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Promotion of Antigen-specific Antibody Production in Murine B Cells by a Moderate Increase in Histone Acetylation

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ABSTRACT. By employing the specific histone deacetylase inhibitor trichostatin A (TSA), we investigated whether histone acetylation modulates the production of antigen-specific antibodies in murine splenocytes *in vitro*. TSA caused a marked increase in both anti-sheep red blood cell (SRBC) and anti-trinitrophenyl (TNP) plaque-forming cell (PFC) responses in splenocytes at much lower concentrations than sodium butyrate. It also dose dependently augmented the production of anti-trinitrophenyl antibodies in splenic B cells with a concomitant, moderate increase in the level of histone H4 acetylation. Its optimal concentration for promoting the production of these antibodies was 10 nM. However, to gain such an effect on antibody production, TSA had to be added to cells before Day 2 in culture. Trichostatin C, an analog of TSA and a less potent inducer of Friend leukemia cell differentiation, also increased both the anti-trinitrophenyl PFC response and histone H4 acetylation in B cells, but at higher concentrations than TSA. TSA did not stimulate the production of lipopolysaccharide-induced polyclonal immunoglobulin M in B cells. These results suggest that a moderate increase in histone acetylation may play a significant role in promoting antigen-specific antibody production in B cells. BIOCHEM PHARMACOL **56**;10:1359–1364, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. histone acetylation; antigen-specific antibody production; trichostatin A; trichostatin C; sodium butyrate; murine splenic B cells

Acetylation of the core histones is a ubiquitous, post-transcriptional modification found in animals and plants [1]. Acetylation occurs on specific lysine residues within the basic amino-terminal tail domains of the core histones. Addition and removal of acetate groups are catalyzed by the specific enzymes histone acetyltransferase and histone deacetylase [2]. There is a general correlation between the level of histone acetylation and the transcriptional activity of a chromosomal domain. Hyperacetylated histones accumulate within particular active chromatin domains [3], whereas hypoacetylated histones accumulate within transcriptionally silent domains [4]. Recent findings that the yeast transcriptional adaptor Gcn5p [5], the transcriptional coactivators p300 and CBP [6, 7], and the $TAF_{II}250$ subunit of the transcription initiation factor TFIID [8] are nuclear histone acetyltransferases, directly link histone acetylation to gene activation. Conversely, it has recently been reported that histone deacetylases are linked to some sequence-specific DNA-binding transcriptional repressors via corepressors [9, 10], and this suggests that histone deacetylation leads to transcriptional repression. Thus, histone acetylation presumably plays a role in modulating various cellular functions, including growth and differentiation.

Modulation of immune responses through histone acetylation is poorly understood. We reported previously that antibody production in murine splenocytes that were stimulated with SRBC† or TNP-LPS was markedly enhanced by NaBu in a cytokine-dependent manner [11, 12]. Induction of some B cell differentiation markers in the Burkitt lymphoma cell Raji by NaBu has also been reported [13]. The mechanism of these NaBu actions is unknown, but due to its ability to inhibit histone deacetylase, it has been suggested that NaBu may modulate gene transcription by inducing histone hyperacetylation [14]. As NaBu is a pleiotropic agent and not a specific inhibitor of this enzyme [14], it is thus also possible that other activities of NaBu are involved in the promotion of antibody production. To study whether histone hyperacetylation stimulates the production of antigen-specific antibodies, we used TSA, a potent and specific inhibitor of histone deacetylase [15], and examined its effect on antibody production. TSA is a *Streptomyces* product originally identified as a fungistatic

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† *Abbreviations*: CBP, CREB binding protein; CREB, cAMP responsive element binding protein; IgG, immunoglobulin G; IgM, immunoglobulin M; IL-2, interleukin 2; LPS, lipopolysaccharide; NaBu, sodium butyrate; PFC, plaque-forming cell; SRBC, sheep red blood cell; TNP, trinitrophenyl; and TSA, trichostatin A.

antibiotic [16]. It has been reported that TSA induces the differentiation of Friend leukemia cells [17] and promotes interleukin 4-dependent, immunoglobulin E class switching in LPS-stimulated murine B cells [18]. In this report, we show that TSA markedly increased anti-TNP antibody production in murine, splenic, B cells with a concomitant elevation in the level of histone H4 acetylation. However, TSA did not enhance LPS-stimulated polyclonal IgM production, suggesting that TSA is effective only on B cells stimulated with antigens. These results imply that a moderate increase in histone acetylation may play a significant role in promoting antigen-specific antibody production in B cells.

MATERIALS AND METHODS

Mice

Female BALB/c mice were purchased from Charles River, Japan. They were kept in our animal care facility and used at between 9 to 12 weeks of age.

Reagents

RPMI 1640 medium and fetal calf serum (FCS) were purchased from Flow Laboratories and Life Technologies, respectively. TSA was from Wako Pure Chemical Industries, NaBu from Sigma Chemical Co., and recombinant murine IL-2 from Genzyme. Trichostatin C was a gift from Dr. M. Yoshida (The University of Tokyo, Tokyo, Japan). TSA and trichostatin C were dissolved in methanol and stored at −20°. TNP-LPS was prepared by the reaction of LPS from *Escherichia coli* 055:B5 (Sigma Chemical Co.) with 2,4,6-trinitrobenzenesulfonate, as described by Jacobs and Morrison [19].

Anti-TNP and Anti-SRBC Antibody Production

Murine, whole spleen cells (8×10^6/well) or splenic B cells (3.5×10^6/well) were seeded in triplicate in 24-well multidishes (Nunc). They were cultured with TNP-LPS (5 μg/mL) or SRBC (2×10^6/well, Japan Lamb) in 1.5 mL of RPMI 1640 medium, supplemented with 10% heat-inactivated FCS, 100 units/mL of penicillin G, and 100 μg/mL of streptomycin at 37° under 5% CO_2 and 95% air. TSA, NaBu, or other agents were added to the wells before seeding the cells. And when necessary, appropriate concentrations (less than 0.1%) of methanol were added to control cultures. After 5 days, the number of anti-TNP and anti-SRBC PFCs were determined by the methods of Rittenberg and Pratt [20] and Jerne and Nordin [21], respectively. Splenic B cells were prepared according to the method of Leibson *et al.* [22]. Briefly, spleen cells from mice that had been injected 2 days before sacrifice with i.p. 20 μL of anti-thymocyte serum (Wako Pure Chemical Industries) were incubated on plastic dishes for 90 min at 37°. The nonadherent cells were then collected and, following treatment with an anti-Thy-1.2 antibody (1/500, Serotec) for 30 min at 4°, were incubated with low toxic, rabbit complement (1/15, Cedarlane Laboratories) for a further 40 min at 37°. Thy-1.2^+ cells in this preparation were usually less than 3% as determined by flow-cytometric analysis.

Extraction of Cellular Histones and Analysis of Acetylated Histone H4

Histones from B cells were isolated according to the procedure of Cousens *et al.* [23]. B cells from 2 wells of the 24-well multidishes 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 $MgCl_2$, 8.6% sucrose, pH 6.5), and centrifuged at 1,000 *g* for 10 min. The resulting nuclei were washed three 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 H_2O, and to give a concentration of 0.4 N, concentrated H_2SO_4 was added to the suspension. After incubation at 0° for 1 hr, the suspension was centrifuged for 5 min at 15,000 rpm using a microfuge, and the supernatant was mixed with 10 volumes of acetone. After an overnight incubation at −20°, the precipitate was collected by centrifugation and air dried. This acid soluble histone fraction was dissolved in H_2O. Protein content was determined by the method of Lowry *et al.* [24]. The acetylation of histones was analyzed by acid-urea-Triton gel electrophoresis [23]. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250, dried, and photographed. The level of histone H4 acetylation was determined using a densitometer (Molecular Dynamics 300 A-T), and calculated as the sum of the percentage of an appropriate histone H4 form × the number of acetylated lysine residues per molecule/100.

Polyclonal IgM Production

Murine splenic B cells (3.5×10^6/well) were seeded in triplicate in 24-well multidishes (Nunc) and cultured with LPS (5 μg/mL) in 1.5 mL of the medium as described above. Culture supernatants were collected after incubating for 5 days and stored at −20° for later IgM and IgG ELISAs. IgM and IgG levels were assayed by a sandwich ELISA as described previously [18]. The calibration curve was made using mouse IgM and IgG.

RESULTS

Effect of TSA on Anti-SRBC and Anti-TNP PFC Responses

Murine, whole splenocytes were stimulated with SRBC or TNP-LPS for 5 days in the presence of various concentrations of TSA, and the number of antibody-producing cells was measured. TSA, like NaBu, caused a marked increase in both anti-SRBC and anti-TNP PFC responses (Fig. 1A and 1B). Its optimal concentration for both responses was 10 nM, which is much lower than that of NaBu (0.4–0.6 mM)

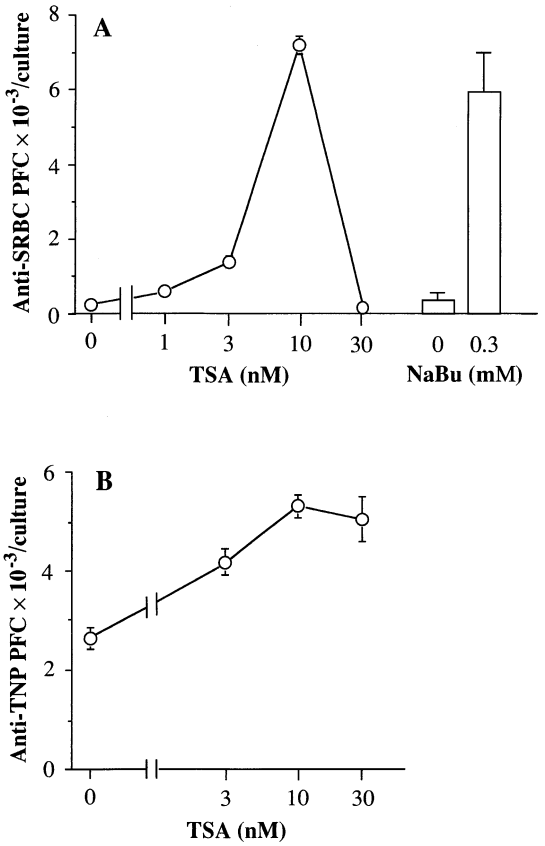

FIG. 1. Effect of TSA on the anti-SRBC and anti-TNP PFC responses in whole splenocytes. Whole splenocytes were stimulated with SRBC (2×10^6) (A) or TNP-LPS (5 μg/mL) (B) in the presence of varying concentrations of TSA or 0.3 mM NaBu. After 5 days, the number of anti-SRBC and anti-TNP PFCs was determined. The data are representative of three independent experiments and expressed as the means ± SD of triplicate wells.

[11, 12]. TSA is also known as an inhibitor of cell-cycle progression [25]. However, at this dose of TSA, the number of viable cells did not decrease, although it did at 30 nM, particularly in the cultures stimulated with SRBC (data not shown). Figure 2 shows the effect of TSA on anti-TNP antibody production in splenic B cells. The maximal response was again obtained at the concentration of 10 nM and was approximately four times that of control cells (Fig. 2A). As reported previously [12], the NaBu-induced promotion of the anti-TNP PFC response in B cells required the presence of IL-2 (Fig. 2B). In contrast, the stimulating effect of TSA was detectable even in the absence of interleukin-2 (Fig. 2B). Trichostatin C, an analog of TSA and a less potent inducer of Friend leukemia cell differentiation [17], also stimulated the anti-TNP PFC response in B cells without IL-2, although its effect was maximal at 100 nM (Fig. 2C).

Figure 3 shows the effect of TSA added on different days after seeding. Compared to Day 0, the promoting effect of TSA was considerably reduced when added on Day 1, and no stimulation was detected when it was added on days thereafter.

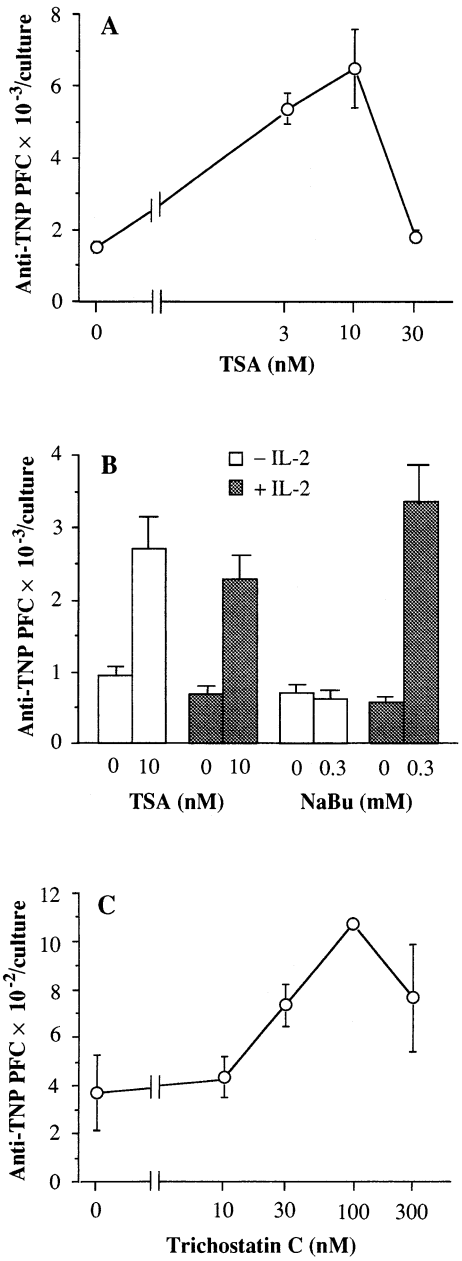

FIG. 2. Effects of TSA and trichostatin C on the anti-TNP PFC response in splenic B cells. Splenic B cells were stimulated with TNP-LPS (5 μg/mL) in the presence of varying concentrations of TSA (A), 10 nM TSA, and 0.3 mM NaBu with or without IL-2 (100 U/mL) (B), and trichostatin C (C). After 5 days, the number of anti-TNP PFC was determined. The data are representative of three independent experiments and expressed as the means ± SD of triplicate wells.

Effects of TSA and NaBu on Histone H4 Acetylation

The effect of TSA on the levels of acetylated histone H4 in B cells was then examined. Extracted histones were analyzed by acid-urea-Triton gel electrophoresis. Murine B cells, stimulated with TNP-LPS alone, showed the major nonacetylated form of histone H4, a minor monoacetylated form, and a trace amount of a diacetylated form (Fig. 4A).

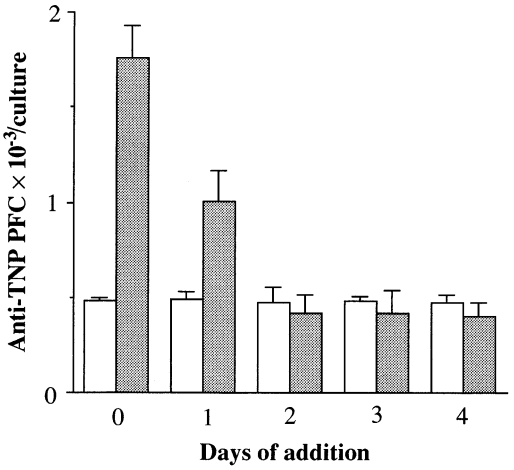

FIG. 3. Effect of the delayed addition of TSA on the anti-TNP PFC response in splenic B cells. Splenic B cells were stimulated with TNP-LPS (5 μg/mL). TSA (10 nM) (shaded) or a vehicle only (0.01% methanol, open) was added on the different days after seeding, as indicated. The number of anti-TNP PFC was determined 5 days after seeding. The data are representative of two independent experiments and expressed as the means ± SD of triplicate wells.

Fresh B cells showed a similar pattern of histone H4 forms (data not shown). In the cells exposed to TNP-LPS plus different doses of TSA for 12 hr, monoacetylated and diacetylated forms of histone H4 significantly increased with a concomitant decrease in the amount of the non-acetylated form (Fig. 4A). Densitometric analysis indicated that TSA dose dependently elevated the acetylation level of histone H4 (Fig. 4B). Figure 4B also shows a dose-response of TSA-induced enhancement of antibody production that was determined in another parallel multidish. Both parameters were significantly stimulated by concentrations as low as 3 nM TSA and were further increased at 10 nM. NaBu and trichostatin C also caused an increase in the acetylation level of histone H4 at the doses optimal for the enhancement of antibody production (Fig. 5). IL-2 did not affect the NaBu-induced elevation in the acetylation level of histone H4 (Fig. 5).

Effect of TSA on Polyclonal IgM Production in B Cells

The results presented above demonstrate that the production of antibodies that are specific for antigens, such as SRBC and TNP-LPS, in B cells was increased by TSA. We investigated whether TSA was also effective in promoting polyclonal antibody production in B cells. Murine B cells were stimulated with LPS for 5 days in the presence or absence of TSA, and secreted IgM and IgG were measured. As shown in Fig. 6, neither polyclonal IgM nor IgG production was stimulated by TSA.

DISCUSSION

Our results show that TSA markedly stimulated the production of antigen-specific antibodies in murine splenocytes. The following lines of evidence support the notion that the effect of TSA is presumably mediated through the moderate increase in the level of histone acetylation caused by its ability to inhibit histone deacetylase: 1) TSA increased both the anti-TNP PFC response and histone H4 acetylation at the same, extremely low concentrations; 2) The effective TSA concentration for enhancing anti-TNP antibody production was similar to the K_i value (3.4 nM) of TSA for histone deacetylase [15]; and 3) trichostatin C, an analog of TSA, also caused increases in both the anti-TNP PFC response and the histone H4 acetylation level. The effective concentrations of TSA were lower than those of NaBu by four orders of magnitude. It has been suggested that TSA binds the deacetylase at a specific binding site [15], while NaBu interacts in a nonspecific manner like a detergent [14, 23]. The TSA-induced increase in the level of histone acetylation in B cells was significant, but moderate. However, as separate classes of nucleosomes exist that are especially accessible or inaccessible to acetylation [23], and the turnover rates of acetate groups of histones

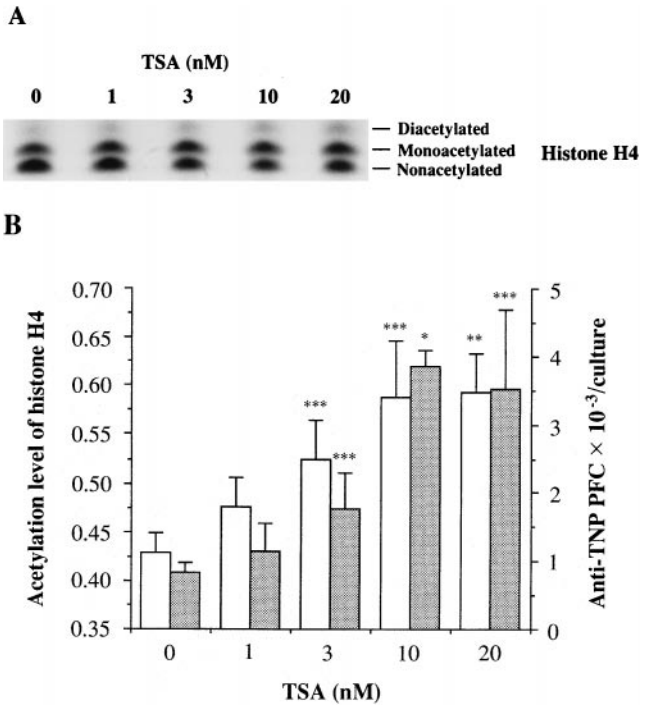

FIG. 4. Dose-response of TSA-induced histone H4 acetylation and promotion of anti-TNP antibody production in splenic B cells. Splenic B cells were cultured with TNP-LPS (5 μg/mL) and varying concentrations of TSA as indicated for 12 hr. Fifteen micrograms of the histones isolated were subjected to acid-urea-Triton gel electrophoresis. (A) The gel was stained with Coomassie blue. The positions of nonacetylated, monoacetylated, and diacetylated histone H4 forms are indicated. (B) The level of histone H4 acetylation (open) was analyzed using a densitometer. The number of anti-TNP PFC (shaded) was determined using B cells cultured in another, parallel, multidish for 5 days. The data are expressed as the means ± SD of three independent experiments. Values that are significantly different from those of the control are indicated by: *$P < 0.001$; **$P < 0.01$; ***$P < 0.05$. The statistical significance was analyzed by the Student's *t*-test.

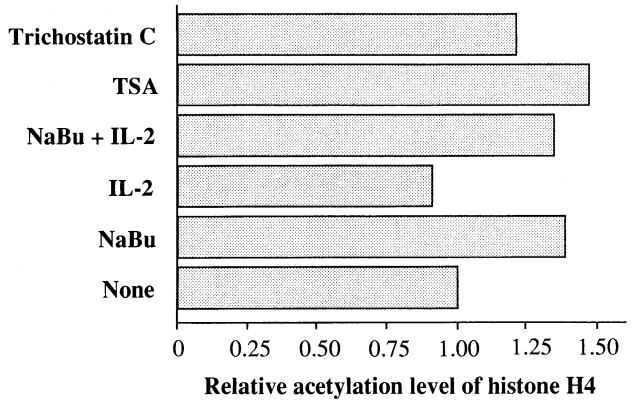

FIG. 5. Effect of NaBu and trichostatin C on the level of histone H4 acetylation in splenic B cells. Histones of B cells, cultured for 12 hr with TNP-LPS (5 μg/mL) and the agents indicated, were isolated, and 10 μg of histones were subjected to acid-urea-Triton gel electrophoresis. After staining, the level of histone H4 acetylation was analyzed using a densitometer. The agent concentrations were: 0.3 mM NaBu, 10 nM TSA, 100 nM trichostatin C, and 100 U/mL of IL-2. The data are representative of three independent experiments and expressed as fold-change relative to the control cultures, which were incubated with only TNP-LPS .

vary among nucleosomes [26], then more histone acetylation could occur at specific sites.

The mechanism by which TSA-induced histone acetylation affects antibody production is still unknown. However, it might not directly stimulate IgM production because polyclonal IgM production in B cells stimulated with LPS was not enhanced by TSA and the addition of the inhibitor to the cells after Day 1 in culture was ineffective. It seems likely that TSA enhances antibody production in B cells activated by cross-linking antigen receptors. TSA-induced histone acetylation could influence an event(s), which occurs during an early phase of B cell activation via antigen receptors and is involved in the differentiation of

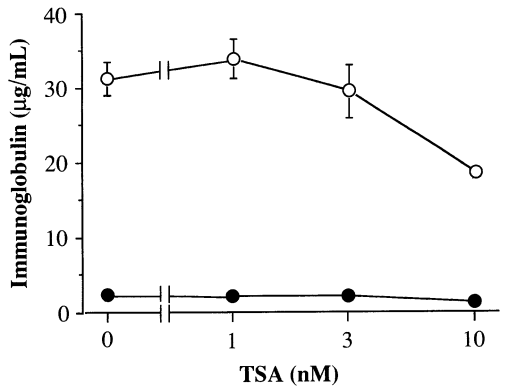

FIG. 6. Effect of TSA on polyclonal IgM and IgG production in splenic B cells. Splenic B cells were stimulated with LPS (5 μg/mL) in the presence of varying doses of TSA. After 5 days, the IgM (○) and IgG (●) secreted were determined by ELISA. The data are representative of three independent experiments and expressed as the means ± SD of triplicate wells. Where SD bars are not shown, the SD was smaller than the symbol.

the B cells to plasma cells. It has been suggested that histone acetylation is a major influence on gene expression [2]. Recent findings that the yeast transcriptional adaptor Gcn5p, the transcriptional coactivators p300 and CBP, the cellular p300/CBP-associated factor (P/CAF), and the $TAF_{II}250$ subunit of the transcription initiation factor TFIID are all nuclear histone acetyltransferases [5–8, 27], directly link histone acetylation to gene activation. The binding of transcription factor IID to the promoters is the first important step in the formation of functional pre-initiation complexes [28]. Gcn5p, p300/CBP, and P/CAF may be recruited onto specific promoter elements via protein–protein interactions, thereby inducing the targeted acetylation of specific chromatin domains. Targeted histone acetylation could contribute to promoter activation by altering or disrupting the repressive chromatin structure. Conversely, it has recently been reported that histone deacetylases are linked to some sequence-specifc DNA-binding transcriptional repressors via corepressors [9, 10]. Therefore, it is possible that the increase in histone acetylation caused by TSA promotes antibody production by activating transcription of some factor(s) that is involved in the differentiation of B cells stimulated with antigens. It has been reported that the expression of approximately 2% of cellular genes is changed in response to TSA treatment [29]. The question whether or not histone acetylation increases in B cells activated via their antigen receptors might be raised. No increase was detected in the splenic B cells stimulated with only TNP-LPS (Kohge T, Gohda E, Okamura T and Yamamoto I, unpublished data). However, because only a small population of B cells responds to this antigen, to answer this question it is necessary to study B cells stimulated with anti-immunoglobulin.

Unless interluekin-2, which alone showed no stimulatory effect, was added to the culture, NaBu alone promoted anti-TNP antibody production in splenocytes, but not in splenic B cells [12]. Thus, the IL-2 requirement was a difference between the effects of TSA and NaBu. The reason for this discrepancy is not yet known. NaBu, however, is a pleiotropic agent and has some activities other than inhibiting histone deacetylase [14]. Therefore, it is not inconceivable that IL-2 may antagonize an inhibitory activity of NaBu, leading it to exhibit only a positive promoting activity.

TSA and butyrate are also known as inhibitors of the cell-cycle progression, blocking it at both the G1 and G2 phases [14, 25]. TSA and NaBu decreased the viable cell numbers at the concentrations of 30 nM and 0.6 mM, respectively, leading to a reduction in antibody production (Fig. 1 and 2A) [12]. Thus, it seems likely that excess histone acetylation causes the inhibition of cell growth. In fact, in HeLa cells the exogenous expression of P/CAF, which has an intrinsic histone acetyltransferase activity as described above, inhibits the progression of the cell cycle and counteracts the mitogenic activity of the adenoviral oncoprotein E1A [27]. Excess histone acetylation may lead

to the induction of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ [30] and other growth inhibitory proteins. Therefore, a precise regulatory mechanism of histone acetylation in B cells will be required to control antigen-specific antibody production.

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