialic acids on IFN. Indeed, Grabenhorst et al. (1995) have demonstrated, in the case of the β-trace protein produced by BHK cells transformed for the expression of the α2,6-ST, that Man-3-arms of glycans were exclusively occupied by α2,3-linked sialic acids. At last, since it is known that CHO cells can release sialidases causing a partial degradation of glycoproteins (Lao et al., 1996; Munzert et al., 1996) including IFN (Gu et al., 1997), the presence of cellular sialidases may also explain the discrimination between α2,3- and α2,6-linked sialic acids. Indeed, Gramer et al. (1995) have shown that CHO sialidases have higher affinity to α2,3- than α2,6-linked sialic acids.

Our results demonstrate how a combined approach of genetic and biochemical engineering can improve both the sialylation status and the production of a recombinant drug by mammalian cells. Further studies are now necessary to elucidate the influence of the sodium butyrate on the intracellular glycosylation pathways, and the entire carbohydrate structure of IFN as well as its biological and pharmacokinetic properties which may also be altered by Na-butyrate addition.

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