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ENHANCEMENT OF BUTYRATE-INDUCED DIFFERENTIATION OF HT-29 HUMAN COLON CARCINOMA CELLS BY 1,25-DIHYDROXYVITAMIN D_3*

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Abstract—The individual and combined effects of sodium butyrate (NaB) and 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) on inhibition of cell growth and initiation of enterocytic differentiation were investigated in established HT-29 human colonic adenocarcinoma cells. 1,25-$(OH)_2D_3$ alone caused some growth restriction but failed to induce differentiation of HT-29 carcinoma cells into a more benign enterocytic phenotype, as assessed by the appearance of mucin-producing colonocytes (goblet cells), increased alkaline phosphatase activity, and the generation of flat foci. NaB, in contrast, produced considerable biochemical and morphologic differentiation along the enterocyte maturation pathway. Combined exposure of HT-29 cells to both NaB and 1,25-$(OH)_2D_3$, however, significantly augmented the frequency of differentiated colonocytes, growth inhibition, extent of goblet cell maturation attained, and level of alkaline phosphatase activity over that seen with NaB alone. These data suggest that the *in vitro* "differentiation" response of human carcinoma cells is a complex process which, like normal cell maturation within the colonic crypts *in vivo*, is modulatable (both qualitatively and quantitatively) as a function of inducer composition.

The common occurrence of the 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$)¶ receptor in various tumor cells [1, 2] suggests that the active form of vitamin D_3 may have pharmacological actions impacting on cell proliferation and differentiation. It has been shown, in fact, that 1,25-$(OH)_2D_3$ restricts growth of HL-60 myeloid leukemia cells with subsequent induction of differentiation along the monocyte/macrophage lineage [3, 4]. Although many carcinoma cells possess the 1,25-$(OH)_2D_3$ receptor and exhibit growth restriction in response to 1,25-$(OH)_2D_3$ treatment [1, 5, 6], modulation of differentiation in non-hematopoietic tumor cells has not been demonstrated conclusively. Assessment of the differentiated stages of epithelial cells, with a few exceptions, is not as readily done as in the hematopoietic system. Among these exceptions are certain lines of human colon carcinoma cells which exhibit inducible characteristics of enterocytic differentiation such as construction of a brush border, elaboration of surface microvilli, secretion of mucin and increased alkaline phosphatase (AP) activity [7, 8]. We have investigated the effect of 1,25-$(OH)_2D_3$ on expression of differentiated traits by HT-29 human colonic adenocarcinoma cells, an established line in which differentiation has been found to be inducible by polar compounds such as sodium butyrate (NaB) [7] and by alteration of nutritional conditions [9]. We report here that 1,25-$(OH)_2D_3$ alone did not induce differentiation of HT-29 cells, as assessed by functional (appearance of mucin secreting cells), biochemical (increase of AP activity) and morphological (generation of flat foci and goblet cell maturation) criteria, and only slightly reduced cell growth rate. However, 1,25-$(OH)_2D_3$ greatly enhanced the butyrate-induced differentiation of HT-29 cells into a more benign enterocytic phenotype, resulting in augmentation of both the frequency of differentiated colonocytes and the extent of maturation achieved.

MATERIALS AND METHODS

Compounds. 24*R*,25-Dihydroxyvitamin D_3 (24,25-$(OH)_2D_3$) and 1,25-$(OH)_2D_3$ were gifts from Dr Uskokovic, Hoffmann-La Roche (Nutley, NJ). 25-Hydroxyvitamin D_3 (25-OHD_3) and vitamin D_3 (D_3) were purchased from Duphar B.V., Veenendall, Holland, and the Sigma Chemical Co. (St Louis, MO) respectively. NaB was a product of Pfaltz & Bauer (Waterbury, CT).

Cell culture. HT-29 cells (a gift of Dr J Fogh, Sloan-Kettering Institute, Rye, NY) were propagated in RPMI-1640 (GIBCO, Grand Island, NY) containing 10% (v/v) heat-inactivated fetal bovine serum at 37° in a humidified atmosphere of 5% CO_2:95% air. For induction of differentiation, medium in a 30% confluent 35 mm culture dish was changed to fresh medium containing either vitamin D (concentration range as indicated in text), NaB (2 or 5 mM), or both. Vitamin D metabolites were dissolved in ethanol and added to medium before filter-through sterilization (final concentration of ethanol in medium was

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¶ Abbreviations: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25-OHD_3, 25-hydroxyvitamin D_3; D_3, vitamin D_3; NaB, sodium butyrate; AP, alkaline phosphatase; and DMSO, dimethyl sulfoxide.

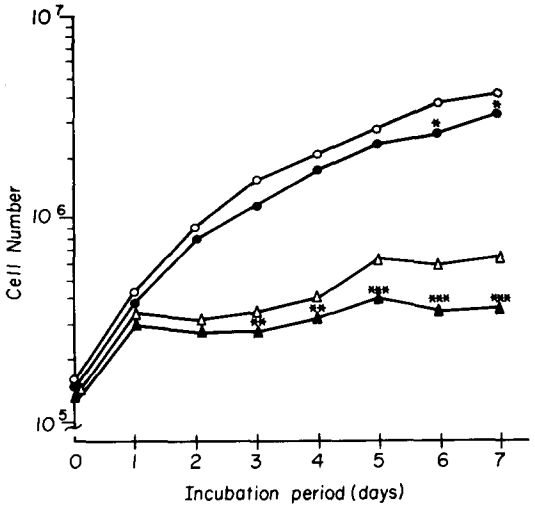

Fig. 1. Restriction of cell growth by NaB and/or 1,25-$(OH)_2D_3$. HT-29 cells were incubated in the medium for 24 hr and then propagated in a control or experimental medium for up to 7 days. The cell number at day 0 indicates cell density after 24 hr of incubation in the medium. Each value is the mean from six cultures. Standard deviations were less than 13% for all points. Key: (○—○) control; (●—●) 10^{-7} M 1,25-$(OH)_2D_3$; (△—△) 2 mM NaB; and (▲—▲) 10^{-7} M 1,25-$(OH)_2D_3$ and 2 mM NaB. Significantly different from corresponding control level (* $P < 0.01$); significantly different from corresponding NaB level (** $P < 0.01$, and *** $P < 0.001$).

0.1%). Control and NaB-treated cultures also contained 0.1% ethanol. All media were changed every 3 days. The extent of cell differentiation was assessed between days 4 and 7, after the change-over to experimental medium.

Mucin production. Assessment of percent mucin-producing cells was done by direct observation of unstained monolayers by phase-contrast microscopy, or by staining of formalin- or methanol-fixed cells with Giemsa [10]. That the mature goblet-like cells so detected were actually producing mucins was confirmed on representative cultures by the metachromatic staining procedure [7] and by indirect immunofluorescence detection using anti-mucin antibodies [11].

Alkaline phosphatase assay. AP activity was measured by the hydrolysis of *p*-nitrophenyl phosphate [12] and expressed in units per milligram of protein. Protein was determined according to the method of Lowry *et al.* [13].

Morphological change. The extent of goblet cell differentiation, the generation of "flat foci", dome formation (indicative of transepithelial fluid transport), and the incidence of "rough foci" consisting of piled cells were quantitated microscopically using criteria described previously in detail [7].

RESULTS

Effects of NaB and 1,25-$(OH)_2D_3$ *on cell growth.* Growth assessments utilized cultures exposed to control medium (0.1% EtOH), and media containing 1,25-$(OH)_2D_3$ (10^{-7} M), NaB (2 mM), or both compounds (10^{-7} M 1,25-$(OH)_2D_3$ and 2 mM NaB) for 1–7 days (Fig. 1). All cells continued to propagate for

Table 1. Enhancement of NaB-induced mucin production by 1,25-$(OH)_2D_3$

Compound given	Concentration (M)	Mucin secreting cells (% of total cells)
Control		3 ± 2 (5)
1,25-$(OH)_2D_3$	1×10^{-8}	3 ± 2 (6)
	1×10^{-7}	5 ± 4 (6)
	1×10^{-6}	2 ± 1 (6)
NaB	2×10^{-3}	44 ± 4* (5)
	5×10^{-3}	39 ± 9* (4)
NaB + 1,25-$(OH)_2D_3$	2×10^{-3} + 1×10^{-7}	91 ± 9*† (9)

HT-29 cells were incubated in experimental medium for 6 days, and mucin secretion was observed. The media of control and NaB experiments contained 0.1% ethanol. Two hundred cells/culture were counted for mucin production. Each value is the mean ± SD from the number of cultures shown in parentheses.

* Significantly different from control, $P < 0.001$.

† Significantly different from NaB, $P < 0.001$).

24 hr after the change-over to experimental medium (day 1). At a concentration of 10^{-7} M, 1,25-$(OH)_2D_3$ produced a slight, but significant ($P < 0.01$), growth inhibition (20–30% reduction in population density compared to corresponding control levels). In contrast, greatly restricted growth of NaB-treated cells was evident as early as day 2 of treatment and thereafter. Exposure to both NaB and 1,25-$(OH)_2D_3$ further inhibited cell growth after day 3 of treatment (~20% greater inhibition of growth compared to cells culture in NaB alone) followed by a more prominent reduction after day 5 (~40% inhibition compared to NaB-treated cells).

Induced mucin accumulation and morphologic alterations. The majority of HT-29 cells (~97%) cultured under control conditions (i.e. ethanol alone) failed to accumulate significant deposits of mucin. Exposure to 1,25-$(OH)_2D_3$ alone at concentrations ranging from 10^{-6} to 10^{-8} M did not alter the incidence of mucin-producing colonocytes; NaB, in contrast, increased the fraction of mucin-producers (and the extent to which mucin accumulated, see below) to 44% of total cells (Table 1). Experiments using various concentrations of NaB indicated that NaB-treated commitment to mucin production was maximal in medium containing 2 mM NaB and could not be enhanced by increasing the NaB concentration (data not shown, see also Table 1). The exact reason for this is not clear but may relate to similar findings in various cell types implicating the NaB concentration range of 2–5 mM as optimal for induction of proliferative restriction and differentiated function [14–16]. Whether 1,25-$(OH)_2D_3$ will enhance the response of HT-29 cells to submaximal concentrations (i.e. less than 2 mM) of NaB remains to be determined. When both NaB and 1,25-$(OH)_2D_3$ were used together at a concentration of 2 mM and 10^{-7} M, respectively, the number of mucin secreting HT-29 cells was greatly increased from the level induced by NaB alone (an average of 2.4-fold) after day 4. Combined use of both agents did not alter the time course of appearance of mucin secreting cells

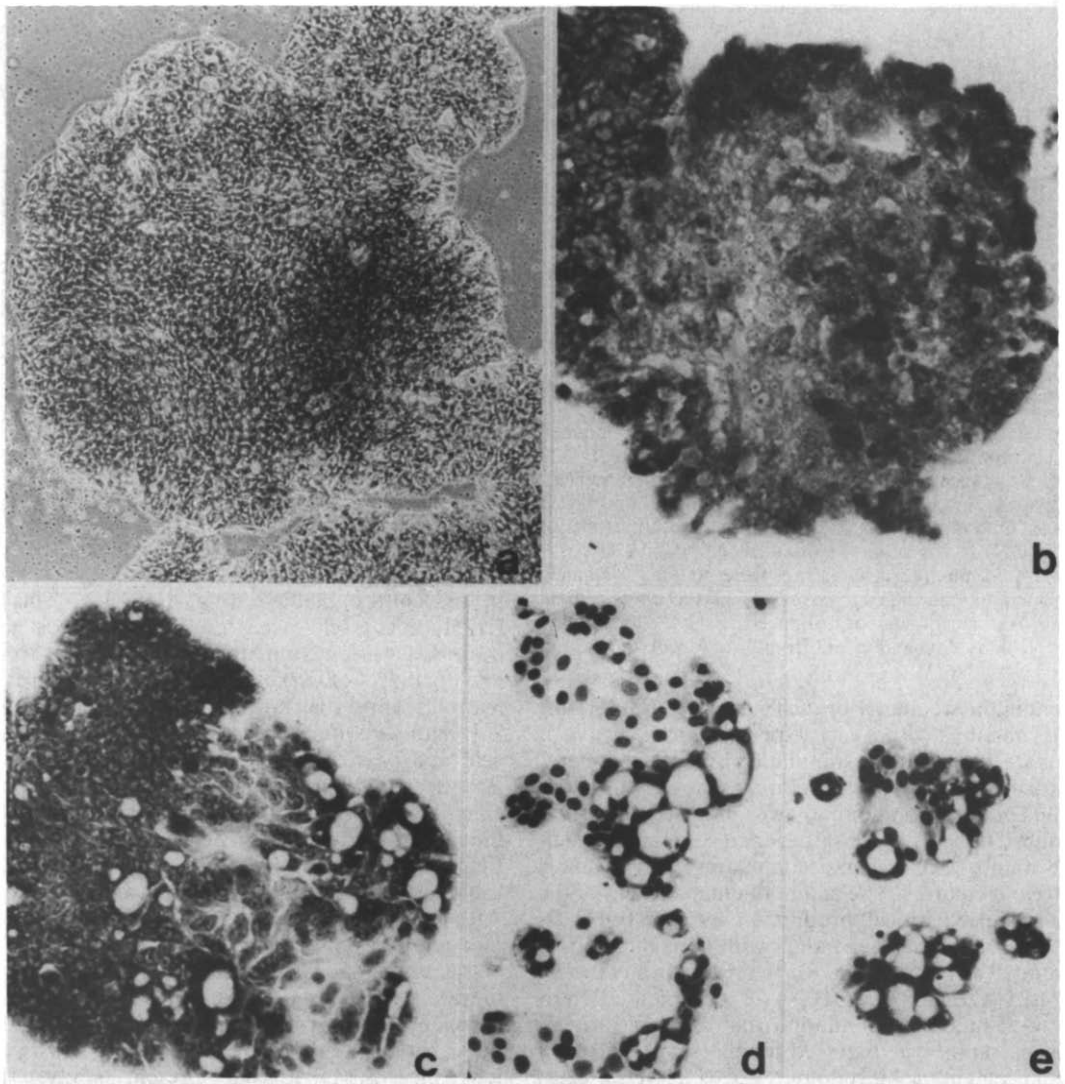

Fig. 2. Morphological response of HT-29 human colon carcinoma cells to NaB, 1,25-$(OH)_2D_3$, or the combination of both agents. (a) Untreated control cells; growth is in tight compacted clusters, individual cells are distinguishable only by phase contrast microscopy, and there is no evidence of either goblet cell differentiation or tendency to generate flat foci. (b) 10^{-7} M 1,25-$(OH)_2D_3$ for 9 days; morphology is very similar to control cells. (c) 2 mM NaB for 9 days; a flat focus is evident in the right half of this colony, and numerous mucin deposits are found in a number of cells indicative of initiation of goblet cell differentiation. (d and e) 2 mM NaB + 10^{-7} M 1,25-$(OH)_2D_3$ for 9 days; extensive goblet cell differentiation is evident, occurring frequently at the periphery of a flat focus. Both the frequency of goblet-like cells and the extent of morphologic maturation are consistently increased in cultures treated with both compounds. (a) Phase-contrast microscopy; (b–e) Giemsa stain, brightfield microscopy. All panels × 130.

induced by NaB alone (data not shown). Morphological changes were assessed for four groups of cultures: (a) untreated control, (b) 1,25-$(OH)_2D_3$ (10^{-7} M) treated cells, (c) NaB (2 mM) treated cells, and (d) cells treated with both 1,25-$(OH)_2D_3$ (10^{-7} M) and NaB (2 mM) for 9 days (Fig. 2). Control and 1,25-$(OH)_2D_3$-treated cells did not exhibit evidence of goblet cell differentiation nor form flat foci. Mucin-accumulating colonocytes were quite obvious in NaB-treated populations approaching an incidence of 44% (Table 1); such differentiated cells were generally found within or at the periphery of flat foci and only rarely within rough foci (which appeared to consist of undifferentiated stem cells). The combined treatment with NaB and 1,25-$(OH)_2D_3$ resulted in dramatic increases in both the frequency (Table 1) and extent (Fig. 2) of goblet-like enterocyte maturation.

Specificity of 1,25-$(OH)_2D_3$ *to enhance NaB-induced mucin production.* We next examined whether the effect of 1,25-$(OH)_2D_3$ on NaB-induced mucin production was dose dependent and whether 1,25-$(OH)_2D_3$ was a specific inducer for the enhancement. Although the incidence of NaB-induced mucin-accumulating cells differed from culture to culture (15–45% of population), the 2- to 3-fold enhance-

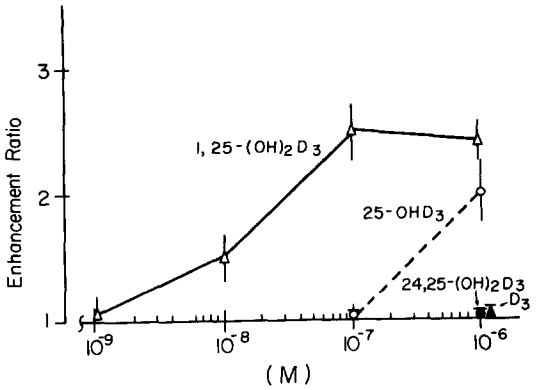

Fig. 3. Dose-dependent enhancements of NaB-induced mucin production by 1,25-$(OH)_2D_3$ and 25-OHD_3. HT-29 cells were incubated in the medium for 24 hr and then propagated in experimental medium containing 2 mM NaB alone, or both 2 mM NaB and various concentrations of 1,25-$(OH)_2D_3$ or other metabolites of vitamin D for 7 days. Two hundred cells/culture were counted for mucin production. The data are expressed as the enhancement ratio over mucin secretion induced by NaB alone. Each value is the mean ± SD from 3 to 7 incubations.

ment in the frequency of such cells by 1,25-$(OH)_2D_3$ was consistent within each experiment irrespective of the rate of maturation stimulation by NaB treatment. Data were expressed, therefore, as the enhancement ratio of test compounds over mucin production induced by NaB alone rather than comparing absolute numbers of mucin-accumulating cells in many different cultures. The extent of enhancement of the NaB-induced mucin production by 1,25-$(OH)_2D_3$ was concentration dependent with a maximal effect obtained at 10^{-7} M (Fig. 3). A precursor metabolite of 1,25-$(OH)_2D_3$, 25-OHD_3, also augmented NaB-induced mucin accumulation, but a 10-fold higher concentration was required for the same degree of enhancement achieved by 1,25-$(OH)_2D_3$. Morphological changes (data not shown) and mucin production induced by 25-OHD_3 correlated well with each other. A metabolite of 25-OHD_3, 24,25-$(OH)_2D_3$, and vitamin D_3 itself did not show any enhancing activity at a concentration of 10^{-6} M. None of these metabolites induced the appearance of mucin secreting cells or morphological changes when added alone (data not shown).

AP activity. Activity of a marker enzyme of HT-29 differentiation, AP, was next examined for cell cultures treated with 1,25-$(OH)_2D_3$ alone, NaB alone or the combined use of both agents. Treatment with 1,25-$(OH)_2D_3$ did not increase AP activity from the control level, whereas NaB greatly increased (1042% above control) enzyme activity (Table 2). When both agents were added, enzyme activity was increased further (2641% above control), to a level, compared with the NaB control as 100%, of 240%.

Table 2. Enhancement of NaB-induced increase of alkaline phosphatase activity by 1,25-$(OH)_2D_3$

Compound given	AP activity (units/mg protein)	(%)
Control	0.95 ± 0.12	100
1,25-$(OH)_2D_3$ (10^{-7} M)	1.08 ± 0.08	113
NaB (2 mM)	10.85 ± 0.55*	1142
NaB (2 mM) + 1,25-$(OH)_2D_3$ (10^{-7} M)	26.04 ± 0.92*†	2741‡, 240§

HT-29 cells were incubated in control or experimental medium for 7 days and AP activity was measured by the method of Herz and Nitowsky [12]. Each value is the mean ± SD from three cultures.

* Significantly different from control, $P < 0.001$.
† Significantly different from NaB, $P < 0.001$.
‡ Against control.
§ Against NaB.

DISCUSSION

HT-29 human colon adenocarcinoma cells can be induced to differentiate into mature enterocytes (of both absorptive columnar and goblet cell phenotypes) upon removal of glucose or addition of NaB to the culture medium (e.g. Ref. 17). This differentiation is characterized by monolayer polarization, development of an apical face brush border, and high level of expression of intestinal microvillar hydrolases and structural proteins such as villin [18–20]. Although dimethyl sulfoxide (DMSO), retinoic acid and *N,N*-dimethylformamide have significant effects on various growth properties of colorectal carcinoma cells including prolongation of doubling time, decreased saturation density and *in vitro* tumorigenicity as treated by growth in soft agar, only NaB causes marked changes in cellular morphology and AP activity when added to glucose-containing culture medium [21–23].

It has been shown that 1,25-$(OH)_2D_3$ inhibits proliferation of a variety of tumor cell types including those derived from malignant melanoma [6, 24], hepatic carcinoma [25], breast carcinoma [6] and myeloid leukemia [3, 4]. In our present study, slight reductions in final population density in 1,25-$(OH)_2D_3$-treated cultures occurred. In contrast, NaB greatly inhibited the growth of HT-29 cells after a lag period of 24 hr. Such a lag period was also observed in NaB-induced growth inhibition of HeLa S3 cells [26] and rat hepatic carcinoma cells [10]. Restricted proliferation, typically observed during *in vitro* treatment of tumor cells with millimolar concentrations of this 4-carbon fatty acid, is mediated (at least in part) by a block in the G_1 phase of the cell cycle [27, 28], with an arrest, specifically, in exit from the G_{1A} substrate [29], effectively preventing transition through the G_1 phase. Combined use of NaB and 1,25-$(OH)_2D_3$ resulted in a slight but significant reduction of final population density compared to that achieved by NaB alone. In contrast to the striking synergistic effect of a combined use of NaB and 1,25-$(OH)_2D_3$ on HT-29 cell differentiation, however, the reduced cell density induced by the combination seemed only additive.

There was no evidence of cell differentiation induced by 1,25-$(OH)_2D_3$ in the present study; neither increase of mucin production and AP activity, nor morphological changes characteristic of enterocytic maturation occurred in HT-29 cells with

1,25-$(OH)_2D_3$ alone. It was apparent, however, that both the frequency of mucin-producing enterocytes and the extent of differentiation along the goblet cell pathway were greatly increased in cultures treated with both NaB and 1,25-$(OH)_2D_3$ compared to cells exposed to NaB alone. Brehier and Thomasset [30] reported that treatment of HT-29 cells with 1,25-$(OH)_2D_3$ alone increases maltase activity. Since only maltase activity was measured for assessment of differentiation trait in their study and HT-29 clones may vary in 1,25-$(OH)_2D_3$ receptor number and sensitivity, it is difficult to draw a conclusion as to how their results relate to the lack of enterocytic differentiation by 1,25-$(OH)_2D_3$ in our present study. There have been some studies on cell differentiation induced by the combination of NaB and another agent. The glucocorticoid-induced increase of AP in HeLa S3 cells is synergistically enhanced by NaB [15]. Increased AP activity in HT-29 cells treated with NaB is greatly enhanced when the treatment is achieved in hyperosmolar medium [16]. Yoneda *et al.* [14] reported that treatment of clonal rat osteosarcoma cells with 1,25-$(OH)_2D_3$ alone increases AP activity and NaB enhances the effect. The effect induced by the combination of the two agents, however, seems additive rather than synergistic.

AP in intestinal cells is located on brush border membranes; its activity is considerably higher in more differentiated villus cells as compared to the less differentiated cells at the crypt base [22]. Therefore, AP can serve as a marker enzyme for assessment of the differentiated status of colon carcinoma cells. AP synthesized by HT-29 cells is of the intestinal type [16] in contrast to the HeLa S3 cell line which produces the term-placental type [31]. In certain conditions, 1,25-$(OH)_2D_3$ can stimulate AP activity in rat intestine, although the increase in enzymic activity does not correlate with the time–course of intestinal calcium transport induced by 1,25-$(OH)_2D_3$ [32]. Similar to the observation that AP produced by HT-29 is refractory to modulation by glucocorticoids [16], AP activity produced by HT-29 in the present study was not altered by 1,25-$(OH)_2D_3$.

It has been speculated that 1,25-$(OH)_2D_3$ interacts with a specific receptor in intestinal cytosol and the complex is translocated to the nucleus, resulting in transcription of specific mRNAs that code for calcium transport proteins [33, 34]. Tumor cell lines responsive to 1,25-$(OH)_2D_3$ for inhibition of cell growth, including HT-29 [30], possess the 1,25-$(OH)_2D_3$ receptor [6, 24], suggesting that the actions of 1,25-$(OH)_2D_3$ on neoplastic cells also involve nuclear events. Elucidation of mechanisms underlying the enhanced enterocyte differentiation in HT-29 cells induced by interaction of 1,25-$(OH)_2D_3$ and NaB, however, is complicated by uncertainties as to the mode of action of NaB in cell maturation. Modulation of a variety of hormone receptors by NaB has been described, suggesting varied effects in different cell types. Glucocorticoid-induced increase in AP activity in HeLa S3 cells is synergistically augmented by NaB treatment despite a reduced number of glucocorticoid receptors [15]. Both the functional response to 1,25-$(OH)_2D_3$ assessed by induction of 25-OHD_3-24-hydroxylase and 1,25-$(OH)_2D_3$ receptor are reduced by NaB treatment in pig kidney cells [35]. In contrast, treatment of rat osteosarcoma cells with NaB increases both nuclear 1,25-$(OH)_2D_3$ receptor binding sites and 1,25-$(OH)_2D_3$-induced increase in AP activity [14]. Thus, the effect of NaB on 1,25-$(OH)_2D_3$ receptor in HT-29 remains to be investigated.

While NaB affects nuclear histone composition, and transcription of particular genes [36], the relevance of such events to subsequent cytoarchitectural events associated with the generation of a more-differentiated phenotype is less well-understood. The morphologic response of HT-29 cells to NaB, however, can be modulated by altering growth medium calcium concentrations (Higgins, manuscript in preparation). Data from several cell systems suggest that alterations on calcium transport may play a crucial role in induction of differentiation of neoplastic as well as normal cells. DMSO-induced commitment of murine erythroleukemia cells to erythrocyte differentiation can be inhibited by ethylene glycol-bis(aminoethyl ether)-tetraacetic acid (EGTA), while treatment of cells with DMSO in the presence of ionophore A23187 causes cells to initiate commitment without the lag normally observed after treatment with DMSO alone [37]. A relatively high concentration of extracellular Ca^{2+} is required for terminal differentiation of mouse epidermal cells [38]. The erythropoietin-sensitive formation of normal murine erythroid colonies is stimulated by A23187 treatment and inhibited by EGTA [39]. It is possible, therefore, that 1,25-$(OH)_2D_3$ enhances NaB-induced cell differentiation by augmenting cellular calcium transport. Indeed, 1,25-$(OH)_2D_3$ alters the lipid composition of brush border membrane [40]. This may result in an augmented fluidity state of the brush border membrane and, thus, greater activity of calcium transport. We, therefore, cannot exclude the possibility that 1,25-$(OH)_2D_3$ enhances NaB-induced differentiation as the consequence of altered calcium regulation resulting from the membrane effect of 1,25-$(OH)_2D_3$.

Certain intestinal flora lower the incidence of colon cancer in experimental animals [41]. Butyrate is a natural fermentation product of colonic bacterial flora and its concentration in lumen of the large bowel is likely to be as high as that used in our study [42]. It is possible, therefore, that the combination of NaB and 1,25-$(OH)_2D_3$ plays an important role in the regulation of colonic cell differentiation and, perhaps, transformation *in vivo*.

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