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BUTYRATE SUPPRESSES HYPOXIA-INDUCIBLE FACTOR-1 ACTIVITY IN INTESTINAL EPITHELIAL CELLS UNDER HYPOXIC CONDITIONS

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ABSTRACT—Interaction between the products of intestinal bacteria and the intestinal epithelial cells is a key event in understanding the biological, physiological, and pathological functions of the intestinal epithelium. Here, we examined the effect of butyrate, one of the major intestinal bacterial products, on hypoxia-inducible factor-1 (HIF-1) activity under hypoxic conditions in intestinal epithelial cells. HIF-1 activity was assessed by luciferase assay using cytoplasmic extracts of intestinal epithelial cells, Caco-2, and IEC-6 cells. These cells were transiently transfected with hypoxia response element (HRE)-luciferase reporter plasmids and cultured under hypoxic conditions in the presence or absence of sodium butyrate (NaB). The effect of NaB on HRE DNA binding activity in Caco-2 cells under hypoxic conditions was assessed by electrophoretic mobility shift assay. Expression of a hypoxia-responsive gene encoding intestinal trefoil factor (ITF) in Caco-2 cells after NaB treatment was assessed using reverse-transcription PCR. The barrier function of Caco-2 cells under hypoxic conditions was also evaluated by transepithelial electrical resistance measurement. NaB suppressed up-regulation of HIF-1 transcriptional activity under hypoxic conditions in Caco-2 and IEC-6 cells. In parallel, NaB reduced HRE DNA binding activity under the same conditions. Furthermore, NaB down-regulated enhanced transcription of *ITF* gene. Addition of NaB under hypoxic conditions delayed recovery of transepithelial electrical resistance of the monolayers after hypoxia-reoxygenation treatment. These findings indicate that NaB suppresses HIF-1 transcriptional activity on hypoxia-responsive genes by reducing the HRE DNA binding activity under hypoxic conditions in intestinal epithelial cells.

KEYWORDS—Caco-2, hypoxia, intestinal trefoil factor, short-chain fatty acid

INTRODUCTION

Interaction between intestinal bacteria or their products and intestinal epithelial cells is an important event in understanding the biological, physiological and pathological functions of the intestinal epithelium. One of the short-chain fatty acids, butyrate is a bacterial product caused by fermentation of hydrocarbons and exists in the lumen of human intestine at a concentration of 2 to 24 mmol/kg (1). Butyrate has been reported to have a variety of effects on intestinal epithelial cells. It has been reported to alleviate damage to the intestinal mucosa caused by thermal and detergent injury (2) or inflammatory bowel disease (3, 4). In addition, several reports have demonstrated that butyrate suppresses tumorigenicity of tumor cells through its apoptosis-inducing function (5, 6).

Butyrate has been shown to modulate histone conformation by inhibition of histone deacetylases (HDAC) (7). Recently, a specific HDAC inhibitor, trichostatin A, was reported to prevent hypoxia-inducible factor-1 (HIF-1) activity under hypoxic conditions (8). HIF-1 is a heterodimeric basic helix-loop-helix transcription factor consisting of HIF-1α and HIF-1β subunits (reviewed in 9). HIF-1α is induced by hypoxia, whereas HIF-1β is constitutively expressed. It is one of the pivotal transcriptional factors by which cells in diverse phyla adapt to hypoxic conditions. HIF-1 transregulates transcription of a panel of genes containing genes encoding growth factors such as vascular endothelial growth factor (VEGF) (10), cytokines such as erythropoietin (11), and glycolytic enzymes such as aldolase A, phosphoglycerate kinase-1 (PGK-1), β-enolase, pyruvate kinase M, and glyceraldehyde-3-phosphate dehydrogenase (12–15). Transcription of intestinal trefoil factor (ITF), which plays critical role in the mucosal barrier function, has been also proved to be regulated by HIF-1 (16). Induction of ITF was reported to partially protect endothelial cells from hypoxia-elicited barrier disruption (16). Short-chain fatty acids including butyrate have been reported to inhibit ITF gene expression in colon cancer cells (17).

These accumulating findings prompted us to investigate whether butyrate suppresses HIF-1 activity in human intestinal epithelial cells when it is exposed to hypoxic environments. In this study, we used a human intestinal epithelium-like cell line, Caco-2 cells, and a rat small intestinal epithelial cell line, IEC-6 cells. We also examined whether butyrate affects the barrier function under hypoxic conditions by measuring the transepithelial electrical resistance (TEER) of Caco-2 cells.

MATERIALS AND METHODS

Construction of hypoxia response element reporter plasmids

pHREpgkLuc and pHREpaiLuc, reporter plasmids for hypoxia response element (HRE), were constructed as follows. Three copies of a double-stranded oligonucleotide that contains HRE of the 5′-flanking region of human PGK-1 (13), 5′-TCGAGACGTGCGGGACGTGCGC-3′, or HRE of the 5′-flanking region of rat plasminogen activator inhibitor-1 (PAI-1; 18), 5′-TCGACACGTACACACGTGTCGCGC-3′, (the underlined portions indicate HRE) were inserted just upstream of minimum SV40 promoter of pGL3-Promoter (Promega, Madison, WI), resulting in pHREpgkLuc and pHREpaiLuc, respectively. The nucleotide sequences of the resultant plasmids were confirmed by dideoxy sequencing with ABI PRISM 310 Genetic Analyzer (Applied Biosystems; Foster City, CA). Large-scale purification of plasmids was conducted with Qiagen plasmid mega kit system (Qiagen, Chatsworth, CA).

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Cell culture

A human colonic epithelial cell line, Caco-2 cells, and a rat small intestinal epithelial cell line, IEC-6 cells, were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (DMEM/10FBS) was used in all experiments. For experiments, 1×10^5 cells were seeded onto the polycarbonate Transwell filters (diameter 12.0 mm) with a pore size of 0.4 μm (Costar, Corning, NY). Forty-eight hours after seeding, the medium was changed to DMEM without FBS in the upper chamber and with 10% FBS in the lower chamber to mimic the physiologic condition *in vivo*.

Transient transfection and luciferase assay

For reporter assays, transient transfection was performed using the high-efficiency calcium-phosphate methods previously described (19). Briefly, 1 μg of the reporter plasmid, pHREpgkLuc or pHREpaiLuc, and 1 μg of pRL-TK (a control for transfection efficiency) were suspended in 50 μL of 0.25 M $CaCl_2$. After vigorous shaking for 15 s, 50 μL of 2× BES [N,N-bis (2-hydroxyethyl)-2-aminoethanesulfonic acid]-buffered saline (BBS) was added to the solution and incubated for 15 min at room temperature. The mixed solution was then added dropwise to confluent cells. The cells were incubated for 12 h in 3% CO_2 atmosphere at 37°C and then washed twice with PBS. Twenty-four hours after transfection, cells were washed with PBS, and the medium was replaced with DMEM/10FBS containing NaB (Wako Chemical, Tokyo, Japan) or sodium propionate (NaPr) (Wako Chemical) at the indicated concentration, or with DMEM/10FBS alone. Then, the cells were subjected to hypoxic conditions or remained in normoxic conditions and incubated for the indicated time. Preparation of cell extracts and luciferase assay were performed using aliquots of cell extracts with the PicaGene luciferase assay system (Toyo Ink, Tokyo, Japan) following the manufacturer's procedure. Luciferase activity was measured with an OPTOCOMP II luminometer (MGM Instruments, Inc., Hamden, CT). The relative luciferase activities were normalized with the activities of *Renilla reniformis* luciferase by cotransfected pRL-TK (Promega).

Hypoxic conditions

For experiments in hypoxia, the culture plates were placed into a modular incubator chamber (Billups-Rothenberg, Del Mar, CA). The chamber was packed with gas consisting of 5% CO_2, 1% O_2, and 94% N_2. The valves were then closed, and the chamber was placed in a conventional CO_2 incubator maintained at 37°C as described previously (20).

Semiquantitative reverse-transcription PCR

Caco-2 cells were harvested, and total RNA was prepared from the cells by Isogen RNA extraction solution (Nippon Gene, Tokyo, Japan). Single-stranded cDNA was synthesized with Molony murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) and then used for PCR analysis. Primers used for human *ITF* and *β-actin* cDNA detection were as follows. For the *ITF* gene (21), 5′-CCAGGCACTGTTCATCTCAG-3′ and 5′-GGAGCATGGGACCTT-TATTC-3′; for *β -actin* gene, 5′-GGCGGCAACACCATGTACCCT-3′ and 5′-AGGGGCCGGACTCGTCATACT-3′.

PCR conditions are 23 cycles of 92°C for 1 min; 60°C, for 30 s; 72°C for 30 s. The strength of primer-specific bands was quantified with AE-6900M densitograph (ATTO, Tokyo, Japan). The density of *ITF*-specific bands was normalized by dividing by that of *β-actin*-specific bands. Results were expressed as relative band densities as compared with the density of *ITF*-specific band at normoxia without NaPr or NaB treatment (= 100).

Electrophoretic mobility shift assay

Nuclear extracts of Caco-2 cells were prepared according to the method of Dignam et al. (22). Briefly, Caco-2 cells were washed twice in cold PBS and then allowed to swell in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM dithiothreitol) for 10 min on ice and pelleted at 1500 rpm for 10 min. Cells were suspended in two pellet volumes of buffer and then lysed by 20 strokes of a type B pestle. The homogenates were centrifuged at 2000 rpm for 10 min. The pellets (crude nuclei) were resuspended in 200 μL of buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 25% glycerol, 10 μg/mL leupeptin, 10 μg/mL pepstatin) by homogenation with 20 strokes of a type B pestle. The suspensions were gently rocked for 30 min and then centrifuged for 45 min at 15,000 rpm. The resulting supernatants were dialyzed against 50 volumes of buffer D (20 mM HEPES, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL pepstatin) for 2 h. Protein concentrations were measured by BioRad protein assay kit (Bio-Rad Laboratories, Herculus, CA). For EMSA, nuclear extracts (3 μg) were preincubated with 2 μg of poly(dI)·(dC) (Pharmacia Fine Chemicals, Piscataway, NJ) in 20 μL of M buffer (10% glycerol, 2% polyvinyl alcohol, 20 mM HEPES, pH 7.9, 40 mM KCl, 7 mM $MgCl_2$, 1 mM dithiothreitol) for 10 min at room temperature. As a control, a 50-fold molar excess of cold HRE competitor oligonucleotide or TPA (12-O-tetradecanoylphorbol-13-acetate) response element (TRE) oligonucleotide was added during preincubation. After preincubation, 0.5 ng of ^{32}P-end-labeled HRE oligonucleotide probe was added to the reaction mixture and incubated for an additional 30 min. The reaction mixture was loaded onto a 4% polyacrylamide gel containing 0.25-fold TBE. Electrophoreses were carried out at 120 V for 3 h. Gels were dried, exposed to a Fuji imaging board, and analyzed using the BAS1000 system (Fuji Photo Film, Tokyo, Japan).

Transepithelial electrical resistance measurement

Measurement of transepithelial electrical resistance (TEER) was performed in culture medium by using a monolayer of cells grown in 12-mm inserts with a surface area of 1.0 cm^2 as described previously (20). We usually cultured Caco-2 cells for 3 to 4 days before they reached confluence. To measure TEER, 100-μA current pulses (1 s) were passed via Ag-AgCl electrodes. The resultant voltage deflections were detected using a separate pair of Ag-AgCl electrodes and a resistance meter (model EVOM, World Precision Instruments, New Haven, CT). Fluid resistance was subtracted, and net resistance was expressed as ohm–square centimeter (Ωcm^2). Average values from triplicate measurement were shown for each time point.

Statistical analysis

Data from multiple experiments were expressed as the mean ± SE. Statistical analyses were performed by using the StatView-J 4.02 statistics program (Abacus Concepts, Berkeley, CA). Data were analyzed by the Student's two-tailed *t* test for two groups, or by one-factor analysis of variance (ANOVA) followed by Fisher's Protected Least Significant Difference (PLSD) test for three or more groups.

RESULTS

HIF-1 activity is augmented under hypoxic conditions in Caco-2 and IEC-6 cells

First, we examined whether HIF-1 activity in Caco-2 and IEC-6 cells is induced under hypoxic conditions by using pHREpgkLuc and pHREpaiLuc reporter plasmids, respectively. The cells were transiently transfected with the reporter plasmids and were subjected to hypoxic conditions for 36 h. Cell lysates were then prepared, and the luciferase activities were analyzed. As shown in Figure 1, HIF-1 activity was

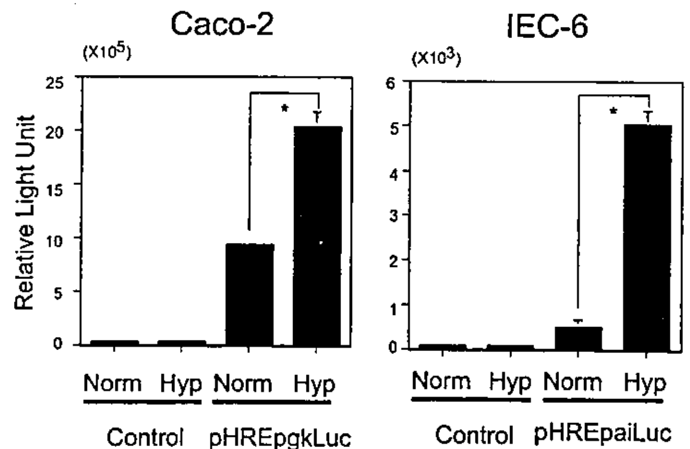

FIG. 1. **HRE activity under hypoxic conditions in Caco-2 and IEC-6 cells.** Left panel, Caco-2 cells were transiently transfected with pHREpgkLuc or pGL3-Promoter (control) plasmid. The cells were then subjected to normoxic (Norm) or hypoxic (Hyp) conditions for 36 h. The relative luciferase activities were measured by using the cell lysates. Right panel, IEC-6 cells were transiently transfected with pHREpaiLuc or pGL3-Promoter plasmid. The same experiments as in left panel were performed. The means ± SE of triplicate determinations are shown. Asterisks indicate statistical significance ($P \leq 0.001$) compared with the groups transfected with pGL3-Promoter plasmid (control).

clearly observed when the cells were cultured under hypoxic conditions but not under normoxic conditions. When a control plasmid that does not contain HRE, pGL3-Promoter, was transfected, no detectable level of luciferase activity was observed even under the hypoxic conditions. When the cells were cultured for more than 10 days and became confluent, HIF-1 activity was always observed even under the normoxic conditions. This tendency increased as the culture periods lengthened (data not shown).

Caco-2 cells have been reported to have characteristics of normal intestinal epithelial cells, but the cells are derived from human colon adenocarcinoma cells. The tumorigenicity of the cells may have some relevance to the observed HIF-1 activity. Therefore, we also examined another cell line, IEC-6, which is derived from rat small intestinal epithelial cells and is considered to reflect the characteristics of normal intestinal epithelial cells without tumorigenicity (23). Increased HIF-1 activity was observed in IEC-6 cells when the cells were subjected to hypoxic conditions, suggesting that HIF-1 activity in Caco-2 cells is not caused by the tumorigenic character of the cells but reflects the character of normal intestinal epithelial cells.

Butyrate, but not propionate, down-regulates HIF-1 activity under hypoxic conditions in Caco-2 and IEC-6 cells

Next, we investigated the influence of the short-chain fatty acids butyrate and propionate on HIF-1 activity in Caco-2 and IEC-6 cells under hypoxic conditions. As shown in Figure 2, NaB suppressed induction of HIF-1 activity under hypoxic conditions. NaB also suppressed HIF-1 activity under normoxic conditions, although HIF-1 reporter activity is much lower in normoxic conditions. NaPr did not show any effects under the experimental conditions examined. In contrast, NaPr had a tendency to augment HIF-1 activity, especially in IEC-6 cells. The HIF-1 activity observed in IEC-6 cells was significantly smaller than that in Caco-2 cells.

Butyrate down-regulates HIF-1 activity specifically under hypoxic conditions in concentration-, and time-dependent manners

Subsequently, we examined the effects of timing and doses of administration of NaB. Treatment of the cells with 2 mM of NaB for 36 h significantly down-regulated induction of HIF-1 activity under hypoxic conditions in the cells (Fig. 3A). Treat-

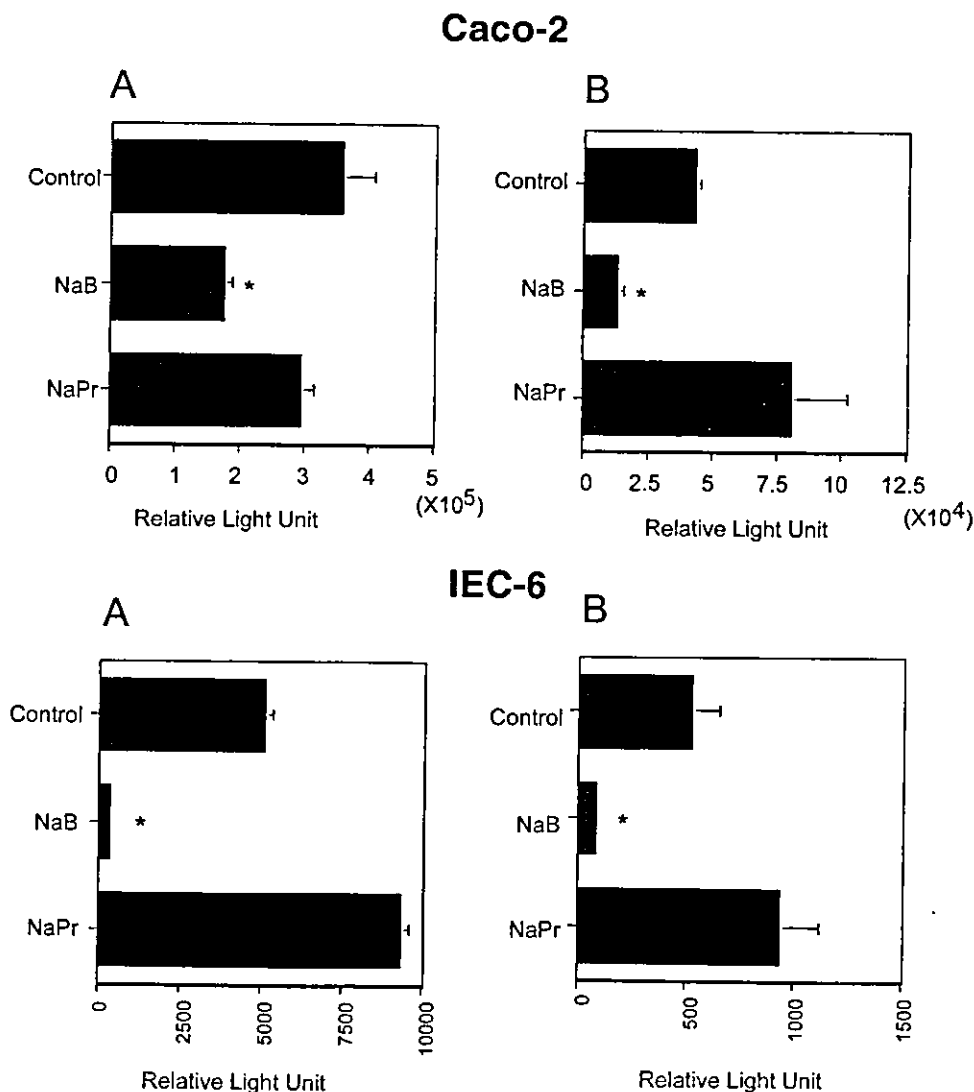

FIG. 2. **Butyrate, but not propionate, suppresses HIF-1 activity under hypoxic conditions in Caco-2 and IEC-6 cells.** Caco-2 or IEC-6 cells were transiently transfected with pHREpgkLuc or pHREpaiLuc plasmid, respectively. The cells were subjected to hypoxic (A) or normoxic conditions (B) for 36 h in the presence of 5 mM of NaB or NaPr. The relative luciferase activities were measured by using the cell lysates. The means ± SE of triplicate determinations are shown. Asterisks indicate statistical significance ($P \leq 0.001$) compared with the untreated groups (control).

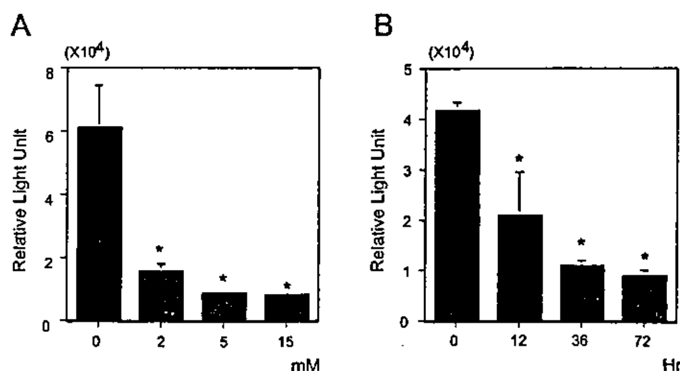

FIG. 3. **Butyrate down-regulates HIF-1 activity under hypoxic conditions in dose-, and time-dependent manners in Caco-2 cells.** Caco-2 cells were transiently transfected with pHREpgkLuc plasmid. (A) The cells were exposed to 0, 2, 5, or 15 mM NaB under hypoxic conditions for 36 h. The relative luciferase activities were measured by using the cell lysates. Asterisks indicate statistical significance ($P \leq 0.001$) compared with cells without NaB (0 mM control). (B) The cells were added with 5 mM of NaB and incubated for 0, 12, 36, or 72 h. Then, they were subjected to hypoxic conditions. The relative luciferase activities were measured by using the cell lysates. Asterisks indicate statistical significance ($P \leq 0.001$) compared with cells incubated without NaB (0 h control).

ment of the cells with 5 mM NaB for 12 h significantly reduced the luciferase activities by HRE reporter transfection. The reduction was more remarkable when the treatment was prolonged (36 and 72 h) (Fig. 3B).

Butyrate inhibits HRE DNA binding activity under hypoxic conditions in Caco-2 cells

To examine whether butyrate treatment indeed inhibits protein binding to HRE DNA in Caco-2 cells under hypoxic culture conditions, we performed EMSA using nuclear extracts of the cells. As shown in Figure 4, we detected faint HRE DNA–protein complex under normoxic conditions. Addition of NaB under normoxic conditions lost the band. Under hypoxic conditions, we could detect a strong band of HRE DNA–protein complex. Excess unlabeled HRE, but not TRE oligonucleotide, inhibited the HRE mobility shift, indicating that the DNA–protein complex is specific. Addition of NaB weakened the band, indicating that butyrate suppresses HRE DNA–protein complex formation.

Butyrate down-regulates expression of ITF *gene*

ITF plays a critical role in the mucosal barrier function and was reported to be regulated by HIF-1 (16). We therefore examined whether butyrate treatment affects the expression of *ITF* mRNA in Caco-2 cells. As shown in Figure 5, NaB decreased *ITF* gene expression under normoxic and hypoxic conditions. The degree of down-regulation was more obvious in hypoxia than in normoxia. Addition of NaPr also affected the expression of *ITF* mRNA under normoxic and hypoxic conditions, although the effect was much weaker than that of addition of NaB.

Butyrate impaired recovery of the intestinal barrier function after hypoxia

Recently, HIF-1 was reported to protect the barrier function through induction of ITF (16). We therefore examined the effect of butyrate on intestinal permeability, i.e., the barrier function, of Caco-2 cells. We measured TEER of Caco-2 cells

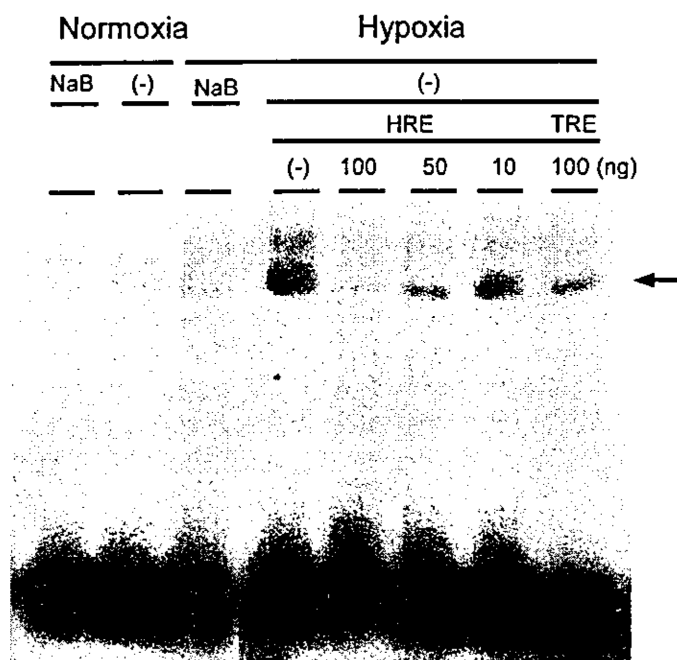

FIG. 4. **Butyrate suppresses HRE DNA–protein complex formation under hypoxic conditions in Caco-2 cells.** Nuclear extracts of Caco-2 cells cultured for 24 h under normoxic conditions (normoxia) with (NaB) or without (–) NaB treatment, and nuclear extracts of Caco-2 cells cultured for 24 h under hypoxic conditions (hypoxia) with (NaB) or without (–) NaB treatment, were analyzed. Different amounts of unlabeled HRE [HRE 100, 50, 10 (ng)] or TRE [TRE 100 (ng)] probes were also added to nuclear extracts of Caco-2 cells cultured under hypoxic conditions without NaB treatment. An arrow indicates HRE DNA–protein complex-specific bands.

after the cells formed a confluent monolayer under normoxic and hypoxic conditions in the presence or absence of NaB for 36 h. Then, the cells were reoxygenated. TEER of the cells that were exposed to hypoxic conditions without NaB treatment recovered to normal levels immediately (within 15 min). On the other hand, addition of NaB decreased TEER recovery in a dose-dependent manner (Fig. 6). Two millimolar NaB significantly reduced recovery of the barrier function after reoxygenation. Addition of 15 mM NaB completely suppressed the recovery.

DISCUSSION

The present study showed that butyrate has suppressive effects on the up-regulation of HIF-1 transcriptional activity, HRE–DNA binding activity, and transcription of *ITF* gene under hypoxic conditions in intestinal epithelial cells and also that butyrate modulates the barrier function of the cells.

In this study, we mainly used Caco-2 cells as a model of intestinal epithelial cells because these cells develop the morphologic characteristics of normal enterocytes and have been widely studied as an excellent model of intestinal epithelial cells (24). The cells were reported to be subjected to differentiation after seeding and to mimic the differentiation of enterocytes *in vivo* (24). Intestinal epithelial cells differentiate during the process of elevating from the crypt fossa toward the apex of villi in approximately 4 days. The cells are exposed to the contents of the intestinal lumen, mainly bacteria and bacterial products, during the differentiation process. Taking this

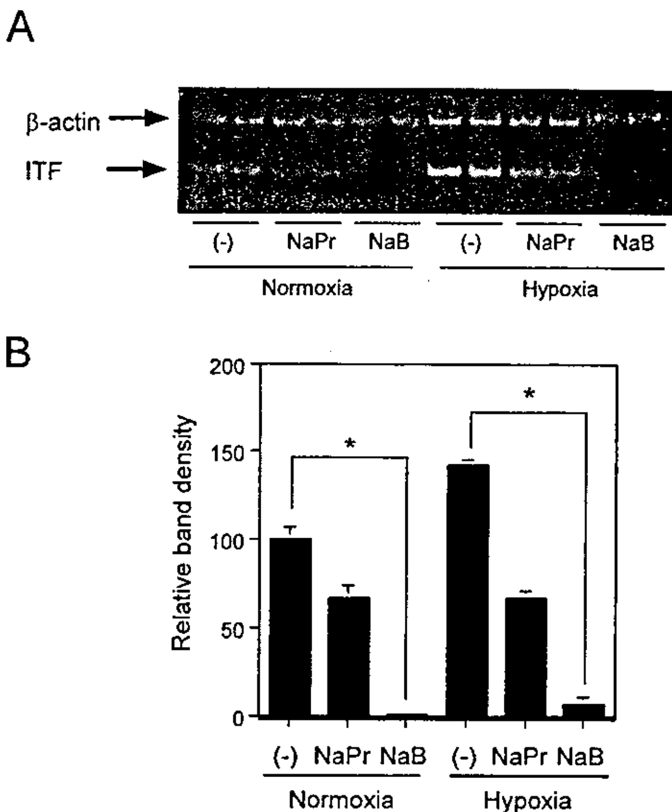

Fig. 5. **Butyrate affects expression of *ITF* gene.** Thirty-six hours after Caco-2 cells were incubated under normoxic or hypoxic conditions in the presence or absence of 5 mM of NaPr or NaB, total RNA was prepared, and semiquantitative RT-PCR was performed with primers specific for *ITF* gene. Five millimolar NaB or NaPr was used. As a control, the same RT-PCR was performed with primers for *β-actin* gene. The means ± SE of triplicate determinations of relative band densities are shown (the strength of *ITF*-specific band in normoxia without NaB or NaPr treatment is 100). Asterisks indicate statistical significance ($P \leq 0.001$) compared with the untreated group (-).

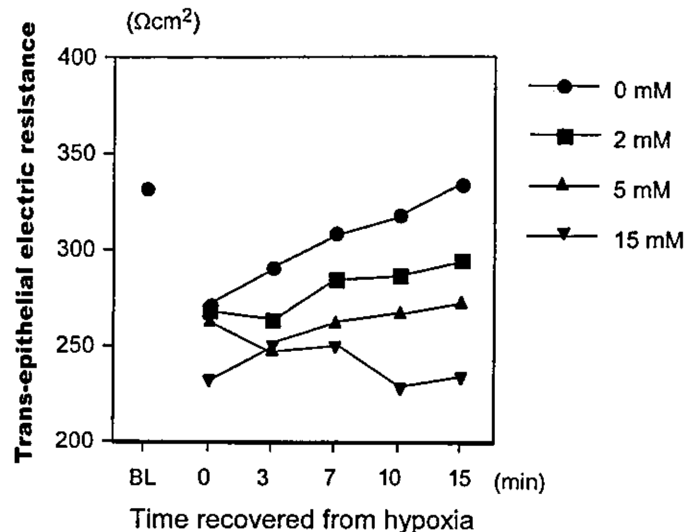

Fig. 6. **Butyrate suppresses recovery of TEER after reoxygenation treatment of Caco-2 cells.** Caco-2 cells were untreated (0 mM) or treated with different concentration of NaB (2, 5, or 15 mM). Immediately, the cells were subjected to hypoxic conditions and cultured for 36 h. Then, the cells were reoxygenated for 0 to 15 min. During reoxygenation, TEER was measured. Average value from triplicate measurement was shown for each point. BL denotes the baseline, the TEER level of Caco-2 cells without NaB treatment in normoxia.

into consideration, we set up a model to study the effects of butyrate under hypoxic conditions on HIF-1 activity, arranging timing and doses of butyrate administration so as to mimic the physiological conditions of enterocytes. Caco-2 cells have been also shown to be suitable as a model system to study the barrier function by measuring the TEER values after the cells form cobblestone-like monolayers on Transwell polycarbonate membranes (25). We measured TEER as an indicator for intestinal barrier functions in this study.

We examined the effects of 2 to 15 mM of butyrate, which concentrations are higher than those used in previous studies (26, 27) but are equal to actual values in the human intestinal lumen (1). Mucinous secretory substances have been suggested to prevent butyrate in the lumen from direct contact with enterocytes *in vivo* (26). That may be the case under normoxic conditions. However, goblet cells, the main source of mucus secretion, do not restitute under the ischemic state or after ischemia-reperfusion injury (28). In addition, hypoxia may increase the number of butyrate-producing anaerobic bacteria such as *Corynebacteria* and *Fusobacteria* in the intestinal tract. Therefore, enterocytes might be exposed to butyrate at higher concentrations than that speculated in a previous work (26).

Butyrate has been reported to favor the barrier function of intestinal epithelial cells under normoxic conditions (26, 27, 29). Supporting that, Verkartraman et al. (2) reported that butyrate hastens restoration of the barrier function after thermal and detergent injury to the rat distal colon *in vitro*. We also found that butyrate reduced paracellular permeability when butyrate (up to 15 mM) was added to Caco-2 cells under normoxic conditions (data not shown). However, we showed in this study that butyrate disturbs the recovery of the barrier function after exposure to hypoxia. Under normoxic conditions, butyrate has been reported to induce differentiation of Caco-2 cells, and the differentiated state of the cells is important for enhancement of the barrier function (24). On the other hand, butyrate may act under hypoxic conditions in a different manner from under normoxic conditions. In this study, we found that butyrate remarkably down-regulated HIF-1 activity and decreased *ITF* gene expression in hypoxia. Because ITF plays a pivotal role in epithelial integrity, we reasoned that this might be the main reason for butyrate to modulate the barrier function in hypoxia.

The expression level of ITF may be one of the critical factors influencing the barrier function of intestinal epithelial cells. Tran et al. (17) showed that butyrate inhibits *ITF* gene expression in colon cancer cells under both normoxic and hypoxic conditions. We confirmed their findings in this study (Fig. 4). They identified the butyrate response element (BRE) in the promoter region of the human *ITF* gene and showed that the BRE is essential for butyrate to influence *ITF* gene expression. On the other hand, Furuta et al. (16) claimed to identify a previously unappreciated HIF-1 binding site (HRE) in the promoter of human *ITF* gene, although we could not identify the site at the promoter region that Seib et al. (21) reported. Here, we showed that butyrate decreases the HIF-1 activity of intestinal epithelial cells in hypoxia. Taken together, either the pathway through BRE or HRE, or both pathways, may be responsible for the effect of butyrate on *ITF* gene expression.

Butyrate has been considered to regulate an array of genes.

This effect has been mainly attributed to the inhibition of histone deacetylases. Recently, Kim et al. (8) reported that a specific HDAC inhibitor, trichostatin A, down-regulates HIF-1α activity and *VEGF* gene expression. Therefore, we reasoned that the suppressive effects of butyrate on HIF-1 activity in intestinal epithelium cells in hypoxia might be attributable to its ability to inhibit HDAC in these cells. However, Andoh et al. (30) reported that the effect of butyrate on several intestinal epithelial cell lines is different from that of trichostatin A. Further study is needed to clarify the effects of butyrate on HDAC under hypoxic conditions. Alternatively, the suppressive effects of butyrate on HIF-1 activity might be mediated by nitric oxide. We observed that butyrate induces expression of the gene for inducible nitric oxide synthase (iNOS) in Caco-2 cells (data not shown). It has been reported that administration of nitric oxide donors or introduction of *iNOS* gene inhibits HIF-1 activity in several cell lines (31–33). So, butyrate may induce production of nitric oxide and consequently inhibit HIF-1 activity in intestinal epithelium cells.

Ding et al. (34) cocultured ileal mucosal membranous tissue and *Escherichia coli* with an Ussing chamber system and showed by measuring the membranous electrical resistance that the combination of hypoxia–reoxygenation plus *E. coli* bacterial challenge caused irreversible mucosal damage. In their work, the cytokines produced by intestinal epithelial lymphocytes, bacteria, and/or bacterial products might have accounted for the barrier failure. Here, we focused on studying the pivotal transcriptional factor HIF-1 in intestinal epithelial cells. We showed that butyrate inhibits HIF-1 activity and modulates the barrier function in the cells. Supporting our results, Zgouras et al. (35) recently reported that butyrate decreases HIF-1α DNA-binding activity by inhibiting HIF-1α nuclear translocation in Caco-2 cells, which subsequently reduce VEGF secretion levels. Mariadason et al. (36) reported that butyrate disrupts the intestinal barrier function under some conditions. It seems that butyrate possesses either beneficial or deleterious effects on the barrier function of intestinal mucosa depending on the situation. We studied here the effect of butyrate on TEER of Caco-2 cell line under hypoxic conditions. Treatment with butyrate delayed recovery of TEER after hypoxic conditions. This effect may be caused by apoptotic changes of Caco-2 cells from butyrate treatment. The TEER values observed here were higher than those observed by some other investigators (24–27). The reason for this discrepancy is not clear, but it is possible that the number of Caco-2 cells used in this study was more than the number that is necessary to form a monolayer culture in the plates. Changes in TEER observed in this study *in vitro* have to be evaluated carefully because the study shown here has some limitations in terms of the clinical implications. The limitations include a 36-h period of hypoxia (clinical hypoxic insults are much shorter), the limited degree of changes in TEER, and the short period of time it was measured (i.e., 15 min after a 36-h insult).

A clinical study (37) reported that luminal irrigation of the whole intestine and flushing of intestinal contents were associated with improved survival of critically ill patients in intensive care units. Irrigation of the lumen of the intestine may remove intestinal bacteria and bacterial products, beneficially affecting the physical conditions of patients, even though the study shown here has limitations on the clinical implications as mentioned before. Further study *in vivo* is absolutely required for clarifying the effects of butyrate *in vivo*.

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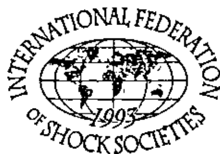

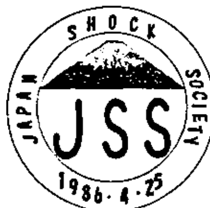

3．Ag85分子DNAワクチンによる抗結核細胞性免疫の誘導

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key words　antigen 85 complex, DNA vaccine, *Mycobacterium tuberculosis*, T-cell epitope, bacterial carrier system

動　向

コッホが結核菌を発見して以来，100年以上を経過しているが，世界的な視野で見ると結核は依然として感染症の中で重要な地位を占めている．毎年，800万人の結核患者が発生し，200万人が結核で死亡している[1]．これは単一の感染症による死因としては最も多い．また，結核菌に曝露された人の多くは細胞性免疫によりこの菌を抑え込み，潜伏させている[2]．この場合には再燃の危険性をはらんでいる．さらに，最近ではエイズの重症な合併症としても結核は問題となっている．結核に対するワクチンとして使われている弱毒生ワクチンである *Mycobcterium bovis* BCGは小児期には有効であるが，10代を過ぎるとその有効性が失われるとされている[3]．このため，BCGに代わるより有効なワクチンの開発が求められている．現在，結核菌弱毒株，DNAワクチン，成分ワクチンなどの研究が進められている．中でも，DNAワクチンは液性免疫のみならず細胞性免疫を誘導できることから，特に感染防御に細胞性免疫を必要とする結核菌に対する新しいワクチンとして注目を集めている．DNAワクチン（プラスミド）のもつCpGモチーフによるアジュバント効果が強力な細胞性免疫誘導能に関与すると考えられる[4]．結核に対するDNAワクチンの構築には，適切な防御抗原を用いる必要がある．主要な防御抗原としては結核菌の分泌蛋白および細胞表面蛋白が候補となる．本稿では結核菌の主要な分泌蛋白であるAg85ファミリー分子を用いたDNA成分ワクチンについて概説する．

A．Ag85分子複合体

表1に結核菌の主要蛋白を示す[5]．Nagaiら[6]の定義によるとMPBという用語は *M. bovis* BCG由来の蛋白質に用いられ，MPTという用語はこれに対応する結核菌由来の蛋白質に用いられる．LI（localization index）は分泌効率を示す[7]．上記のように成分ワクチンに用いる防御抗原の候補としては分泌蛋白があげられるが，Ag85分子複合体（Ag85A，Ag85B，Ag85C）は結核菌の主要な分泌蛋白であることが，報告されている．Fukuiら[8]は結核菌培養液中の蛋白質の41％をAg85分子が占めると報告している．また，De Bruynら[9]は14～15％，Wikerら[5]は60％を占めると報告している．これらの結果の相違は培養中の結核菌の溶菌によるものと考えられる．すなわち，溶菌により結核菌の細菌内蛋白が培養液中

表1　結核菌および *M. bovis* BCG由来蛋白の分類（文献5より改変）

グループ (kDa)	名称	CIE[a]抗原 No.	他の名称	LI[b]	N-末アミノ酸配列
分泌蛋白					
41	MPT32			90	DPEPA
31.5	MPT45	Ag85C		20	FSRPG
31	MPT44	Ag85A	P32	150	FSRPG
30	MPT59	Ag85B	ag6，α-ag	70	FSRPG
27	MPT51			25	APYEN
26	MPT64			40	APKTY
23	MPB70	70		1,000	GDLVG
18	MPT63				AYPIT
15	MPT53				DEXLQ
熱ショック蛋白（Hsp）					
71	DnaK	63		＜0.03	ARAVG
65	GroEL	82		0.0004	KTIAY
12	GroES		BCG-a，MPT57	＜0.04	AKVNI
リポ蛋白					
38	PhoS	78		5	CGSKP
19					CSSNK
酵素					
40	EC1.4．1.1				MRVGI
23	SOD[c]	62			AEYTL

[a] CIE: crossed immunoelectrophoresis
[b] LI: localization index
[c] SOD: superoxide dismutase

に混入すると，当然のことながら，Ag85分子複合体の占める割合は低くなる．Wikerら[5]は溶菌を起こしにくい結核菌株（*M. tuberculosis* H37Rv）では，上記のようにAg85分子複合体が全分泌蛋白中の60%を占め，Ag85Bの方がAg85Aよりも若干多いことを証明している．Ag85分子複合体はヒトフィブロネクチンに結合能を示す．また，これはコードファクターの生成に必要なmycolyl transferase酵素活性を示す[10]．因みに，Ag85AはP32と，Ag85Bはα抗原と同一の分子である（表1）．

Oharaら[11]はAg85A分子をコードする遺伝子の下流179bpにMPT51（MPB51）分子をコードする遺伝子が存在することを報告している．MPB51分子はAg85分子複合体と37～43%の相同性を示すため，Ag85ファミリー分子に属すると我々は考えている（図1）．

B. Ag85分子DNAワクチン

Ag85A分子は結核菌に感染した健常人のリンパ球を刺激し，強い増殖性反応およびIFN-γ産生を誘導する[12]．従って，予想された通り，Ag85分子は主要な感染防御抗原である可能性が強い．実際，マウスにAg85AまたはAg85B分子を発現するDNAワクチンを筋注接種すると，特異的液性免疫，細胞性免疫〔T細胞増殖反応，Th1細胞反応，細胞傷害性T細胞（CTL)〕が誘導されるのみならず，結核菌の経静脈感染に感染防御能が賦与される[13,14]．しかしながら，Ag85C

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              1        10        20        30        40        50        60        70       80
BCG Ag85A     MQLVDRVRGAVTGMSRRLVVGAVGAALVSGLVGAVGGTATAGAFSRPGLPVEYLQVPSPSMGRDIKVQFQSGGANSP-ALY
M.t.Ag85A     MQLVDRVRGAVTGMSRRLVVGAVGAALVSGLVGAVGGTATAGAFSRPGLPVEYLQVPSPSMGRDIKVQFQSGGANSP-ALY
BCG Ag85B     MTDVSRKIRAWGRRLMIGTAAAVVLPGLVGLAGGAATAGA---FSRPGLPVEYLQVPSPSMGRDIKVQFQSGGNNSP-AVY
M.t.Ag85B     MTDVSRKIRAWGRRLMIGTAAAVVLPGLVGLAGGAATAGA---FSRPGLPVEYLQVPSPSMGRDIKVQFQSGGNNSP-AVY
BCG MPB51     MKGRSALLRALWIAALSFGLGGVAVAAEPTAKA---------------APYENLMVPSPSMGRDIPVAFLAGG---PHAVY
M.t.MPT51     MKGRSALLRALWIAALSFGLGGVAVAAEPTAKA---------------APYENLMVPSPSMGRDIPVAFLAGG---PHAVY
                       90       100       110       120       130       140       150      160
BCG Ag85A     LLDGLRAQDDFSGWDINTPAFEWYDQSGLSVVMPVGGQSSFYSDWYQPACGKAGCQTYKWETFLTSELPGWLQANRHVKP
M.t.Ag85A     LLDGLRAQDDFSGWDINTPAFEWYDQSGLSVVMPVGGQSSFYSDWYQPACGKAGCQTYKWETFLTSELPGWLQANRHVKP
BCG Ag85B     LLDGLRAQDDYNGWDINTPAFEWYYQSGLSIVMPVGGQSSFYSDWYSPACGKAGCQTYKWETLLTSELPQWLSANRAVKP
M.t.Ag85B     LLDGLRAQDDYNGWDINTPAFEWYYQSGLSIVMPVGGQSSFYSDWYSPACGKAGCQTYKWETFLTSELPQWLSANRAVKP
BCG MPB51     LLDAFNAGPDVSNWVTAGNAMNTLAGKGISVVAPAGGAYSMYTNWEQD-------GSKQWDTFLSAELPDWLAANRGLAP
M.t.MPT51     LLDAFNAGPDVSNWVTAGNAMNTLAGKGISVVAPAGGAYSMYTNWEQD-------GSKQWDTFLSAELPDWLAANRGLAP
                      170       180       190       200       210       220       230      240
BCG Ag85A     TGSAVVGLSMAASSALTLAIYHPQQFVYAGAMSGLLDPSQAMGPTLIGLAMGDAGGYKASDMWGPKEDPAWQRNDPLLNV
M.t.Ag85A     TGSAVVGLSMAASSALTLAIYHPQQFVYAGAMSGLLDPSQAMGPTLIGLAMGDAGGYKASDMWGPKEDPAWQRNDPLLNV
BCG Ag85B     TGSAAIGLSMAGSSAMILAAYHPQQFIYAGSLSALLDPSQGMGPSLIGLAMGDAGGYKAADMWGPSSDPAWERNDPTQQI
M.t.Ag85B     TGSAAIGLSMAGSSAMILAAYHPQQFIYAGSLSALLDPSQGMGPSLIGLAMGDAGGYKAADMWGPSSDPAWERNDPTQQI
BCG MPB51     GGHAAVGAAQGGYGAMALAAFHPDRFGFAGSMSGFLYPSNTTTNGAIAAGMQQFGGVDTNGMWGAPQLGRWKWHDPWVHA
M.t.MPT51     GGHAAVGAAQGGYGAMALAAFHPDRFGFAGSMSGFLYPSNTTTNGAIAAGMQQFGGVDTNGMWGAPQLGRWKWHDPWVHA
                      250       260       270       280       290       300       310      320
BCG Ag85A     GKLIANNTRVWVYCGNGKPSDLGGNNLPAKFLEGFVRTSNIKFQDAYNAGGGHNGVFDFPDSGTHSWEYWGAQLNAMKPD
M.t.Ag85A     GKLIANNTRVWVYCGNGKPSDLGGNNLPAKFLEGFVRTSNIKFQDAYNAGGGHNGVFDFPDSGTHSWEYWGAQLNAMKPD
BCG Ag85B     PKLVANNTRLWVYCGNGTPNELGGANIPAEFLENFVRSSNLKFQDAYKPAGGHNAVFNFPPNGTHSWEYWGAQLNAMKGD
M.t.Ag85B     PKLVANNTRLWVYCGNGTPNELGGANIPAEFLENFVRSSNLKFQDAYNAAGGHNAVFNFPPNGTHSWEYWGAQLNAMKGD
BCG MPB51     SLLAQNNTRVWVWSPTNPGASDPAAMI-GQAAEAMGNS--RMFYNQYRSVGGHNGHFDFPASGDNGWGSWAPQLGAMSGD
M.t.MPT51     SLLAQNNTRVWVWSPTNPGASDPAAMI-GQAAEAMGNS--RMFYNQYRSVGGHNGHFDFPASGDNGWGSWAPQLGAMSGD
                      330     338
BCG Ag85A     LQRALGATPNTGPAPQGA
M.t.Ag85A     LQRALGATPNTGPAPQGA
BCG Ag85B     LQSSLGSG
M.t.Ag85B     LQSSLGSG
BCG MPB51     IVGAIR
M.t.MPT51     IVGAIR
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図1　Ag85A，Ag85BおよびMPT/MPB51分子のアミノ酸配列

下線の部分はシグナル配列，太字はT細胞エピトープを示す．

のDNAワクチンでは結核菌に対する感染防御能を誘導できない[14]．このDNAワクチンはマウスに液性免疫応答を誘導できることから[15]，Ag85Cは免疫原性を保持しているが，結核菌からの分泌量が少ないため，感染防御能を誘導できないと推察される．

結核菌に対する感染防御能の誘導にはDNAワクチンの免疫ルートも関与することが報告されている．Tangheら[16]はC57BL/6およびBALB/cマウスにAg85A DNAワクチンを筋注法と遺伝子銃法で接種した．その結果，筋注で免疫したC57BL/6マウスのみが結核菌の経静脈感染に抵抗性を示し，遺伝子銃法は無効であった．これは筋注法ではTh1細胞が，遺伝子銃法ではTh2細胞が誘導されるという結果と一致するものであった（遺伝子銃の標的細胞となる表皮抗原提示細胞であるLangerhans細胞は所属リンパ節に移行するとCCL22ケモカインを発現する．CCL22はCCR4のリガンドであり，CCR4を発現しているTh2細胞を感作すると推察される[17]）．この場合，BALB/cマウスでは遺伝子銃法で強いCTL活性が誘導できていることから，この結核感染実験ではTh1細胞が感染防御に重要であると考えられる．しかしながら，結核菌の感染防御にはTh1細胞のみならずCTLも関与することが報告されている[18,19]．Th1細胞は感染早期に，CTLは感染後期に働く可能性があり[19]，このような結果になったとも考えられる．我々の研究結果[20]によると，遺伝子銃法は筋注法に比べて免疫誘導の再現性がよく，必要なプラスミドの量も少なくて済む（遺伝子銃法: 1～2μg，筋注法: 50～100μg）ので，IL-12の発現プラスミドを同時投与するな

どの工夫を加えて，Th1細胞を誘導できるようにすれば，優れたワクチン接種法となろう．

Ag85A，Ag85B DNAワクチンの有効性は報告により，ばらつきが認められる．その要因として，上記の免疫ルートの他に，免疫スケジュール，プラスミドのCpGモチーフ（抑制性または中和CpGの存在），結核菌の感染ルート（経静脈またはエアロゾル感染），用いた結核菌のビルレンスの差などがあげられる．

マウスはヒトに比べると結核菌感染に抵抗性であり，ヒトの感染モデルとしては適当でないかも知れない．モルモットはヒトよりも感受性である．Baldwinら[21]はAg85Aを発現するDNAワクチンをモルモットに接種し，感染実験を行った．このワクチンはモルモットの生存期間を延長したが，BCGよりも効果は劣ると報告している．カニクイザルはヒトと同等の結核菌に対する感受性を示し，ヒトの感染モデルとして適している[22]．しかし，個体差が大きく実験結果にばらつきがみられるという憾みがある（このことはヒトにも当てはまるが）．

DNAワクチンを結核の治療に用いる試みが行われている．Lowrieら[23]は結核菌静注後8週にDNAワクチンを接種し，治療効果を検討した．その結果，Hsp65 DNAワクチンが脾臓，肺での結核菌数を有意に減少させることができた．また，結核の再燃モデルであるCornell型モデル（結核菌感染後，抗結核薬を投与し，その後にステロイドを投与し，再燃させる）でもこのDNAワクチンは有効であったと報告している．しかしながら，Taylorら[24]は肺結核のモデルである結核菌のエアロゾル感染後においてはHsp65 DNAワクチンは所謂"コッホ反応"を起こし，重症な肺壊死を引き起こすと報告している．また，Cornell型モデルでも肺の菌数を減少させることはできなかった．このことは，すでに結核菌に対する免疫が成立している宿主へのDNAワクチン接種には注意を払う必要があることを示唆している．結核菌のHsp（熱ショック蛋白）は酸欠状態で培養した場合に多量に産生される．肺においては，酸素量が多いためHspの産生は少ないとも考えられる．また，しばしば議論の対象となるように結核菌のHsp65はヒトのそれと40～50%のアミノ酸配列の相同性を示すので，これをワクチンに用いると自己免疫疾患を惹起する可能性も否定できない．

C. Ag85ファミリー分子のT細胞エピトープ

Ag85ファミリー分子のT細胞エピトープを知ることは，①各Ag85分子ワクチンで誘導される細胞性免疫の質（Th1細胞，CTL），②テトラマー法などによるAg85分子特異的T細胞の解析，③エピトープワクチンの開発などに有用である．Ag85A，Ag85B，Ag85Cは高い相同性を示し，Ag85AはAg85Bと約77%，Ag85Cとは約71%の相同性を示す．従って，これら分子間には共通のT細胞エピトープが存在することが推察される．実際，Ag85AとAg85Bの161～169アミノ酸残基（図1ではシグナル配列43アミノ酸を含むので204～212）は共通して，BALB/c（$H\text{-}2^d$）の$CD8^+$T細胞エピトープであることが報告されている[25]．また，BALB/cマウスではAg85A（aa 61～80; 図1では104～123）にもおよびAg85A（aa 145～153）に$H\text{-}2^d$拘束性の$CD8^+$T細胞拘束性のエピトープがある[25]．このうち，Ag85A（aa 60～68）はK^d拘束性[26]，Ag85A（aa 70～78）はL^d拘束性の可能性が指摘されている[25]．さらに，Ag85A（aa 101～120）には$H\text{-}2E^d$拘束性のCD^+4 T細胞のエピトープが存在する[27,28]．C57BL/6（$H\text{-}2^b$）マウスにおいてはAg85A（aa 261～280）に$CD4^+$T細胞エピトープが存在する[27]．また，Ag85B（aa 240～254; 図1では283～297）は$I\text{-}A^b$拘束性$CD4^+$T細胞エピトープで

表2　Ag85分子のヒトT細胞エピトープ

ペプチド	HLA拘束性	反応性T細胞
Ag85Bペプチド（aa 10～27）	HLA-DR3, DR52, DR53	CD4
Ag85Bペプチド（aa 51～70）	HLA-DR3	CD4
Ag85Bペプチド（aa 19～36）	HLA非拘束性	CD4
Ag85Bペプチド（aa 91～108）	HLA非拘束性	CD4
Ag85Cペプチド（aa 204～212）	HLA-B35	CD8
Ag85Bペプチド（aa 199～207）	HLA-A*0201	CD8

あり，このT細胞は$V\beta 11$を発現している[29]．これらT細胞エピトープの同定には，マウスをDNAワクチンで感作する方が，結核菌，BCGで感作するよりも，明確な結果が得られることがわかっている[25]．以上より，C57BL/6マウスではAg85A分子を認識する$CD8^+$T細胞エピトープがないことがわかる．実際，H-2^{b}ハプロタイプの$CD4^+$T細胞KOマウスではAg85A DNAワクチンが結核菌感染に無効であるが，$CD8^+$T細胞KOマウスでは有効であるとの報告がある[30]．一方，BALB/cマウスではAg85A DNAワクチンで強力に特異的CTLが誘導される[16]．

以上のように，Ag85AおよびAg85B分子のT細胞エピトープはよく研究されている．図1に示すように，MPB51分子はAg85分子複合体と37～43%の相同性を示すため，我々はAg85ファミリー分子に属すると考えている．そこで，現在MPB51分子のT細胞エピトープの同定を行っている．C57BL/6マウスではドミナント，サブドミナントの2つの$CD4^+$T細胞エピトープを同定した．また，BALB/cマウスでは1つの$CD8^+$T細胞エピトープを同定している．さらに，HLA-A*0201トランスジェニック（Tg）/H-2クラスI KOマウスを用いることにより，MPB51分子のHLA-A*0201拘束性エピトープを同定している．HLA-A*0201拘束性T細胞エピトープに関しては，Ag85B（aa 199～207）が同定されている[31]．Ag85BおよびAg85CのヒトT細胞エピトープを表2にまとめた．興味あることにHLA非拘束性（promiscuous）エピトープが認められている[32]．これらはエピトープワクチンおよび結核の診断に有用であると考えられる．

D. DNAワクチンの接種法

1. 細胞内寄生菌弱毒株をキャリヤーとしたDNAワクチン

DNAワクチンの接種法としては，上記した筋注法および遺伝子銃法が一般的である．一方，DNAワクチンを効率よく，宿主細胞内へ導入するため，細胞内寄生菌の弱毒株をキャリヤーとして用いる方法が試みられている．*Shigella flexneri*[33]および*Salmonella typhimurium*[34]の栄養素要求株に真核細胞の発現ベクターを使ったDNAワクチンを導入し，動物に接種するものである（図2）．

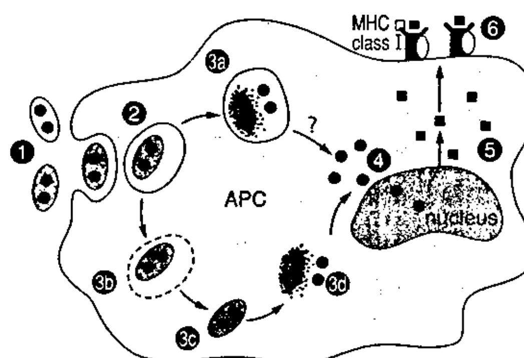

図2　細胞内寄生菌をキャリヤーとしたDNAワクチン（文献53より改変）
3aはサルモネラ，3b，3cはリステリア，赤痢菌によるDNAワクチンの運搬経路を示す．

サルモネラをキャリヤーにした場合は，宿主細胞の食胞に取り込まれ，その後，不明のメカニズム（おそらくはcross-primingに関わる機構）を介して，細胞質，核内に移行し，DNAワクチン（プラスミド）の遺伝子発現が行われると考えられる．一方，リステリアはlisteriolysin O（LLO）を産生し，食胞から細胞質に移行するため，そこでDNAワクチンを放出すると考えられる．赤痢菌も同様に細胞質に移行する性質をもっている．Mollenkopfら[35]は*S. typhimurium*弱毒株をキャリヤーとして，ESAT-6をほ乳類発現ベクターに組込んだDNAワクチンをマウスに免疫した．その結果，結核に対する感染防御能を誘導できたと報告している．我々は*S. typhimurium*をキャリヤーとしたDNAワクチンを作製したが，期待通りの結果は得られなかった．*S. typhimurium*は上記したように宿主の抗原提示細胞（APC）に取り込まれた後，食胞内で壊れ，DNAワクチンを放出するため，転写，翻訳に支障をきたす可能性がある．また，グラム陰性菌をキャリヤーとして用いるとLPSがプラスミドからの蛋白合成を阻害する可能性がある．

そこで，我々はグラム陽性細胞内寄生菌であるリステリアの弱毒株をキャリヤーとして，DNAワクチンを宿主細胞（APC）内に導入することを計画した．リステリアは食胞から細胞質内へ移行する点，リステリアが産生するlisteriolysin O（LLO）が感作T細胞をTh1へシフトさせる点からも結核ワクチンの目的に適っている．弱毒リステリア株としてはレシチナーゼオペロン（*mpl, actA, plcB*）を欠失した弱毒リステリア株を用いた．これにリステリア溶菌性ベクター（リステリアに対するファージのリジン遺伝子（PLY118）の上流にactAプロモーターを配したプラスミド）に結核菌の主要な防御抗原であるAg85A，Ag85B，MPT51をコードする遺伝子をおのおの挿入し，エレクトロポレーションでリステリアに導入した．この組換えリステリアは宿主細胞の食胞からLLOにより細胞質に移行した時点で，actAプロモーターが活性化するため溶菌し，DNAワクチンを細胞質に放出するようにデザインしてある（図2; 3d）．この組換えリステリア株をC57BL/6マウスに腹腔投与したところ，結核菌特異的Th1細胞の誘導に成功した．また，結核菌の経静脈感染に対する抵抗性を誘導した．このワクチンは点鼻投与可能で，結核菌の感染防御に重要と考えられるBALT（bronchus-associated lymphoid tissue）ホーミング性T細胞（$\alpha_4\beta_1$インテグリン; VLA-4陽性）の誘導に有効である可能性もある[36]．

2．組換えレトロウイルス導入樹状細胞（DC）ワクチン

DCはナイーブT細胞を有効に感作するプロフェッショナルAPCである．DCにペプチドをパルスして免疫する試みは腫瘍免疫の分野で盛んに行われている[37]．結核に対しても，BCGを感染させたDCで免疫する試みや，Ag85Aの$CD8^+$および$CD4^+$T細胞エピトープ（ペプチド）をパルスしたDCでマウスを免疫する試みが行われており，両者とも結核菌感染の防御に一定の効果をあげている[26]．我々はDCワクチンをさらに改善するため，抗原遺伝子をレトロウイルスでDCに導入し，効率よく抗原提示を行うDCの作製法を確立した[38]．プラスミドpMXとパッケージ細胞株Phoenixを用いてリステリアのCTLエピトープLLO91-99を発現するレトロウイルスを作製した．これをBALB/cマウスの骨髄由来DCに感染させ，マウスに経静脈的に投与した．我々の方法によるレトロウイルスのDCへの導入効率は34.8%であった．この免疫法で誘導される特異的IFN-γ産生能，CTL活性および感染防御能はDNAワクチンによるものより強力であった（図3）．現在，Ag85AおよびAg85Bの組換えレトロ

ウイルスベクターを作製し，これを感染させた．DCをマウスに免疫し，感染防御能を検討している．

3. 感作-ブースト prime-boostヘテロ免疫法

DNAワクチンで最近最もホットな話題は感作-ブースト・ヘテロ免疫法である．これは感作とブーストを同じ抗原を含む異なった組換えワクチンで行う方法である[39]．これにより，同じワクチンで感作-ブーストを行った場合に比し，約50～100倍のCTL活性を誘導できる．この方法は主にHIV[40]，マラリア原虫[41]感染に対するワクチン研究で実施されており，通常DNAワクチンで感作した後，組換えワクシニアウイルス（MVA: modified vaccinia Ankara，Ankara株は高度に弱毒化したワクシニア）でブーストが行われる．ブーストに組換えfowlpox（鶏痘）ウイルスが使われる場合もある[42]．しかしながら，この逆，すなわち感作を組換えワクシニアで行い，ブーストをDNAワクチンで行った場合には増強効果は認めない．

結核に対するワクチンでも同様の試みが行われている．McShaneら[43]はESAT-6とMPT63のDNAワクチンで感作し，MVAでブースターを行い，BCGと同等の結核菌感染防御を得ている．一方，Skinnerら[44]はESAT-6とAg85AのDNAワクチンで感作し，BCGかWag520（新規弱毒株）でブースターしたところ，特異的IFN-γ産生はDNAワクチンまたは弱毒株ワクチン単独よりも増強した．しかし，結核菌のエアロゾル感染に対する防御能はBCGまたはWag520単独免疫で誘導されるものと差がなかった．Tangheら[45]はAg85AのDNAワクチンでC67BL/6マウスを感作し，その後にこの蛋白でブースターすることで，DNAワクチン単独よりも強いTh1細胞と結核菌の経静脈感染に対する防御能を得ている．Ag85A DNAワクチンによる感染防御能は

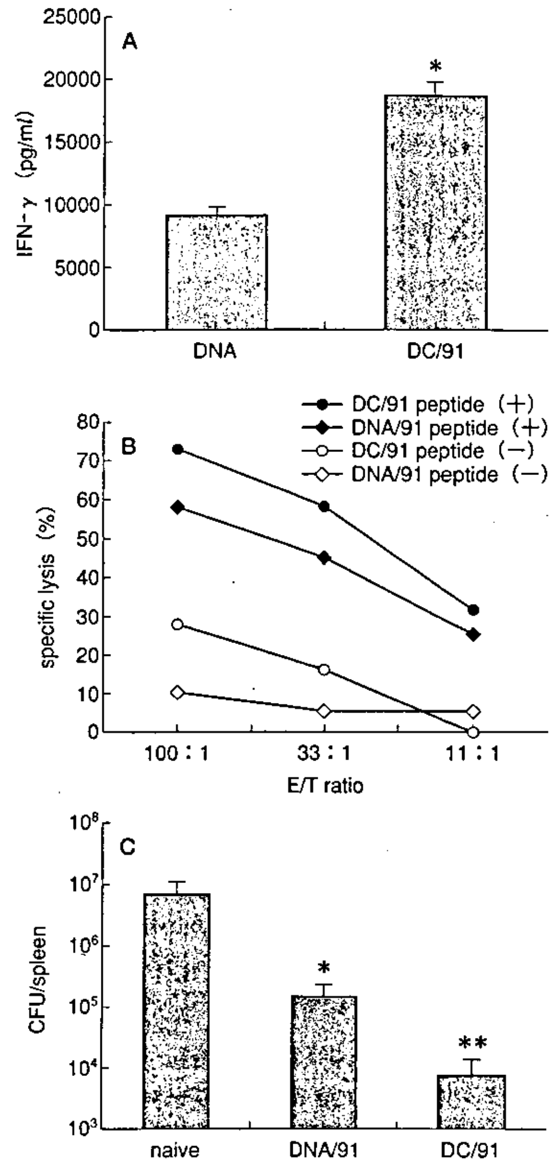

図3 DNAワクチンと組換えレトロウイルス導入樹状細胞(DC)ワクチンによる免疫誘導能の比較

BALB/cマウスにリステリアのLLO91-99を発現するDNAワクチン（DNA）またはレトロウイルスベクターでLLO91-99遺伝子を導入したDCで免疫した(DC/91)．免疫8週後に脾細胞を回収し，LLO91-99ペプチドで刺激しIFN-γ産生能(A)およびJ774細胞をLLO91-99ペプチドでパルス（+）または非パルス（−）したものを標的細胞として，CTL活性を測定した(B)．免疫および非免疫（naive）マウスに3×10^4 CFUのリステリアを感染させ，72時間後の脾臓中の菌数を測定した(C)．*$p < 0.01$（naiveとの比較），**$p < 0.01$（DNA/91との比較）

結核菌感染1週間に強く，その後減弱することから，この感作-ブースト法は有効かも知れない．しかし，Tangheらの方法で誘導された感染防御能はBCGで得られたものと同程度であった．

E. DNAワクチンの効果増強

これまで述べてきたDNAワクチンによって誘導される感染防御能は程度の差こそあれ，BCGによって誘導される防御能を越えることはなく，初期の目的を達成していない．そこで，様々な試みが行われている．たとえば，①融合蛋白発現DNAワクチン，②アジュバントの使用，③サイトカインの同時投与などが報告されている．Minionら[46]は結核菌の主要な防御抗原であるESAT-6と結核菌の表面抗原であるP71の融合蛋白ESAT-6: P71を発現するDNAワクチンを作製した．これはマウスに強いTh1細胞反応を誘導することを報告している．Olsenら[47]はESAT-6とAg85Bの融合蛋白をアジュバントとともに接種することにより，強い感染防御能を誘導できることを証明している．このワクチンの特徴は記憶T細胞を維持できる点にあり，結核菌感染後30週後では特に肺における結核菌数はBCG免疫マウスのそれに比し，有意に少ない．これはDNAワクチンにも応用できよう．アジュバントとしてはプラスミドを陽イオン脂質（VC1052: DPyPE）（Vaxfection）または中性脂質（GAP-DLRIE: DOPE）で包埋したものが使われている．当初，これらはDNAワクチンによる抗体産生は増強するが，細胞性免疫は増強しないとされていた．しかしながら，D'Souzaら[48]はAg85AのDNAワクチンにVaxfectinを用いてマウスに筋注すると，抗体産生のみならずTh1細胞の活性増強を認めている．また，興味あることに，この筋注とAg85A/GAP-DLRIE: DOPEを点鼻すると，脾臓のみならず，肺での特異的Th1細胞活性の増強が認められている．

サイトカインに関してはIL-12をコードするDNAワクチンをAg85BまたはMPT64 DNAワクチンと同時に接種すると，強いリンパ球増殖性反応とIFN-γ産生能の誘導が認められている[49]．しかし，感染防御能の顕著な増強は認めなかった．また，Kamathら[50]はGM-CSFがAg85AまたはMPT64のDNAワクチンにより誘導される細胞性免疫を約2倍増強するが感染防御能は増強しないことを報告している．IL-18もIL-12と同様にTh1細胞を誘導することが知られている．しかし，Triccasら[51]によればIL-18はAg85B-DNAワクチンによって誘導される肺結核に対する防御能を増強しない．その他，記憶CD8$^+$T細胞の維持に重要な役割を示すIL-15やIL-7は両者とも結核菌感染マウスの生存率を増加させる[52]ことから，これらサイトカインとの同時投与はDNAワクチンの効果を増強させるかも知れない．

むすび

Ag85分子などの結核菌の主要防御抗原を用いたDNAワクチンは，結核の感染防御に必要なTh1細胞およびCTLを強力に誘導することから，大きな期待を集めている．しかし，マウスを用いた研究では多くの場合，BCGによって誘導される感染防御能を越えることはできない．DNAワクチンとして用いるプラスミドの改良，融合蛋白発現DNAワクチンの研究，より有効な感作-ブーストのプロトコールの開発などが求められよう．一方，DNAワクチンはマウスでは有効であるがヒトを含めた霊長類では無効であるとの噂もある．これはマウスでアジュバント効果を示すCpGモチーフとヒトで有効なCpGモチーフの配列が異なることによるかも知れない．今後は，ヒトへの応用を視野に入れたカニクイザルなど霊長類での研究が求められる．

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