An Increase in Histone Acetylation and IL-2 Antagonizing the Immuno inhibitory Effect Are Necessary for Augmentation by Butyrate of in Vitro Anti-TNP Antibody Production

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We investigated the role of histone acetylation in the promotion of antigen-specific antibody production in murine B cells induced by sodium butyrate (NaBu) plus interleukin 2 (IL-2). NaBu dose dependently increased the acetylation levels of histone H4 at concentrations which effectively enhanced anti-trinitrophenyl (TNP) antibody production in the presence of IL-2. Among other short-chain fatty acids and NaBu analogs, propionate, valerate and vinylacetate were effective in the presence of IL-2 in increasing both antibody production and the histone H4 acetylation level, but acetate, α-, β- and γ-hydroxybutyrates and α-, β- and γ-aminobutyrate were not effective, even in the presence of IL-2. The effect of the specific histone deacetylase inhibitor trichostatin A (TSA), which enhances anti-TNP antibody production without IL-2, was markedly inhibited by adding NaBu simultaneously. However, the effect of TSA was neither inhibited nor potentiated by NaBu in the presence of IL-2. Splenic B cells treated with NaBu, TSA and both together in the presence or absence of IL-2 showed almost the same increased acetylation level of histone H4. These results suggest that the NaBu-induced enhancement of anti-TNP antibody production in the presence of IL-2 is mediated through a moderate increase in the level of histone acetylation and that NaBu has both stimulating and inhibiting activities for anti-TNP antibody production, the latter of which is overcome by IL-2.

Key words: butyrate; antibody production; histone acetylation; interleukin 2; trichostatin A

Butyrate, a physiologically occurring agent, induces the differentiation of various types of cells. However, only a limited amount of information exists about its effect on the differentiation of immune cells. Butyrate induces the Burkitt lymphoma cell Raji to differentiate towards plasmablast or plasma cell morphology, and also causes an increase in the expression of some B-cell differentiation markers. It also induces B-cell antigens in the chronic myeloid leukemic cell line K562. Although the mechanism of these butyrate actions is largely unknown, due to its ability to inhibit histone deacetylase, it has been suggested that butyrate modulates gene transcription by inducing histone hyperacetylation. Butyrate, however, is a pleiotropic agent and is not a specific inhibitor of histone deacetylase. Thus, not all the data are consistent with a mechanism in which histone hyperacetylation is involved in cell differentiation caused by butyrate.

We previously reported that antibody production in murine splenocytes which had been stimulated with sheep red blood cell or trinitrophenyl-lipopolysaccharide (TNP-LPS) was markedly enhanced by sodium butyrate (NaBu). In murine splenic B cells, however, NaBu does not promote anti-TNP antibody production, unless interleukin 2 (IL-2), which alone has no effect, is added to the culture. However, trichostatin A (TSA), a more specific inhibitor of histone deacetylase, markedly increases anti-TNP antibody production in both whole splenocytes and splenic B cells without adding IL-2. Thus, the IL-2 requirement differs between the effects of NaBu and TSA. The effect of TSA is accompanied with a concomitant, moderate increase in the level of histone acetylation in B cells, suggesting that an increase in histone acetylation has a role in promoting anti-TNP antibody production. NaBu also moderately increased the level of histone acetylation in B cells both in the presence and absence of IL-2. The question exists as to whether the increase in histone acetylation is involved in the NaBu-induced enhancement of anti-TNP antibody production in the presence of IL-2. We report here that although a discrepancy in the IL-2 requirement exists between the effects of the 2 inhibitors, the effect of NaBu is also presumably mediated through a moderate increase in the level of histone acetylation. We also show data that may explain this discrepancy in the IL-2 requirement.

MATERIALS AND METHODS

Mice Female BALB/c mice were purchased from Charles River Japan (Yokohama). They were kept in our animal care facility and were used between 9 and 12 weeks of age.

Reagents RPMI 1640 medium and fetal calf serum were purchased from ICN (Aurora, OH, U.S.A.) and Life Technologies (Rockville, MD, U.S.A.), respectively. NaBu, 1-α-amino-n-butryic acid, 1-β-amino-n-butryic acid, γ-amino-n-butryic acid, d-(-)-β-hydroxy-n-butryic acid sodium salt and γ-hydroxy-n-butryic acid sodium salt were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). TSA and dL-α-hydroxy-n-butryic acid sodium salt were purchased from Wako Pure Chemical Industries (Osaka). Propionic acid and n-valeric acid were from Tokyo Chemical Industry (Tokyo). Vinlylactic acid was from Sigma-Aldrich Japan (Tokyo), and recombinant murine IL-2 was from Genzyme (Cambridge, MA, U.S.A.). All acids diluted with phosphate-buffered saline (PBS) were neutralized with NaOH, adjusted to 1 m, and filtered before adding them to the cultures. TNP-LPS was prepared by reacting LPS from Escherichia coli 055: B5 (Sigma Chemical Co.) with 2,4,6-trinitrobenzenesulfonate, as described by Jacobs and Morrison.

Anti-TNP Antibody Production B cells (3.5×10⁶/well) or whole cells (8×10⁶/well) prepared from the spleens of 3–4 mice were seeded in triplicate in 24-well plates (Nunc, Roskilde, Denmark). They were cultured with TNP-LPS

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(5 μg/ml) in 1.5 ml of RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml of penicillin G and 100 μg/ml of streptomycin at 37 °C in 5% CO₂ and 95% air. NaBu, TSA or other agents were added to the wells before seeding the cells. After 5 d, the number of anti-TNP plaque-forming cells (PFC) was determined by the method of Rittenberg and Pratt. Splenic B cells were prepared according to the method of Leibson et al. Briefly, spleen cells from mice that had been injected intraperitoneally with 20 μl of anti-thymocyte serum (Wako Pure Chemical Industries) 2 d before the mice were killed were incubated on plastic dishes for 90 min at 37 °C. The nonadherent cells were then collected, and after treating them with anti-Thy-1.2 antibody (1/500, Serotec, Kidlington, UK) for 30 min at 4 °C, they were incubated with low toxic rabbit complement (1/15, Cedarlane Laboratories, Hornby, Canada) for a further 40 min at 37 °C. Thy-1.2⁺ cells in this preparation were usually less than 3% when determined by flow cytometric analysis. Typical data from several repeated experiments were presented.

**Extraction of Cellular Histones and Analysis of Acetylated Histone H4** B cells prepared from the spleens of 3—4 mice were cultured for 12 h as described above. Histones from B cells were isolated according to the procedure of Cousins et al. B cells from 2 wells of the 24-well plates were pooled and centrifuged. Cell lysates were prepared in an ice-cold lysis buffer (10 mm Tris—HCl, 25 mm sodium metabisulfite, 1% Triton X-100, 10 mm MgCl₂, 8.6% sucrose, pH 6.5), and were centrifuged at 1000×g for 10 min. The resulting nuclei were washed 3 times with the lysis buffer and once with 10 mm Tris—HCl (pH 7.4) containing 13 mm EDTA. The pellets were then suspended in ice-cold water, and concentrated H₂SO₄ was added to the suspension to give a concentration of 0.2 m. After incubation at 0 °C for 1 h, the suspension was centrifuged for 5 min at 15000 rpm using a microfuge, and the supernatant was mixed with 10 volumes of acetone. After an overnight incubation at −20 °C, the precipitate was collected by centrifugation and was air-dried. This acid soluble histone fraction was dissolved in water, and its protein concentration was determined by the method of Lowry et al. The acetylation of histones was analyzed by acid—urea—Triton gel electrophoresis. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 and dried. The level of histone H4 acetylation was determined using a densitometer (Molecular Dynamics 300 A-T), and was calculated as the sum of the percentage of an appropriate histone H4 form multiplied by the number of acetylated lysine residues per molecule/100. Data from 3 independent experiments or typical data from at least 2 repeated experiments were presented.

**RESULTS**

We reported previously that NaBu increased the acetylation level of histone H4 in murine splenic B cells at a dose optimal for enhancing antibody production. The dose-response of butyrate-increased histone H4 acetylation was compared with that of anti-TNP antibody production enhanced by butyrate in the presence of IL-2 (Fig. 1). The degree of histone H4 acetylation was estimated by electrophoretic analysis on acid—urea—Triton polyacrylamide gels that resolve differentially acetylated forms of histone H4 (Fig. 1A). In the absence of NaBu, the nonacetylated and monoacetylated forms of histone H4 were dominant over a faint band corresponding to diacetylated histone H4. Adding NaBu led to a dose-dependent increase in the amount of the diacetylated form of histone H4. Figure 1B shows the levels of histone H4 acetylation calculated and anti-TNP antibody production estimated in other parallel cultures. NaBu dose dependently increased the acetylation levels of histone H4 at concentrations effective in enhancing anti-TNP antibody production.

Propionate and valerate, like NaBu, are effective inhibitors of histone deacetylation. The effects of various saturated short-chain fatty acids on the anti-TNP PFC response in whole splenocytes were examined (Fig. 2). Both propionate and valerate increased anti-TNP antibody production, although their effects were less marked than the effect of NaBu. Acetic acid was ineffective at the same concentrations. Figure 3A shows the effects of these fatty acids on anti-TNP antibody production in splenic B cells. Propionate and valerate, like NaBu, increased the anti-TNP antibody response in the presence of IL-2 which alone has no stimulating effect, but no increase occurred in the absence of IL-2. Vinylacetate, a NaBu analog, was also effective only in the presence of IL-2, but acetate was ineffective even in the presence of IL-2. Figure 3B shows the levels of histone H4 acety-
Fig. 2. Effects of Various Short-Chain Fatty Acids on the Anti-TNP PFC Response in Whole Splenocytes

Murine splenocytes were stimulated with TNP-LPS (5 μg/ml) in the presence of varying concentrations of short-chain fatty acids as indicated. After 5 d, the number of anti-TNP PFC was determined. The data are expressed as means of triplicate wells. Bars indicate S.D.

(A) Vinylacetate
(B) Valerate
(C) NaBu
(D) Propionate
(E) Acetate
(F) None

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<th>Anti-TNP PFC × 10³/culture</th>
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<td></td>
<td>0.25</td>
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<tr>
<td>Valerate</td>
<td>0.6</td>
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<tr>
<td>NaBu</td>
<td>0.45</td>
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<tr>
<td>Propionate</td>
<td>0.6</td>
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<td>Acetate</td>
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Fig. 3. Effects of Various Short-Chain Fatty Acids on the Anti-TNP PFC Response and Histone H4 Acetylation in Splenic B Cells

(A) Splenic B cells were stimulated with TNP-LPS (5 μg/ml) in the presence of the indicated short-chain fatty acids with (shaded columns) or without 100 U/ml of IL-2 (open columns). After 5 d, the number of anti-TNP PFC was determined, and the data are expressed as means of triplicate wells. Bars indicate S.D. (B) Splenic B cells were stimulated with TNP-LPS (5 μg/ml) in the presence of the indicated short-chain fatty acids and 100 U/ml of IL-2 for 12 h. Fifteen micrograms of isolated histones were subjected to acid-urea–Triton gel electrophoresis. The gel was then stained with Coomassie blue. The level of histone H4 acetylation was analyzed using a densitometer, calculated as described in Materials and Methods, and expressed as a fold-change relative to the control cultures. The data are expressed as means±S.D. of three independent experiments. Values that are significantly different from those of the control are indicated by: * p<0.01, ** p<0.05. The statistical significance was analyzed by the Student’s paired t-test. The agent concentrations were: 0.6 mM NaBu, 0.75 mM valerate and 0.3 mM vinylacetate.

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<tr>
<td>IL-2</td>
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<tr>
<td>IL-2 + NaBu</td>
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<tr>
<td>IL-2 + Propionate</td>
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<tr>
<td>IL-2 + Acetate</td>
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Fig. 4. Effects of Hydroxbutyrates and Aminobutyrates on the Anti-TNP PFC Response and Histone H4 Acetylation in Splenic B Cells

Splenic B cells were stimulated with TNP-LPS (5 μg/ml) in the presence of the indicated hydroxbutyrates or aminobutyrates with 100 U/ml of IL-2. The agent concentrations were: 0.3 mM NaBu, 0.8 mM hydroxbutyrate and 0.8 mM aminobutyrate. (A) After 5 d, the number of anti-TNP PFC was determined, and the data are expressed as means of triplicate wells. Bars indicate S.D. (B) Fifteen micrograms of isolated histones isolated after 12 h were subjected to acid-urea–Triton gel electrophoresis. The gel was then stained with Coomassie blue. The level of histone H4 acetylation was analyzed using a densitometer, calculated as described in Materials and Methods, and expressed as a fold-change relative to the control cultures.

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<th>Relative acetylation level of histone H4</th>
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<tbody>
<tr>
<td>IL-2</td>
<td>**</td>
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<tr>
<td>IL-2 + γ-Aminobutyrate</td>
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<tr>
<td>IL-2 + β-Aminobutyrate</td>
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<td>IL-2 + α-Aminobutyrate</td>
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<tr>
<td>IL-2 + NaBu</td>
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Figures 4A and 4B show the effects of both hydroxbutyrates and aminobutyrates on the anti-TNP PFC response and the levels of histone H4 acetylation, respectively, in splenic B cells. No α-, β- and γ-hydroxbutyrates or α-, β- and γ-aminobutyrates were effective, even in the presence of IL-2, in increasing either the antibody response or histone H4 acetylation.

All data shown in Figs. 1–4 support the view that the effect of NaBu enhancing anti-TNP antibody production in the presence of IL-2 is presumably mediated through moderate increases in the level of histone acetylation caused by the ability of NaBu to inhibit histone deacetylase, as with the effect of TSA. However, the effect of NaBu differs from that of TSA: unless IL-2 is added to the cultures, NaBu does not promote anti-TNP antibody production in splenic B cells, while TSA does.5,8 To understand this discrepancy, the combined effects of TSA and NaBu were examined (Fig. 5A). In
the absence of IL-2, TSA alone enhanced the anti-TNP PFC response, but this enhancement was markedly inhibited by simultaneously adding NaBu, suggesting that NaBu without IL-2 has an inhibitory effect on the stimulation of the anti-TNP PFC response. However, in the presence of IL-2 the effect of TSA was neither inhibited nor potentiated by NaBu. Thus, the inhibitory effect of NaBu was overcome by adding IL-2. The inhibitory effect of NaBu was exhibited without decreasing the number of viable cells in the cultures.

Figure 5B shows the acetylation levels of histone H4 of these cells. Splenic B cells treated with NaBu, TSA or both together in the presence or absence of IL-2 showed almost the same acetylation level of histone H4.

DISCUSSION

The following evidence supports the role of a moderate increase in histone acetylation in the augmentation of anti-TNP antibody production induced by NaBu plus IL-2 in B cells. First, NaBu increased both antibody production and histone acetylation at the same concentrations in the presence of IL-2. Second, among short-chain fatty acids and NaBu analogs, agents capable of inducing histone acetylation, such as propionate, valerate and vinylacetate, also enhanced anti-TNP antibody production in the presence of IL-2, whereas agents incapable of inducing histone acetylation did not. Third, TSA, a structurally unrelated inhibitor of histone deacetylase, promotes the anti-TNP antibody response with a concomitant, moderate increase in histone acetylation, although this effect of TSA does not require the coexistence of IL-2.8)

Histone acetylation has been suggested to greatly influence gene expression.14) Recent findings that the yeast transcriptional adaptor Gen5p, transcriptional coactivators p300 and CREB (cAMP responsive element binding protein) binding protein (CBP), cellular p300/CBP-associated factor (P/CAF), the TAF1250 subunit of the transcription factor IID, the steroid receptor coactivator SRC-1, and the nuclear receptor coactivator ACTR are all nuclear histone acetyltransferases directly link histone acetylation to gene activation.15–21) The moderate increase in histone acetylation induced by NaBu could activate the expression of a gene(s) involved in the differentiation of antigen-specific B cells to plasma cells.

With the difference in IL-2 requirement between the effects of NaBu and TSA, the Fig. 5A data, in which NaBu inhibited the promoting effect of TSA in the absence of IL-2, suggest that NaBu can also inhibit antibody production. In the presence of IL-2, however, NaBu did not show an inhibitory effect. Therefore, NaBu likely has both stimulating and inhibiting activities for anti-TNP antibody production, the latter of which is antagonized by IL-2. Thus, NaBu may show only a stimulating effect in the presence of IL-2. The IL-2 receptor is expressed on murine splenic B cells stimulated by both anti-immunoglobulin, which cross-links the B cell antigen receptor, and LPS, whereas it is not expressed on cells stimulated by LPS alone.22) IL-1β, but not IL-4, may have an antagonizing effect less effective than IL-2.23) As an immunoinhibitory effect of NaBu, this agent has been shown to downregulate the stimulatory function of antigen-presenting cells in inducing T-cell alloresponses.21)

NaBu had an inhibitory effect without decreasing the number of viable cells (Fig. 5A), although its precise effect on the proliferation of TNP-specific B cells, which were present in too low a concentration in whole B cell populations, is not known. Whether an increase in histone acetylation is involved in the inhibitory activity of NaBu is also not known. However, the fact that TSA did not have an inhibitory effect (Fig. 5A) suggests that the negative effect is unrelated to the increase in histone acetylation. Although several histone deacetylases coexist in mammalian cells,24–30) NaBu and TSA likely have similar inhibition profiles for histone deacetylases: the activity of human histone deacetylase 3 is inhibited by both agents in vitro to the same degree as the activity of human histone deacetylase 1.30) IL-2, which antagonizes the inhibitory activity of NaBu, has no effect on the levels of histone H4 acetylation.8)

Butyrate is a naturally occurring fatty acid. Is it effective in vivo in promoting antigen-specific antibody production? Butyrate is supplied to mammals from 2 main sources: it is a major product of bacterial fermentation of unabsorbed carbohydrates in the colon, and is produced by a diet such as milk fat that contains 3–4% butyrate in a complex of esters of
glycerol. It reaches concentrations of up to 20 mm in the colon and feces of mammals and man, but is only in negligible concentrations (less than 10 μM) in peripheral blood.22 Infused butyrate has a half-life of 6 min in man, resulting in peak blood levels below 0.06 mm.23 Thus, maintaining a plasma concentration of butyrate effective to promote antibody production seems difficult, that is, a minimum of 0.1 mm. Recently, several butyrate pro-drugs, including tributyrin, 3-0-butyryl-1,2-0-isopropyliden-α-0-glucofuranose and pivaloxyxymethyl butyrate (AN-9), have been reported to act longer than butyrate and show antitumor activity in vivo.27,30-32 In vivo studies on the potential immunostimulatory effect of these butyrate pro-drugs are now under way in our laboratory.

REFERENCES