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# Cancer cell sensitization to Fas-mediated apoptosis by sodium butyrate

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## Abstract

**Cancer cells often resist Fas-mediated apoptosis even when the Fas receptor is expressed at the cell surface. We show here that human and rat colon cancer cells undergo massive apoptosis when they are exposed to soluble Fas ligand in the presence of sodium butyrate, an agent that induces by itself only a low rate of apoptosis. Sodium butyrate potentiates Fas-dependent apoptosis in seven out of eight colon cancer cell lines. Sodium butyrate does not increase Fas receptor cell surface expression and does not modify cell levels of Bcl-2, Bcl-$x_L$, Bcl-$x_S$ and Bax. Sodium butyrate also induces tumor cell sensitization to the apoptotic effect of the combination of TNF-$\alpha$ and IFN-$\gamma$, but it does not modify the level of the FADD/Mort1 adaptator molecule, at the connection between Fas- and TNF-dependent apoptosis pathways. Because the clinical toxicity of butyrate is low, its ability to enhance Fas-signal delivery in cancer cells could be of therapeutic interest.**

**Keywords:** apoptosis; colorectal cancer; Fas; sodium butyrate

**Abbreviations:** IFN-$\gamma$, interferon-$\gamma$; TNF-$\alpha$, tumor necrosis factor-$\alpha$.

## Introduction

Fas (APO-1/CD95) is a protein receptor abundantly expressed on the plasma membrane of a variety of cells including epithelial cells of the colon (Krammer, 1994). Cross-linking of this receptor by either agonistic antibodies or its natural ligand, Fas-L, can induce apoptosis in Fas-expressing cells (Yonehara *et al*, 1989; Trauth *et al*, 1989; Suda *et al*, 1993; Nagata and Golstein, 1995). The Fas receptor is often expressed in colon carcinoma cells (Leithäuser *et al*, 1993; Krammer, 1994). However, these cells are usually resistant to Fas-mediated cell apoptosis (Owen-Schaub *et al*, 1994). This resistance might allow these cells to escape the immune clearance since both cytotoxic T cells and NK cells express Fas-L and use the Fas/Fas-L system as one of the key mechanisms to kill their targets (Lowin *et al*, 1994; Kägi *et al*, 1994; Arase *et al*, 1995). We have recently shown that cytotoxic drugs could sensitize colon cancer cells to Fas-mediated apoptosis by upregulating the expression of Fas (Micheau *et al*, 1997). These drugs can induce apoptosis in many cell types in a manner that is not dependent on Fas (Eischen *et al*, 1997). Nevertheless, drug-induced apoptosis of other tumor cell lines was reported to be completely inhibited by anti-Fas antibodies (Friesen *et al*, 1996; Müller *et al*, 1997), suggesting that the resistance of colon cancer cells to Fas-mediated apoptosis might be one of the mechanisms of their resistance to cytotoxic drugs. For these reasons, agents that restore colon carcinoma cell sensitivity to Fas-mediated apoptosis might improve current therapeutic strategies.

Butyric acid is the major short-chain fatty acid produced by fermentation of dietary fibers in the colon (Cummings, 1981). It is avidly absorbed by the colonic epithelium, induces a variety of alterations at the molecular and cellular levels (Kruh, 1982) and acts as a survival factor for freshly isolated colonic epithelial cells (Hass *et al*, 1997). By contrast, sodium butyrate inhibits the proliferation and induces the differentiation of various tumor cell lines including colorectal cancer cells (Barnard and Warwick, 1993; Schroy *et al*, 1994; Augenlicht *et al*, 1995). Sodium butyrate also slightly increases the rate of apoptosis in several colon carcinoma cell lines (Hague *et al*, 1993; Heerdt *et al*, 1994).

In the present study, we demonstrate that sodium butyrate can enhance dramatically the sensitivity of colon carcinoma cell lines to Fas-mediated apoptosis. Because the clinical toxicity of sodium butyrate is low (Miller *et al*, 1987; Perrine *et al*, 1993), this compound is a good candidate for therapeutic trials aiming to improve the efficacy of both chemotherapy and immunotherapy in colon carcinomas.

## Results

### Sodium butyrate dramatically potentiates colon cancer cell sensitivity to soluble Fas ligand or agonistic anti-Fas antibody induced apoptosis

We used the supernatant of Fas ligand-transfected Neuro-2a cells (Rensing-Ehl *et al*, 1995) as a source of soluble Fas ligand and the supernatant of the same cells transfected with the empty vector was used as a control. When rat PROb colon carcinoma cell monolayers were treated with the supernatant of Fas ligand-transfected Neuro-2a cells for up to 3 days, no major change was noticed by phase contrast microscopy relative to cells treated with the control supernatant. When treated with a combination of soluble Fas ligand and 1 to 4 mM

sodium butyrate, these cells exhibited widespread death, manifested by cell rounding, contraction and detachment from the dish (Figure 1A). Staining with terminal deoxynucleotidyl transferase to detect DNA fragmentation (Figure 1B), or with Hoechst 33258 for chromatin labeling (Figures 1C and 2), detected few apoptotic cells in PROb cell monolayers treated with Fas ligand alone, whereas most cells were apoptotic upon combined treatment. In the wells that received supernatant of empty vector-transfected Neuro-2a cells, addition of sodium butyrate decreased PROb cell density, but only few apoptotic cells were observed (Figure 1).

Sodium butyrate also dramatically potentiated Fas ligand-induced apoptosis, as shown after Hoechst 33258 staining, in six out of seven human colon cancer cell lines (Figure 2). Only Caco-2 cells remained resistant to Fas ligand-induced apoptosis, whatever the butyrate concentration used. At a fixed concentration of butyrate, the ratio of apoptosis in sensitive cells was found to be dependent on Fas-ligand concentration (Figure 3). At least 12 h of continuous exposure to both Fas ligand and sodium butyrate were necessary to observe the synergy between these two compounds.

In PROb and HT29 cell lines, extensive cell apoptosis, then an almost complete disappearance of tumor cells, were observed after a 3 day treatment with soluble Fas ligand and 2 mM sodium butyrate (not shown). Toxicity resulting from shorter exposure was determined by using clonogenicity assays. A 24 h exposure to sodium butyrate and soluble Fas ligand almost completely suppressed the capability of human HT29 and rat PROb cells to form colonies after seeding in a new culture flask, whereas exposure to soluble Fas ligand only, or to sodium butyrate only, had a limited effect (Figure 4).

Fas receptor ligation can be obtained not only with soluble Fas ligand, but also with agonistic anti-human Fas monoclonal IgM, such as CH11. On the contrary, the

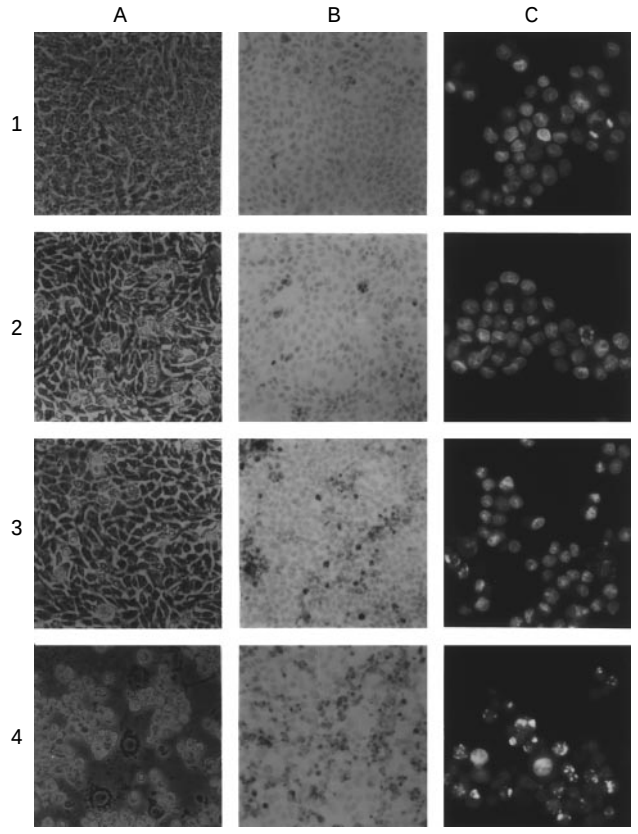


**Figure 1** Effect of sodium butyrate, Fas ligand and a combination of Fas ligand and butyrate on PROb cell monolayer morphology and apoptosis. Established PROb cell monolayers were treated for 3 days with supernatant of Neuro-2a cells transfected with an empty vector (mock supernatant) (1), mock supernatant plus 1 mM sodium butyrate (2), supernatant of Neuro-2a cells transfected with Fas ligand cDNA (soluble Fas ligand) (3) or soluble Fas ligand plus 1 mM sodium butyrate (4). Cell cultures were examined using phase contrast microscopy (**A**), or after tdt-mediated dUTP nick-end labeling (APOPTAG) (**B**). Floating cells and trypsin-detached adherent cells were also pooled, spread on a slide and chromatin was stained with Hoechst 33258 (**C**)

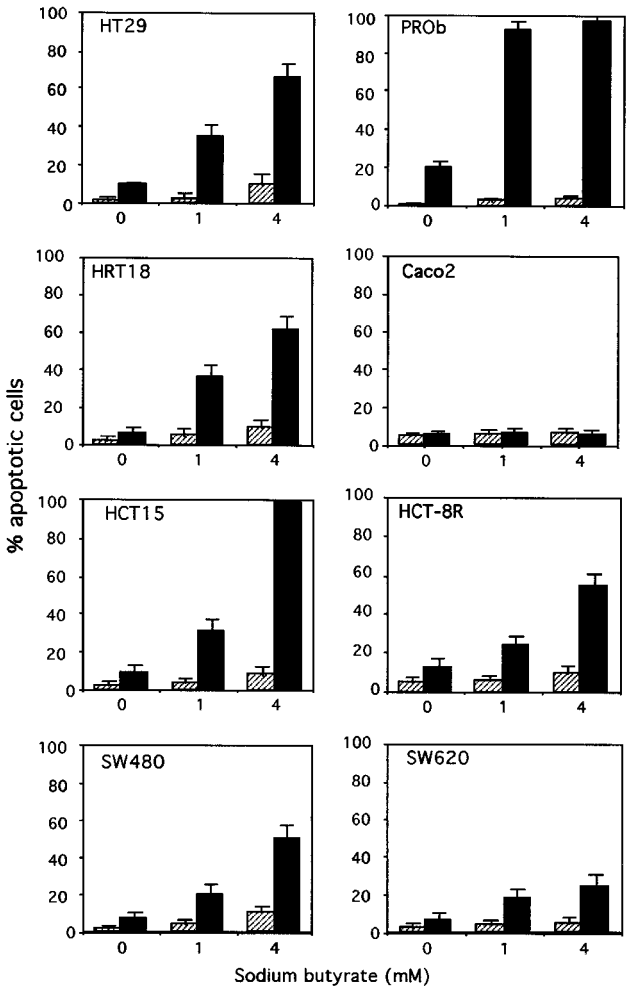


**Figure 2** Sodium butyrate potentiates apoptosis induced by Fas ligand in rat colon cancer PROb cells and in 6 out of 7 human colon cancer cell lines. Cancer cell monolayers were cultured for 24 h with half-diluted mock supernatant (hatched columns) or soluble Fas ligand (solid columns), supplemented or not with 1 or 4 mM sodium butyrate. The percentage of apoptotic cells in pooled attached and floating cells was determined after chromatin staining with Hoechst 33258. Each count was established on 200 or 400 cells. The 95% confidence interval was indicated by a bar

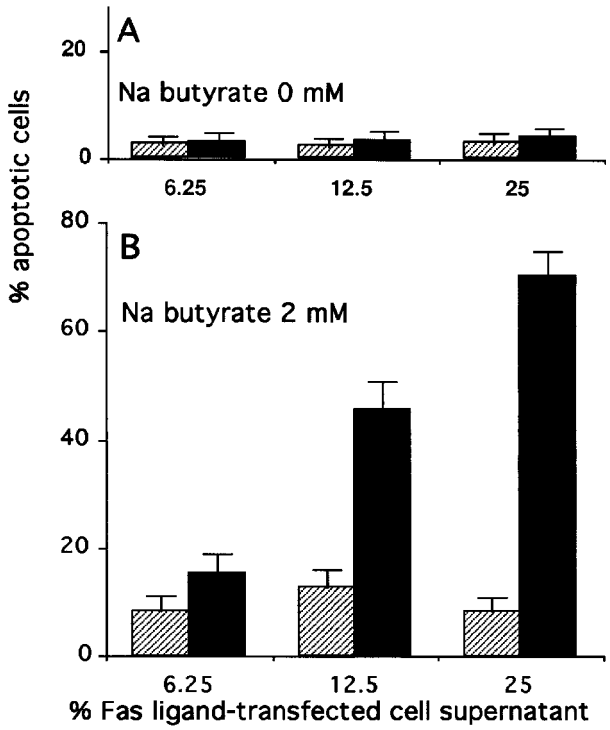


**Figure 3** Effect of sodium butyrate on HT29 cell apoptosis according to the concentration of Fas ligand. HT29 cell monolayers were cultured for 24 h with various dilutions of supernatant of mock-transfected (hatched columns) or Fas ligand-transfected (solid columns) Neuro-2a cells, supplemented or not with 2 mM sodium butyrate. The percentage of apoptotic cells and the 95% confidence interval were determined and represented as in the Figure 2

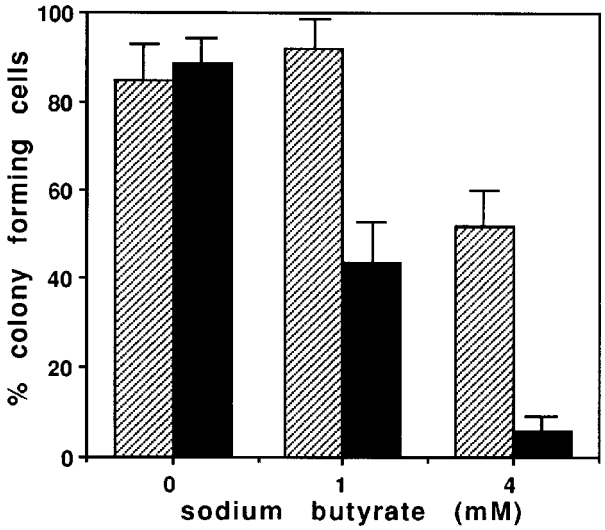


**Figure 4** Effect of sodium butyrate and/or soluble Fas ligand on colony formation by colon cancer cells. HT29 cells ($5 \times 10^4$ per well) were incubated for 24 h in complete culture medium (hatched columns) or in complete culture medium supplemented with soluble Fas ligand (black columns), without sodium butyrate or in the presence of 1 or 4 mM sodium butyrate. At the end of the incubation period, floating and attached cells were recovered, washed and suspended in complete culture medium. A fraction of the cell suspensions was seeded in culture flasks. After 8 days, colonies were counted after methanol fixation and crystal violet staining. The results are expressed as the percentage of clonogenic cells relatively to the initial cell population. The means and standard deviations (bar) were determined from the results obtained from three replicative wells

antagonistic anti-Fas antibody ZB4 binds to human Fas without mediating cytotoxicity and is widely used to suppress apoptosis induced by agonistic anti-Fas antibodies or by Fas ligand (Itoh *et al*, 1991). Addition of 2 mM sodium butyrate increased from 7 to 52% the ratio of apoptotic cells observed after CH11 antibody (200 ng/ml) treatment of HT29 cells (Figure 5A). Addition of anti-Fas antagonistic ZB4 antibody (2.5 $\mu$g/ml) inhibited apoptosis that was observed when HT29 cells were cultured in 2 mM sodium butyrate and either soluble Fas ligand or the agonistic anti-Fas antibody CH11 (Figure 5B).

## Sodium butyrate does not modulate expression of surface-associated Fas, secretion of soluble inhibitors of the Fas-mediated cytotoxic pathway, and levels of apoptosis-modulating proteins Bcl2, Bcl-x or Bax

To determine whether the effect of sodium butyrate was due to an increase in the level of Fas receptor on the tumor cells, Fas expression was analyzed on the surface of HT29 cells by immunolabeling and quantitative flow cytometry. The number of immunoreactive Fas receptor sites did not increase at the surface of HT29 cells after a 24 h treatment with 4 mM sodium butyrate, whereas it increased after exposure to IFN-$\gamma$ (200 IU/ml) (Figure 6). This result was confirmed in two independent experiments. Surface-associated immunoreactive Fas level did not change when cells were treated for 6 h with 1 or 4 mM sodium butyrate.

Several tumor cell lines have been reported to produce and secrete soluble isoforms of the Fas receptor that can contribute to their Fas-resistance (Owen-Schaub *et al*, 1995a). These isoforms are able to inhibit the binding of Fas ligand or agonist anti-Fas antibodies to membrane-associated Fas. To determine whether sodium butyrate decreased the production or the secretion of soluble Fas or other inhibitory factors by tumor cells, we compared the effect of HT29 tumor cell supernatant on the cytotoxic effect of serially-diluted Fas ligand supernatant on Jurkat T cells. These cells are highly sensitive to Fas ligand, which induces extensive apoptosis at dilutions up to 1:160 of Fas ligand supernatant. Addition of HT29 cell supernatant blocked Fas-mediated Jurkat cell apoptosis, but this suppressive effect was not modified when 2 mM sodium butyrate had been added during the tumor cell culture (Figure 7). These data suggest that butyrate does not enhance tumor cell apoptosis by decreasing the secretion of soluble Fas or another factor into the cell culture medium.

Cell sensitivity to apoptosis can be modulated by expression levels of anti- and pro-apoptotic proteins of the Bcl-2 family. We analyzed the effect of butyrate on the expression of Bcl-2, Bax, Bcl-$x_L$, and Bcl-$x_S$ in HT29 cells. Twenty-four hour treatment of HT29 cells with 2 mM butyrate did not modify the cellular content of these proteins (Figure 8).

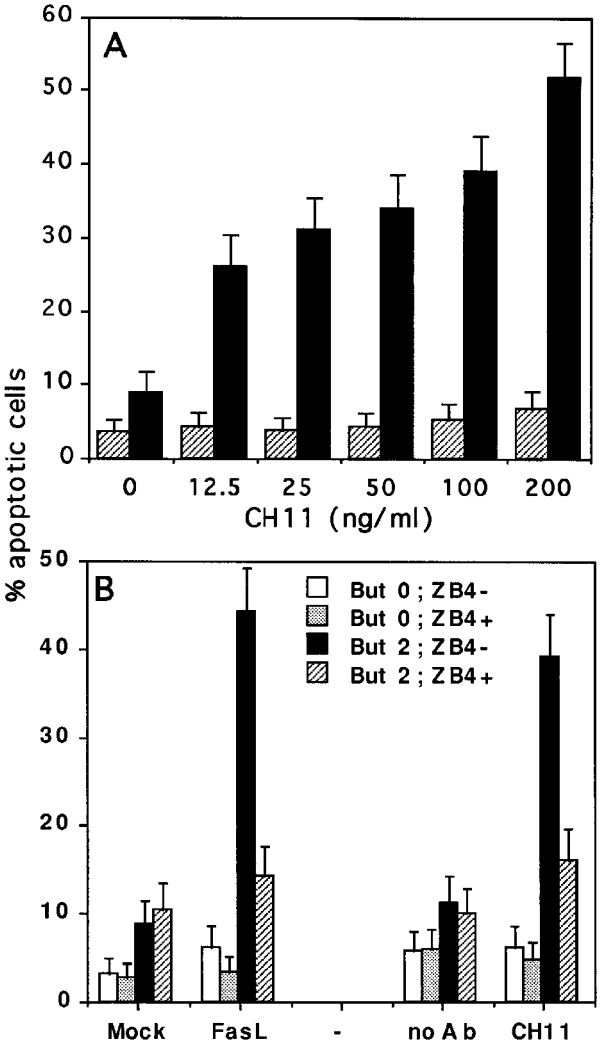


**Figure 5** Effect of agonistic (CH11) and antagonistic (ZB4) anti-Fas antibodies. (**A**) Sodium butyrate (2 mM, black columns) enhances HT29 cell apoptosis when added to agonistic anti-Fas monoclonal antibody CH11. (**B**) Antagonistic anti-Fas monoclonal antibody (ZB4, 2.5 $\mu$g/ml) inhibits HT29 cell apoptosis that is induced by soluble Fas ligand or the agonistic anti-Fas monoclonal antibody (CH11, 100 ng/ml) in the presence of 2 mM sodium butyrate. The percentage of apoptotic cells and the 95% confidence interval were determined and represented as in the Figure 2

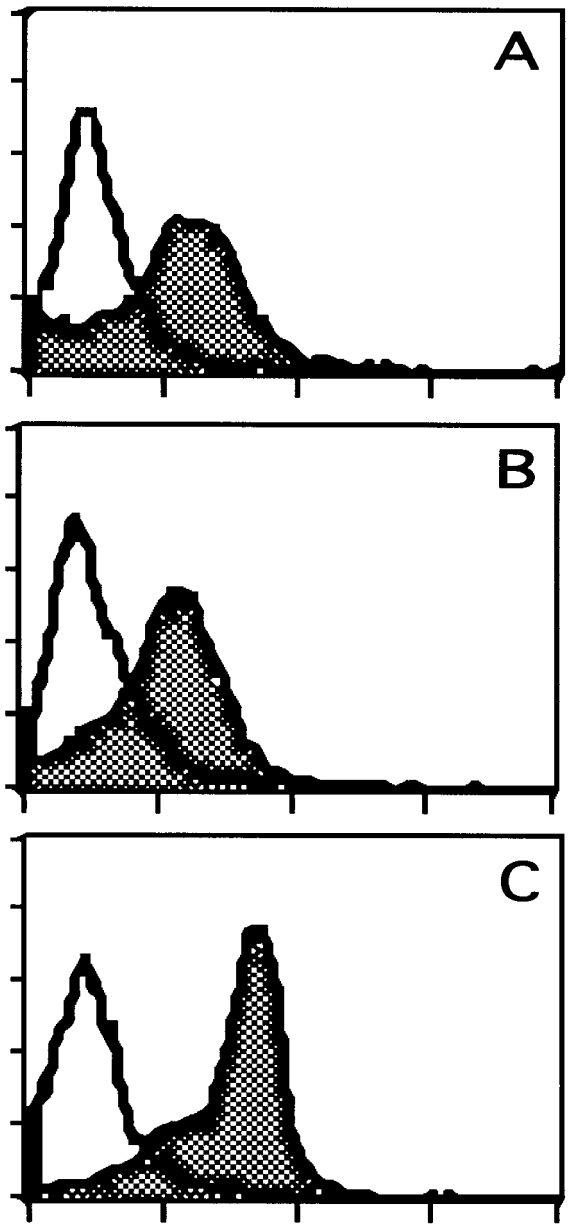


**Figure 6** Effect of sodium butyrate and IFN-$\gamma$ on Fas receptor levels on the HT29 cell surface. HT29 cells were cultured for 24 h in complete culture medium (**A**) that was supplemented with 4 mM sodium butyrate (**B**) or 200 IU/ml rhIFN-$\gamma$ (**C**). Fas receptor expression was determined using flow cytometric analysis (logarithmic scale) after incubation with mouse anti-Fas IgG, clone DX2 (black), or a mouse isotype-matched IgG control (white), then FITC goat anti-mouse IgG. Fas receptor quantification was performed using mouse IgG-loaded beads. The antigen-binding capacity was significantly increased for IFN-$\gamma$-treated cells, but not for butyrate-treated cells, relative to untreated HT29 cells. Similar results were found in another experiment

### Specificity of the effect of sodium butyrate on tumor cell apoptosis

The 55 kDa TNF-$\alpha$ receptor belongs to the same family as Fas and can initiate cell apoptosis through a closely-related signaling pathway (Varfolomeev *et al*, 1996). IFN-$\gamma$ increases TNF-$\alpha$ receptor expression (Yonehara *et al*, 1989). Figure 9 shows that sodium butyrate enhanced apoptosis induced by the combination of human TNF-$\alpha$ and human IFN-$\gamma$ in three human colon cancer cell lines, HT29, HRT18 and HCT15.

FADD/MORT1 is an intracytoplasmic adaptator molecule that connects Fas ligand-induced and TNF-$\alpha$-induced signals leading to cell apoptosis. Using Western blot analysis, we found that exposure to sodium butyrate for 24 h did not modify HT29 cell content in FADD/MORT1 (Figure 8).

## Discussion

The results presented here demonstrate that sodium butyrate synergistically increases Fas ligand-induced apoptosis in the tested cell lines, with the exception of the Caco-2 cells. Caco-2 cell resistance is likely due to the absence of Fas receptor which could not be detected using flow cytometry even after Caco-2 cell exposure to IFN-$\gamma$ (data not shown).

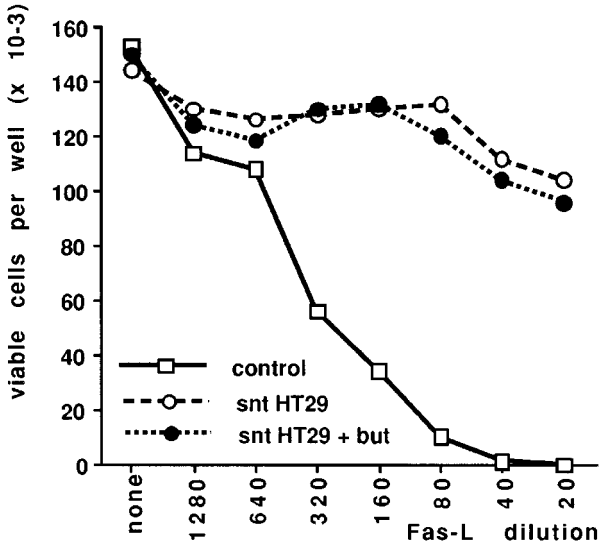


**Figure 7** Effect of HT29 cell culture supernatant on the toxicity of Fas ligand to Jurkat T cells. Jurkat cells ($5 \times 10^4$) were cultured for 3 days with various dilutions of Fas ligand supernatant in the presence of dialyzed and concentrated fresh culture medium (control) or conditioned medium of confluent HT29 cells cultured without (snt HT29) or with (snt HT29+but) 2 mM Na butyrate. Surviving cells were counted at the end of the assay (mean of two replicative wells)

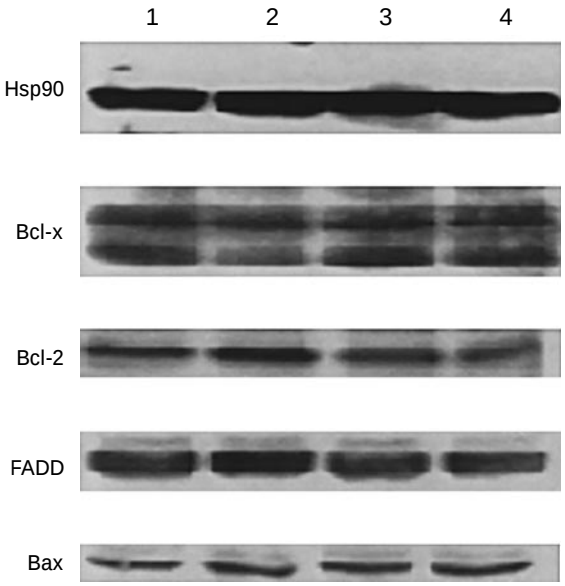


**Figure 8** Effect of sodium butyrate on levels of apoptosis-modulating proteins Bcl2, Bcl-x and Bax, and the adaptator protein FADD in HT29 cell extracts. HT29 cells were either left untreated (lane 1) or treated for 24 h with 2 mM sodium butyrate (lane 2), 25% soluble Fas ligand-containing supernatant (lane 3) or both sodium butyrate and soluble Fas ligand (lane 4). Immunoblots were probed with antibodies to Bcl-2, Bcl-x, Bax or FADD. An antibody against the heat shock protein Hsp90 was used as an internal control for protein loading. The two bands observed with the anti-Bcl-x antibody represent the Bcl-$x_L$ (upper band) and Bcl-$x_S$ (lower band) isoforms

The supernatant of Fas ligand-transfected Neuro-2a cells was used as the source of Fas ligand. Apoptosis due to this supernatant in the presence of sodium butyrate was

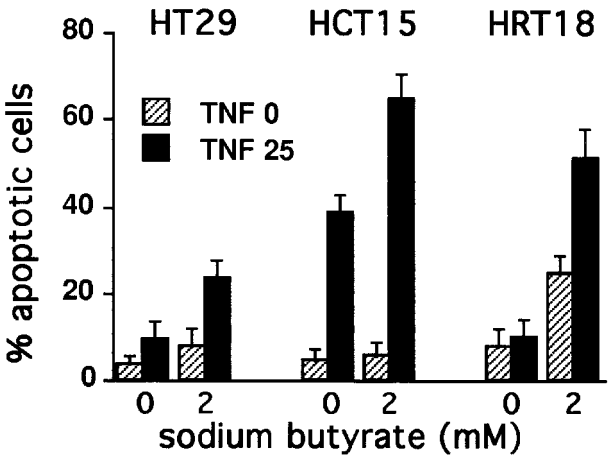


**Figure 9** Effect of butyrate on HT29 cell apoptosis upon treatment with TNF-$\alpha$ and IFN-$\gamma$. HT29 cell monolayers were treated for 24 h with 200 U/mL rhIFN-$\gamma$ and various concentrations of rhTNF-$\alpha$ in the absence (hatched columns), or in the presence (solid columns) of 2 mM sodium butyrate. The percentage of apoptotic cells and the 95% confidence interval were determined and represented as in the Figure 2

inhibited by the antagonistic anti-Fas ZB4 antibody and mimicked by the agonistic anti-Fas CH11 antibody. These experiments confirmed that Fas ligand was responsible of the effects of this supernatant.

Sodium butyrate was reported to induce apoptosis in various cell lines, including human colorectal carcinomas (Hague *et al*, 1993; Herdt *et al*, 1994). In this work, we show that the percentage of apoptotic cells resulting from exposure of various colon cancer cell lines to butyrate alone is low, usually less than 10%. These results are in accordance with a recent report (Heerdt *et al*, 1997) in which only 6% of SW620 human colonic carcinoma cells undergo apoptosis following culture with 5 mM sodium butyrate. In addition to this moderate apoptosis, sodium butyrate induces a blockade of cell proliferation at the early $G_1$ stage of the cell cycle (Barnard and Warwick, 1993; Schroy *et al*, 1994). The combination of butyrate and Fas ligand dramatically enhances Fas-mediated apoptosis and, when maintained for up to 3 days, completely destroys established monolayers of PROb or HT29 cells (not shown).

The role of the anti- or pro-apoptotic proteins of the Bcl-2 family in the Fas pathway is controversial. Bcl-2 expression is not clearly related to sensitivity or resistance to Fas-mediated apoptosis in various cell lines (Debatin and Krammer, 1995; Huang *et al*, 1997; Strasser *et al*, 1995) including colon cancer cells (Owen-Schaub *et al*, 1994). In accordance with recently published data (Moss *et al*, 1996), we found that butyrate treatment did not modify Bcl-2, Bcl-$x_L$, Bcl-$x_S$ and Bax levels in HT29 cells.

Fas expression can be induced in response to various cytokines, including IFN-$\gamma$ and TNF-$\alpha$ (Yonehara *et al*, 1989; Leithäuser *et al*, 1993), to wild-type p53 activity (Owen-Schaub *et al*, 1995b), and to cytotoxic drugs (Müller *et al*, 1997; Micheau *et al*, 1997). Flow cytometric analysis did not reveal any increase in HT29 cell surface-

associated Fas upon butyrate treatment. Thus, the synergistic effect of butyrate on Fas ligand-mediated cell killing could not be explained by an increased expression of the Fas receptor.

Fas-dependent apoptosis may be inhibited by the cellular release of FasΔTM, a soluble isoform of Fas encoded by an alternatively-spliced mRNA lacking the transmembrane domain coding sequence. This isoform may be secreted into the tumor cell supernatant and antagonize Fas ligand killing (Cheng *et al*, 1994; Owen-Schaub *et al*, 1995a; Natoli *et al*, 1995). HT29 cell supernatant suppressed Fas-mediated apoptosis of Jurkat cells. This inhibitory effect was not modified when HT29 cells were cultured with sodium butyrate. Thus, butyrate does not act by suppressing tumor cell secretion of FasΔTM or other molecules that could inhibit Fas-mediated cytotoxicity.

As previously reported by others (Abreu-Martin *et al*, 1995), we observed that colon cancer cells were poorly sensitive to the cytotoxic effect of TNF-$\alpha$, unless IFN-$\gamma$ was added either as a pre-treatment or a co-treatment (not shown). We observed that sodium butyrate significantly increased apoptosis induced by a combination of TNF-$\alpha$ and IFN-$\gamma$ in HT29, HCT15 and HRT18 cell lines. Rat TNF-$\alpha$ was not cytotoxic for PROb cells, even in the presence of rat IFN-$\gamma$ and/or 4 mM sodium butyrate (not shown), which suggested that PROb cells did not express a functional TNF-$\alpha$ receptor.

The common effect of sodium butyrate on Fas ligand- and TNF-$\alpha$-mediated apoptosis suggests that it takes place on the molecular pathway which is shared by the receptors of both ligands. Through its death domain, activated Fas interacts directly with an intracytoplasmic protein, FADD/MORT1, which activates Flice (caspase 8) thus generating the caspase cascade and leads to apoptosis. Activated TNF-$\alpha$ p55 receptor interacts through its death domain with another intra-cellular protein, TRADD. It is believed that TRADD forms a bridge connecting the TNF receptor with FADD/MORT1, allowing cross-talk between the functional expression of the p55 TNF receptor and Fas (Varfolomeev *et al*, 1996). We did not observe any change of FADD/MORT1 level in butyrate-treated HT29 cells. Sodium butyrate could induce a downexpression of the recently reported Flice-inhibitory protein (FLIP), which interacts with the adaptator protein FADD (Irmler *et al*, 1997), or an alteration in the caspase cascade. By inhibiting the histone deacetylase (Sealy and Chalkley, 1978), butyrate leads to progressive hyperacetylation of histones, global change in chromatin conformation, reversible growth inhibition and alteration in multiple gene expression (McBain *et al*, 1997). It has been recently shown that sodium butyrate, through this histone deacetylase inhibition, could induce the conversion of the pro-enzyme form of caspase-3 to the catalytically active effector protease and sensitize tumor cells to induction of apoptosis by staurosporine (Medina *et al*, 1997). In preliminary experiments, we found that caspase-3 activity was strongly increased when HT29 cells were cultured with both Fas ligand and 2 mM sodium butyrate, but did not significantly increase when the cells were incubated with butyrate or Fas ligand alone.

The Fas ligand-dependent pathway is one of the mechanisms by which cytotoxic T lymphocytes and NK cells trigger apoptosis of susceptible target cells. Sodium butyrate enhances the effect of IL-2 immunotherapy on peritoneal PROb cell carcinomatosis and induces complete regression of established tumors (Perrin *et al*, 1994). We have recently shown that the Fas system was also involved in colon cancer cell apoptosis induced by cytotoxic drugs (Micheau *et al*, 1997). Thus, enhancing the sensitivity of cancer cells to Fas ligand could help to eradicate colorectal cancer by potentiating the effect of immunotherapy or chemotherapy. Whether sodium butyrate, a low-toxic way for reducing colorectal cancer cell resistance to Fas-mediated apoptosis, will prove useful in the fight against colorectal carcinoma is an important topic for further research.

## Materials and Methods

### Cancer cell lines

The human HT29, HRT18, HCT15, HCT8R, SW620 and Caco-2 colorectal cancer cell lines were obtained from the American Tissue Culture Collection. SW480 human colon cancer line was a gift of Dr. A. Zweibaum (Villejuif, France). DHD/K12/PROb is a cell line established from a colon carcinoma that was induced by 1,2-dimethylhydrazine treatment in an inbred BD-IX rat (Caignard *et al*, 1985). All the cell lines were cultured as monolayers in a mixture of Ham-F10 and DMEM (1 : 1, vol/vol, Gibco, Paisley, Scotland), supplemented with 10% fetal bovine serum, 40 $\mu$g/ml gentamycin and 2 mM glutamine. Cells were detached using EDTA and trypsin in $Ca^{2+}$- and $Mg^{2+}$-free Hanks medium. The human T lymphoma cell line Jurkat was obtained from the American Tissue Culture Collection and cultured in RPMI1640 medium (Biowhittaker, Fontenay-sous-Bois, France) supplemented with 10% FBS and glutamine.

### Reagents

Fas ligand was obtained as the supernatant of Fas ligand-transfected Neuro-2a cells. The specificity of its toxicity on various cell lines has been reported previously (Rensing-Ehl *et al*, 1995). The supernatant of Neuro-2a cells that were transfected with the geneticin-resistance gene alone was used as a control. Both Fas ligand and control supernatants were collected after a 2 day culture of confluent Neuro-2a cells in DMEM supplemented with 1% fetal bovine serum. Supernatants were stored at $-80^{\circ}$C until used. Mouse monoclonal agonistic CH11 and antagonistic ZB4 antibodies to human Fas were obtained from Biovalley (Rockville MD). Sodium n-butyrate was bought from Sigma (Saint-Quentin Fallavier, France). Recombinant human TNF-$\alpha$ and IFN-$\gamma$ were obtained from Dainippon (Osaka, Japan) and Roussel-Uclaf (Romainville, France), respectively. Recombinant rat TNF-$\alpha$ and IFN-$\gamma$ were obtained from Serotec (Realef, Paris, France).

### Determination of apoptosis and cytotoxicity

Several methods were used for characterizing apoptosis in the treated cell cultures. Using phase contrast microscopy, apoptotic cells were observed as rounded up, irregularly-shaped cells that detached from the monolayer. Apoptotic cells were also labeled using a nick-ended DNA labeling method (APOPTAG, Oncor, Gaithersburgh, MD). The percentage of apoptotic cells was determined by collecting together

floating cells and adherent cells that were detached by EDTA-trypsin treatment. The cells were washed in PBS, then suspended in PBS supplemented with 1% paraformaldehyde and 5 $\mu$g/ml bisbenzimide H 33258 (Aldrich, Steinheim, Germany). Cells were incubated overnight at 4°C, then the percentage of apoptotic cells was determined on an epifluorescence microscope at 400 × magnification. Apoptotic cells were identified by chromatin condensation or fragmentation that clearly distinguished them from mitotic cells. The percentage of apoptotic cells was determined in populations of 200 to 400 cells.

### Western blot analysis

After detachment with trypsin and EDTA, subconfluent cultured cells were lysed in lysis buffer (150 mM NaCl, 1 mM $KH_2PO_4$, 1 mM EGTA, 1 mM $Na_3VO_4$, 5 mM $MgCl_2$ and 10% glycerol containing PMSF 0.1 mM, Aprotinin 0.15 U/ml, Pepstatin 1 $\mu$g/ml), and centrifugated for 15 min at 15 000 × *g*. The protein concentration was measured in the supernatant by using the micro BCA protein assay (Pierce, Asnieres, France). Equal amounts of proteins (75 $\mu$g) were separated by SDS–PAGE using a 12% polyacrylamide gel and electroblotted to PVDF membrane (BioRad, Ivry-sur-Seine, France). Blots were blocked with 5% non-fat milk in PBST (PBS with 0.1% Tween 20) and probed either with mouse monoclonal antibodies to Hsp90 (StressGen, Victoria, Canada) or to FADD (Transduction Laboratories, Lexington, KE) or with a rabbit polyclonal antibodies to Bcl-2, Bcl-x or Bax (Santa Cruz Biotechnology, Santa Cruz, CA). Following three 5 min washes in PBST, the blots were incubated with peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham, Les Ullis, France). The Amersham ECL Western Blotting Analysis System was subsequently used for protein detection. The results are representative of three separate experiments.

### Fas receptor flow cytometry analysis

Human colon cancer cells HT29 ($2 \times 10^6$) were cultured in 75 $cm^2$ culture flasks for two days in EMEM medium supplemented with 10% FBS and 2 mM glutamine (Biowhittaker), and treated or not for 6 or 24 h with sodium butyrate (1 or 4 mM) or rhIFN-$\gamma$ (200 IU/ml) in the same medium. Cells were collected by trypsinization, washed once in PBS containing 0.1% $NaN_3$ and placed on ice for 45 min with the mouse anti-Fas IgG, clone DX2, (Pharmingen, San Diego, CA) or a mouse isotype-matched control IgG (Sigma) diluted 1/800 in PBS containing 0.5% BSA and 0.1% $NaN_3$. After two washes in PBS, cells were incubated for 45 min with FITC-labeled goat anti-mouse IgG and analyzed on a Becton Dickinson flow cytometer for mean fluorescence intensity. Fas receptor antigen quantification was performed using the QIFIKIT assay (Dako, Trappes, France) as described by the manufacturer. Briefly, HT29 cells were compared with beads containing well defined quantities of mouse monoclonal antibody molecules that were used for the construction of a calibration curve representing the mean fluorescence intensity *versus* the antibody-binding capacity.

### Effect of tumor cell supernatants on Fas-mediated cytotoxicity

Fresh complete culture medium and tumor cell culture supernatants were desalted and concentrated 100-fold using an Ultrafree-15 centrifugal filter device with 10K membrane (Millipore, Molsheim, France), then reconstituted at four-times their initial concentration, in RPMI 1640 medium. A fixed volume (50 $\mu$l) was added to wells of 96-well flat-bottomed microculture plates that contained $5 \times 10^4$ Jurkat cells in 100 $\mu$l RPMI 1640 medium supplemented with 2 mM glutamine and 10% FBS. Serial dilutions of Fas ligand supernatant in 50 $\mu$l complete RPMI were added and the plates were cultured for 72 h at 37°C. The cells were counted after staining with trypan blue.

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