INFECTION AND IMMUNITY, Aug. 2011, p. 3149–3158 Vol. 79, No. 8
0019-9567/11/$12.00 doi:10.1128/IAI.01237-10
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

Suppressed Induction of Proinflammatory Cytokines by a Unique Metabolite Produced by *Vibrio cholerae* O1 El Tor Biotype in Cultured Host Cells▽†

Wasimul Bari,[1,2] Yoon-Jae Song,[4] and Sang Sun Yoon[1,2,3]*

Department of Microbiology,[1] Brain Korea 21 Project for Medical Sciences,[2] and Research Institute of Bacterial Resistance,[3] Yonsei University College of Medicine, Seoul, South Korea, and Department of Life Science, Kyungwon University, Seongnam-Si, Kyeonggi-Do, South Korea[4]

Received 19 November 2010/Returned for modification 9 January 2011/Accepted 5 May 2011

***Vibrio cholerae* O1 has two biotypes, El Tor and Classical, and the latter is now presumed to be extinct in nature. Under carbohydrate-rich growth conditions, El Tor biotype strains produce the neutral fermentation end product 2,3-butanediol (2,3-BD), which prevents accumulation of organic acids from mixed acid fermentation and thus avoids a lethal decrease in the medium pH, while the Classical biotype strains fail to do the same. In this study, we investigated the inhibitory effect of 2,3-BD on the production of two proinflammatory biomarkers, intreleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α), in human intestinal epithelial HT29 and alveolar epithelial A549 cells. Cell-free culture supernatants of El Tor strain N16961 grown in LB supplemented with 1% glucose induced a negligible amount of IL-8 or TNF-α, while the Classical O395 strain induced much higher levels of these proinflammatory cytokines. On the other hand, three mutant strains constructed from the N16961 strain with defects in the constitutive 2,3-BD pathway were also able to induce high levels of cytokines. When HT29 and A549 cells were treated with bacterial flagella, known proinflammatory cytokine inducers, and chemically synthesized 2,3-BD at various concentrations, a dose-dependent decrease in IL-8 and TNF-α production was observed, demonstrating the suppressive effect of 2,3-BD on the production of proinflammatory cytokines in epithelial cells. Upon cotreatment with extraneous 2,3-BD, elevated levels of IκBα, the inhibitor of the NF-κB pathway, were detected in both HT29 and A549 cells. Furthermore, treatments containing 2,3-BD elicited lower levels of NF-κB-responsive luciferase activity, demonstrating that the reduced cytokine production is likely through the inhibition of the NF-κB pathway. These results reveal a novel and potential role of 2,3-BD as an immune modulator that might have conferred a superior pathogenic potential of the El Tor over the Classical biotype.**

Cholera, an acute watery diarrheal disease, is caused by toxigenic strains of the enteric pathogen *Vibrio cholerae* and is mainly associated with consumption of contaminated water (39). On the basis of O-antigen typing, *V. cholerae* has more than 200 identified serogroups, among which only O1 and O139 are reported to be toxigenic. Seven recent pandemics of cholera have been caused by O1 serogroup strains, although O139 strains were reported to be associated with Asiatic cholera epidemics (7, 24, 37). The O1 serogroup of *V. cholerae* has further been classified into two biotypes, El Tor and Classical. Among the seven worldwide historical pandemics, the Classical strains are presumed to be the etiologic agent for the first six but are only presumed to be the etiologic agent due to the lack of ample epidemiologic surveillance, while the El Tor strains emerged as the major cause during the ongoing seventh pandemic. The reasons behind the extinction of the Classical biotype and the emergence of the El Tor biotype remain unclear, although several hypotheses have been proposed. The two biotypes show significant differences in their genotypic and phenotypic properties. A comparative genomic study revealed that the Classical biotype strains lack more than 20 genes found in the seventh pandemic El Tor strains, with most of these genes located on two chromosomal islands (12). Furthermore, transcriptional profiling experiments found that 524 genes are differentially expressed between the two biotypes under conditions that induce virulence expression in the Classical biotype. Biochemical behaviors such as hemolysis, hemagglutination of chicken erythrocytes (23), resistance to polymyxin B (18), bacteriophage-mediated lysis (4), and acetoin synthesis (28) are used to distinguish El Tor from Classical biotype strains. Acetoin biosynthesis in the presence of exogenous sugar such as glucose is crucial for biotyping the seventh pandemic El Tor strains and O139 strains (24). El Tor strains have a competitive growth advantage both *in vitro* and *in vivo* in cocultures with Classical strains and express higher levels of virulence factors in the tested cocultures (36).

A recent study showed that *V. cholerae* El Tor biotype strains are associated with the production of 2,3-butanediol (2,3-BD) through the 2,3-BD fermentation pathway, and their growth was enhanced when they were cultured in medium with added glucose. Classical strains, however, showed a sharp decrease in medium pH from accumulation of organic acids, ultimately resulting in a loss of viability. This finding suggested that the ability to produce a neutral metabolite like 2,3-BD might account for the higher evolutionary fitness of *V. cholerae* El Tor

* Corresponding author. Mailing address: Department of Microbiology, Yonsei University College of Medicine, 250 Seongsanno, Seodaemun-gu, Seoul 120-752, South Korea. Phone: 82 2 2228 1824. Fax: 82 2 392 7088. E-mail: sangsun_yoon@yuhs.ac.

† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 16 May 2011.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Serogroup, biotype (serotype)	Relevant characteristic(s)	Reference or source
V. cholerae strains			
N16961	O1, El Tor (Inaba)	Wild type	12
O395	O1, Classical (Ogawa)	Wild type	12
SSY01	O1, El Tor (Inaba)	N16961 VC1590::TnKGL3	48
SSY02	O1, El Tor (Inaba)	N16961 ΔVC1589	48
SSY05	O1, El Tor (Inaba)	N16961 VC2614::TnKGL3	This study
Escherichia coli strain			
DH5α[a]		*supE44* Δ(*lacU169*) (ϕ80d*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Lab collection
Plasmids			
pRL SV40[b]		pRL family of *Renilla* luciferase control reporter vector, Ap^r	Promega Inc.
3× MHC[c]-*luc*		Three copies of MHC I enhancer NF-κB-responsive elements TGGGGGATTCCCCA upstream of the luciferase (*luc*) reporter gene, Ap^r	Lab collection

[a] Used to amplify luciferase reporter plasmids.
[b] SV40, simian virus 40.
[c] MHC, major histocompatibility complex.

compared to the Classical biotype (48). The finding is further supported by the oral rehydration solution (ORS) used to treat *V. cholerae*-infected patients, which contains a large amount of glucose (16). This might aid in *in vivo* selection of El Tor strains that use glucose to produce 2,3-BD, while the same solution might have acted as a bactericidal agent for Classical strains that lack a functional 2,3-BD synthesis pathway. However, whether the ability to produce 2,3-BD provides any additional benefit to El Tor strains in the modulation of the host immune system has never been investigated.

The capability to produce the low-molecular-weight hydrocarbon 2,3-BD from pyruvate is widely distributed among different bacterial species, including all species of *Serratia* and *Enterobacter*, as well as some species of *Klebsiella*, *Erwinia*, *Bacillus*, and *Aeromonas* (44). In *Aeromonas* spp., another member of the *Vibrionaceae* family, it has been shown that 2,3-BD production depended on quorum sensing and that its production prevented lethal acidification of the medium (21). Previous studies showed that 2,3-BD is involved in a variety of physiological activities and is used across a broad spectrum of industrial applications, including as a homeostatic agent of environmental pH when bacterial cells are grown to higher cell densities (21), for regulation of the cellular NAD/NADH ratio (28), as a biomarker of ethanol abuse (34), and as biofuel (30). Although a number of studies have examined and characterized 2,3-BD production, its interaction with mammalian hosts and whether it plays any role in pathogenesis remain unclear. Recently, a study showed that chemically synthesized 2,3-BD can suppress lipopolysaccharide-induced host inflammatory responses in a rat acute lung injury model through inhibition of the nuclear factor κB (NF-κB) signaling pathway (22).

V. cholerae flagellins induce the inflammatory cytokines interleukin-1β (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α) and the chemokine IL-8 in intestinal epithelial cells through the Toll-like receptor 5 (TLR5)-dependent NF-κB and mitogen-activated protein kinase (MAPK) signaling pathway (2, 19, 49). *V. cholerae in vivo* infection in mice demonstrated that the local inflammation was activated, as evidenced by elevated secretion of TNF-α and IL-1β (17), which ultimately mediates a wide range of inflammatory responses (9). Thus, for successful colonization and production of virulence factors, pathogenic organisms must adopt certain mechanisms to bypass or suppress the host inflammatory responses that ultimately lead to the activation of antipathogen defense.

Keeping this in mind, this study was designed to elucidate another crucial physiological role of 2,3-BD with respect to the emergence of the El Tor biotype strains. We hypothesized that the metabolite 2,3-BD, which is exclusively produced in El Tor biotype strains, might be involved in modulating the way in which host immune defense is activated and thereby in contributing to the higher virulence potential of the El Tor over the Classical biotype. We found that 2,3-BD exerted a clear suppressive effect on the induction of two proinflammatory cytokines, IL-8 and TNF-α, in two distinct human epithelial cells. Subsequent analysis also revealed that such reduction occurred most likely through the inhibition of NF-κB signaling. Conversely, Classical strains grown in LB supplemented with 1% glucose (LBG) induced very high levels of IL-8 and TNF-α. Our study provides insight into how a metabolite produced by the El Tor biotype strain is capable of modulating local inflammation at the site of infection. This insight may offer a clue to explain the present dominance of El Tor biotype as a causal pathogen in cholera. A better understanding of this mechanism would answer a long-standing mystery regarding the environmental adaptability and biotype switching of this deadly pathogen.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. All strains and plasmids used in this study are listed in Table 1. Bacterial cultures were grown at 37°C in LB (10 g tryptone, 5 g NaCl, and 5 g yeast extract per liter) and LB medium supplemented with 1% glucose (Sigma Chemical, St. Louis, MO) with shaking, unless otherwise stated.

Preparation of cell-free culture supernatants. Cell-free culture supernatants were prepared as described elsewhere (47). Briefly, 3 ml of an overnight (~16-h) shaking culture of *V. cholerae* strains was grown to an optical density (OD) at 600 nm (OD_{600}) of ~4 (except for the O395 strain grown in LBG medium, which had an OD of ~1.8 at a similar time point) and centrifuged at 13,000 rpm for 10 min, and the supernatant was passed through a 0.22-μm-pore-size syringe filter (Pall Corp., MI). Cell-free supernatants were used immediately or stored at −20°C.

Cell culture, reagents, and stimulation. The intestinal epithelial HT29 and alveolar epithelial A549 cell lines (ATCC, Manassas, VA) were cultured in

Dulbecco's modified Eagle medium (DMEM) and minimum essential medium (MEM), respectively, supplemented with 10% fetal bovine serum (FBS; Wel-GENE, South Korea), 2.5 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C under 5% CO_2 in a water-jacketed CO_2 incubator (Forma Scientific). The 2,3-BD (99.8%) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Fugene6 transfection reagent was from Roche Applied Science (Mannheim, Germany). All expression plasmid constructs were prepared using an Accuprep plasmid extraction kit (Bioneer, South Korea). IκBα rabbit monoclonal antibody was from Cell Signaling Technology (Danvers, MA). Secondary antibody was horseradish peroxidase (HRP)-conjugated goat antirabbit antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). For stimulation with bacterial cell-free culture supernatants, 2×10^4 host cells were seeded in each well of 96-well plates (SPL Life Sciences, South Korea) and cultured overnight. On the next day, culture medium was replaced with serum-free medium before treatment. Ten microliters of prepared culture supernatants from bacterial cultures was added to cultured host cells and the cells were incubated for 6 h at 37°C under 5% CO_2. HT29 and A549 cells were incubated with 2,3-BD, added to the supernatants at a concentration of 1%, 0.5%, or 0.1%, as indicated. Samples for IL-8 and TNF-α ELISAs were collected, and cell viability was measured using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) or 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma Chemical, St. Louis, MO) according to the manufacturer's instructions.

Purification and extraction of *V. cholerae* flagella. Flagella from *V. cholerae* El Tor strain N16961 were prepared following the method described previously (49), with slight modifications. LB agar plates were inoculated with *V. cholerae* N16961 and incubated at 37°C overnight. The plates were scraped, and bacteria were inoculated in 1,000 ml LB broth and grown with continuous shaking for overnight in a shaking incubator. Bacteria were pelleted by centrifugation at 15,600 × *g* for 10 min, and the pellet was resuspended in 50 ml phosphate-buffered saline (PBS; pH 7.4). For flagellum purification, the bacterial cell suspension was mechanically sheared using a Waring blender for 1 min at full speed. Bacterial cells and other components were removed by centrifugation at 10,000 rpm for 10 min (Sorvall Science, Buckinghamshire, United Kingdom). The supernatant containing flagella was further centrifuged at 210,000 × *g* for 90 min at 4°C using an ultracentrifuge with a SW41 rotor (Beckman). The flagellum pellet was resuspended in phosphate-buffered saline and subjected to 1.37-g/ml CsCl density gradient ultracentrifugation at 100,000 × *g* for 18 h at 25°C. On the next day, the flagellum band was isolated by syringe and dialyzed against water overnight. The purity of the flagella was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and the protein concentration was measured by the Bradford method using a Bio-Rad Inc. (CA) protein assay solution with bovine serum albumin as a standard.

Quantification of 2,3-BD in bacterial culture supernatant by GC-MS. 2,3-BD, produced in laboratory culture (LB plus 1% glucose) by the *V. cholera* El Tor N16961 strain, was resolved and quantified by gas chromatography (GC)-mass spectrometry (MS) using an Agilent 6890 Plus gas chromatograph equipped with a 5973N mass selective detector quadrupole mass spectrometer system (Palo Alto, CA). The DB-5 MS capillary column (30 m by 0.25 mm [inner diameter], 0.25-μm film thickness, 5% diphenyl–95% dimethylsiloxane phase) was obtained from J&W Scientific (Folsom, CA). Sample preparation was performed as described elsewhere (38). Briefly, 1 ml of each sample was mixed with 1 ml of a 0.05% aqueous standard solution of 1,4-butandiol, alkalinized to pH 10, extracted with 6 ml of ethyl acetate (Sigma, St. Louis, MO), concentrated to 0.2 ml, and injected (1 μl) in the GC-MS system in the split mode at a splitting ratio of 1:10. The GC oven temperature was maintained at 60°C for 1 min and then ramped to 290°C at 10°C per min. The temperatures of the GC injection port and MS interface were set at 250 and 280°C, respectively. The carrier gas used was helium, set at a constant flow rate of 1 ml/min. The mass selective detector was run in the electron impact (EI) mode, with the electron energy at 70 eV. The silyl derivatization and selected ion monitoring (SIM) mode were used to remove the interferences for the quantification analysis.

IL-8 and TNF-α assay. After treatment, the HT29 and A549 cell culture supernatants were collected and stored at −80°C. The levels of IL-8 and TNF-α production by both cell lines were determined using HU IL-8 and HU TNF-α CytoSet enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen Corp., CA) according to the manufacturer's instruction, and the titers were expressed as pg/ml.

NF-κB reporter assay. HT29 cells were seeded (3×10^5 cells/well) and stably transfected with two luciferase reporter constructs according to the manufacturer's cell line-specific instructions. Optimized amounts of NF-κB-responsive firefly luciferase plasmid (0.2 μg) and transfection control *Renilla* luciferase plasmid (0.1 μg) were used. Cells were allowed to recover for at least 24 h in fresh medium without antibiotics and were stimulated with 50 μl *V. cholerae* culture supernatants (prepared as described above) or various concentrations of purified *V. cholerae* flagella in 24-well plates (SPL Life Science, South Korea). Luciferase activity was measured using a Dual luciferase reporter assay kit (Promega Corp., Madison, WI) according to the manufacturer's protocol. A Victor Plus plate reader (Perkin-Elmer) was used to measure luciferase activity. Firefly luciferase activity was normalized with *Renilla* luciferase activity and expressed as relative light units (RLU).

Western blot analysis. HT29 and A549 cells at ~90% confluence were seeded at 10^5 cells/well in each well of 24-well plates (SPL Life Sciences, South Korea), treated with diverse stimuli for specified time intervals for up to 2 h, and lysed with 1× sample lysis buffer (62.5 mM Tris-HCl, pH 6.8, at 25°C, 2% [wt/vol] SDS, 10% glycerol, 50 mM dithiothreitol, 0.01% [wt/vol] bromophenol blue or phenol red). After lysis, cells were scraped off, kept on ice, and boiled for 5 min. After they were cooled on ice, samples were microcentrifuged for 5 min. Equal amounts of total protein (50 μg proteins per gel lane) were separated by SDS-PAGE on 12% gels and transferred to polyvinylidene difluoride membranes (Amersham Hybond-ECL; GE Healthcare). Membranes were blotted with rabbit anti-human IκBα immunoglobulin G (Cell Signaling) or mouse anti-human β-actin immunoglobulin G (Sigma-Aldrich, Steinheim, Germany) at 4°C overnight, followed by incubation with HRP-conjugated secondary antirabbit or antimouse antibodies (Santa Cruz Biotechnology). The immunoreactive protein bands were then visualized using the enhanced chemiluminescence (ECL) reagent Westsave Up (Ab Frontier).

Fluorometric NO assay. Nitric oxide (NO) production by HT29 cells was determined by measuring the level of nitrite, a stable oxidation product of NO, by use of the Griess reagent (Invitrogen, Eugene, OR). HT29 and A549 cells (2×10^6 cells) were treated with gamma interferon (IFN-γ; 300 U/ml) and TNF-α (100 ng/ml) in the presence or absence of 1% 2,3-BD. The amount of nitrite produced was expressed in μM.

Statistical analysis. Data are expressed as means ± standard deviations (SDs). An unpaired Student's *t* test was used to analyze the data. A *P* value of <0.05 was considered statistically significant. All the experiments were repeated for reproducibility.

RESULTS

Culture supernatants of LBG-grown N16961 induced significantly lower levels of IL-8 and TNF-α in epithelial cells than culture supernatants of O395. *V. cholerae* El Tor strains were represented by strain N16961, and Classical strains were represented by strain by O395. Both strains have been extensively studied, and the genomic sequences are well characterized. Filter-sterilized culture supernatants of N16961 or O395 grown in either LB or LBG medium were used to treat HT29 and A549 cells as described in Materials and Methods. As shown in Fig. 1A and B, 6-h treatments induced production of proinflammatory cytokines in markedly different ways. The culture supernatant from LBG-grown N16961 induced negligible amounts of IL-8 (<40 pg/ml) or TNF-α (~15 pg/ml) in the HT29 cell line. In contrast, the culture supernatant from the LBG-grown Classical O395 strain induced a significantly higher level of IL-8 (>400 pg/ml) and TNF-α (~93 pg/ml), as determined by ELISA, although the organism grew poorly in the medium. At the same time, HT29 cells treated with LB-grown N16961 culture supernatants induced very low levels of IL-8 or TNF-α secretion (Fig. 1A and B), because this treatment showed a clear cytotoxic effect on the epithelial cells. For this reason, this culture supernatant was not used further in this study. To eliminate the possibility that the elevated production of IL-8 (Fig. 1A) is mediated by the effect of acidified medium pH, O395 was cultured in phosphate-buffered LBG medium. The medium pH was found to be stable at about neutral (7.5) after 16 h growth, while the pH of the medium of the LBG-grown Classical strain was 4.5 under the same conditions (data not shown). The culture supernatant from buff-

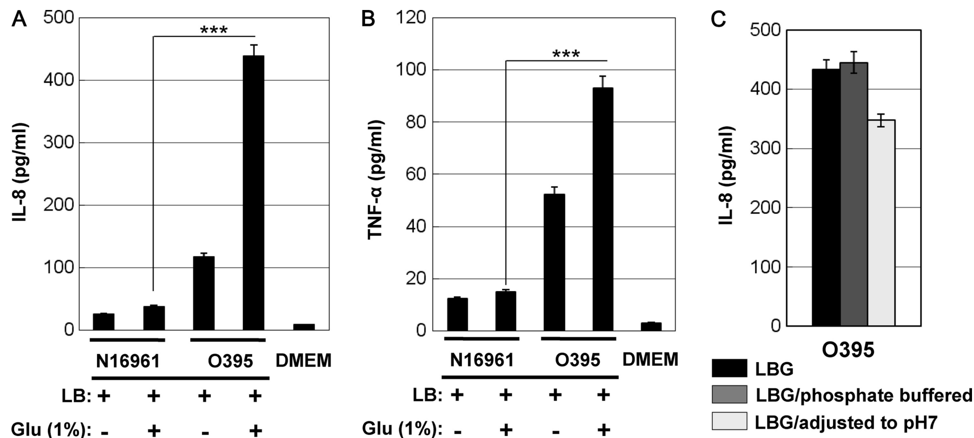

FIG. 1. Differential IL-8 and TNF-α production in HT29 cells in response to the culture supernatants of *V. cholerae* El Tor and Classical biotype strains. HT29 cells seeded in 96-well plates at 2×10^4 cells per well were treated for 6 h with culture supernatants of El Tor N16961 or Classical O395 grown in LB or LBG. IL-8 (A) and TNF-α (B) levels in culture supernatants were measured by ELISA, and values of means ± SDs are displayed in each bar ($n = 4$). ***, $P < 0.001$ for a comparison between LBG-grown O395 and LBG-grown N16961 supernatant treatments. (C) IL-8 production in response to treatment with O395 culture supernatants. O395 was grown in LBG (black bar) or in pH-buffered LBG (gray bar). The pH of the LBG-grown O395 culture supernatant was also adjusted with 0.1 N NaOH before treatment (white bar).

ered medium was still able to induce higher levels of IL-8 (Fig. 1C). In addition, the LBG-grown Classical strain continued to give higher signals for IL-8, even when the medium pH was manually adjusted to neutrality (pH 7.0) after overnight growth by the addition of 0.1 N sodium hydroxide (Fig. 1C). All these experiments were repeated with alveolar epithelial A549 cells, and similar patterns of IL-8 and TNF-α induction were observed (see Fig. S1 in the supplemental material).

Suppressed cytokine production by the LBG-grown N16961 supernatant was not associated with a change in HT29 cell viability. We then examined whether the reduced production of IL-8 and TNF-α in response to the supernatant of LBG-grown N16961 was mediated by the altered host cell viability. As shown in Fig. 2A, the culture supernatant of LB-grown N16961 was completely cytotoxic to HT29 cells, as determined by the CCK-8 cell viability assay. This observation was further verified in our microscopic analysis. After only 2 h of treatment, >90% cells were vacuolated and necrotic death occurred, as shown Fig. 2B. In contrast, such a cytotoxic effect was completely absent or negligible when cells were treated with the supernatant of LBG-grown N16961 or with supernatants of O395 (Fig. 2A). Consistent with the CCK-8 assay results, no difference in cellular morphology was observed in HT29 cells responding to the LBG-grown N16961 supernatant and to the control treatment (Fig. 2C and D). Together, these observations demonstrated that the decreased production of proinflammatory cytokines by the treatment with the LBG-grown N16961 supernatant was not a consequence of any change in host cell viability. Similar to the viability pattern observed with HT29 cells, only the LB-grown N16961 supernatant was cytotoxic to A549 cells (see Fig. S2 in the supplemental material).

Production of higher levels of IL-8 and TNF-α by 2,3-BD-defective N16961 mutants and O395 was reversed by the addition of extraneous 2,3-BD. We then sought to examine whether the El Tor biotype-specific glucose metabolite 2,3-BD was responsible for the downregulated production of IL-8 and TNF-α. To address this question, we tested the immune-stimulating capability of three N16961-derived mutant strains that are defective in 2,3-BD synthesis (48). Detailed information on these mutant strains is listed in Table 1. Similar to the O395 Classical biotype strain, each of these mutant strains was unable to produce 2,3-BD using glucose and thus was significantly affected in its ability to grow in glucose-amended medium (data not shown). Unlike the wild-type N16961 strain, supernatants of these LBG-grown mutant strains invariably stimulated the production of higher levels of the proinflammatory

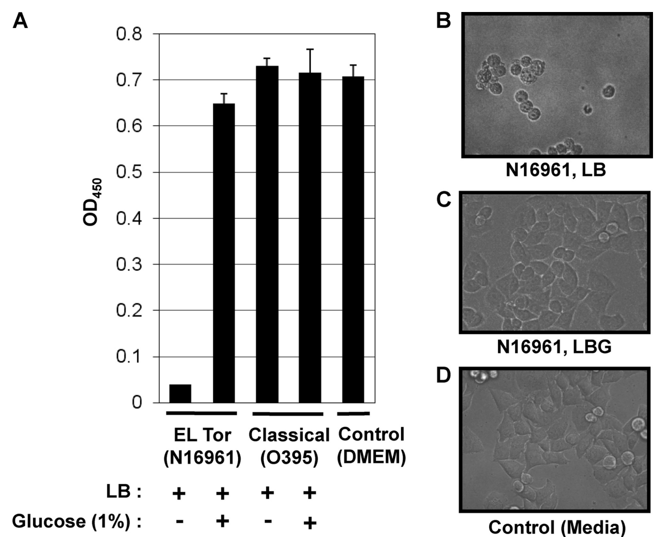

FIG. 2. Viability of HT29 cells in response to treatments with culture supernatants. (A) CCK-8 assay showing HT29 cell viability with indicated treatments for 6 h. Values of means ± SDs are displayed in each bar, and the medium composition is indicated at the bottom. Microscopic images of HT29 cells treated with the supernatant of LB-grown N16961 (B), with the supernatant of LBG-grown N16961 (C), or with DMEM (D).

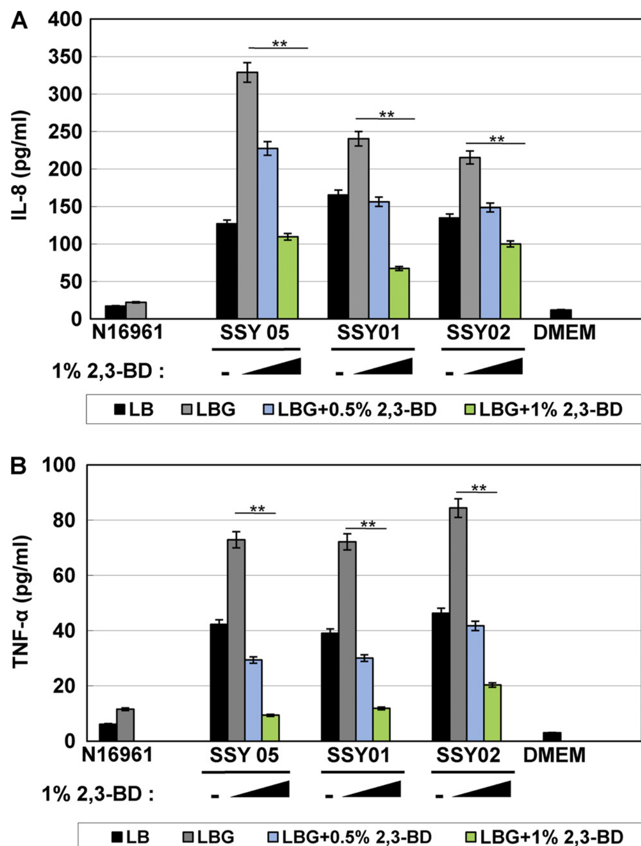

FIG. 3. Treatments with culture supernatants of 2,3-BD-defective N16961 mutant strains resulted in production of higher levels of IL-8 (A) and TNF-α (B). Values of means ± SDs (n = 4) are displayed in each bar. The strain and medium composition are indicated at the bottom. Three mutant strains were grown in LB (black bars) or LBG (gray bars). Aliquots of supernatants of LBG-grown strains were mixed with 2,3-BD to a final concentration of 0.5% (blue bars) or 1% (green bars) prior to the treatment. **, $P < 0.01$ for comparison (i) between values of gray and blue bars and (ii) between values of blue and green bars. The leftmost set of bars shows the levels of cytokines produced by the supernatants of wild-type strain N16961, and the rightmost bar shows the levels for the unstimulated medium control.

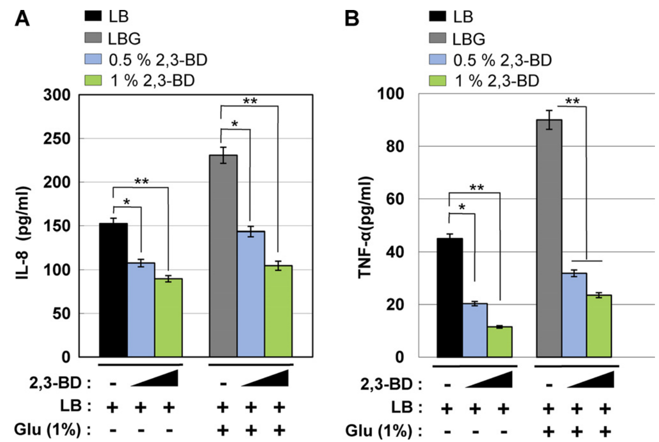

FIG. 4. Suppressed production of IL-8 and TNF-α by O395 supernatants in the presence of extraneous 2,3-BD in HT29 cells. Culture supernatants of LB- and LBG-grown O395 were used to stimulate HT29 cells. Each supernatant was mixed with 2,3-BD at a final concentration of 0.5% or 1%. A statistically significant decrease in IL-8 (A) or TNF-α (B) production was observed when 2,3-BD was added. IL-8 and TNF-α levels were determined by ELISA, and values of means ± SDs (n = 4) are displayed in each bar. *, $P < 0.05$ for comparison between supernatants without 2,3-BD and supernatants reconstituted with extraneous 2,3-BD; **, $P < 0.01$ for comparison between supernatants without 2,3-BD and supernatants reconstituted with extraneous 2,3-BD.

cytokines IL-8 and TNF-α. As shown in Fig. 3A and B, HT29 cells secreted up to >200 pg/ml of IL-8 and up to >70 pg/ml of TNF-α when they were treated with filter-sterilized culture supernatants of these mutant strains (Fig. 3A and B, gray bars). As was observed in O395 (Fig. 1A and B), supernatants of LB-grown strains were not as potent as supernatants of LBG-grown strains in inducing IL-8 and TNF-α production (Fig. 3A and B, black bars). To examine the effect of 2,3-BD on IL-8 and TNF-α production, HT29 cells were treated with the LBG-grown strain supernatants that were devoid of 2,3-BD but reconstituted with 0.5% (blue bars) and 1% (green bars) 2,3-BD. As shown in Fig. 3A and B, levels of IL-8 or TNF-α production were significantly decreased by the addition of extraneous 2,3-BD. Again, no reduction in host cell viability was observed in response to the treatment (see Fig. S3 in the supplemental material), further demonstrating that the reduced production of proinflammatory cytokines was not caused by any change in host cell viability. Use of A549 cells to observe the effects of extraneous 2,3-BD on the production of IL-8 and TNF-α provided similar outcomes, as shown in Fig. S4 in the supplemental material.

Next, we applied 2,3-BD extraneously while treating HT29 cells with culture supernatant of LBG-grown O395 to observe if the added 2,3-BD could also modulate the ability to produce proinflammatory cytokines. After 6 h treatment, we detected a marked decrease in the production of IL-8 and TNF-α, and the decrease was dose dependent (Fig. 4A and B, right portions). Such findings, which were similar to those for the 2,3-BD-defective N16961 mutant strains, further supported the role of 2,3-BD as a negative regulator for the production of proinflammatory cytokines. Again, the supernatant of the O395 strain grown in plain LB, which also induced the production of intermediate levels of IL-8 and TNF-α, was less efficient in doing the same in the presence of extraneous 2,3-BD (Fig. 4A and B, left portions). Together, these data demonstrated the involvement of 2,3-BD in suppressing the production of proinflammatory cytokines *in vitro*. As before, these data were successfully reproduced using A549 cells (see Fig. S5 in the supplemental material).

Quantification of 2,3-BD produced by N16961 in the presence of glucose. We then sought to gain an idea of how much 2,3-BD can be produced biologically from the El Tor *V. cholerae* biotype strain during growth with 1% glucose. To this end, we analyzed the bacterial culture supernatants by GC-MS. As shown in Table 2, ~28 mM 2,3-BD was detected in the LBG-grown N16961 culture supernatant, while a ~20-fold smaller amount of 2,3-BD was detected in the LB-grown N16961 supernatant, further proving that 2,3-BD is produced by glucose fermentation. As expected, negligible levels of 2,3-BD were detected in O395 culture supernatants. Given the fact that 1% glucose is equivalent to ~56 mM, this result indicated that

TABLE 2. Levels of 2,3-BD produced by N16961 and O395 strains grown in different culture media determined by GC-MS

Strain-growth medium	2,3-BD concn (mM)
O395-LB	0.15 ± 0.01
O395-LBG	0.28 ± 0.012
N1696-LB	1.34 ± 0.05
N1696-LBG	28 ± 0.12

about half of the glucose was metabolized to produce 2,3-BD, which represents a metabolic conversion with a considerable yield rate.

2,3-BD downregulated the level of cytokines produced in response to the purified flagella in a dose-dependent manner. We then examined whether 2,3-BD can also suppress the production of IL-8 in response to the purified *V. cholerae* flagella, known inducers of the TLR5-mediated innate immune response (19). Flagella extracted from the *V. cholerae* El Tor N16961 strain were used as a positive control to induce IL-8 production in HT29 cells. When they were treated with increasing concentrations of purified flagella, HT29 cells secreted IL-8 at levels ranging from 200 to 350 pg/ml (Fig. 5, black bars). Again, when HT29 cells were cotreated with various concentrations of 2,3-BD, a pattern of gradual decrease in IL-8 secretion by the addition of increasing amounts of 2,3-BD was observed in all sets of flagellum treatments (Fig. 5, gray and white bars), further demonstrating that 2,3-BD can suppress the proinflammatory cytokine production in cultured host epithelial cells. A similar dose-dependent decrease in IL-8 production was also observed in A549 cells when they were cotreated with 2,3-BD (see Fig. S6 in the supplemental material).

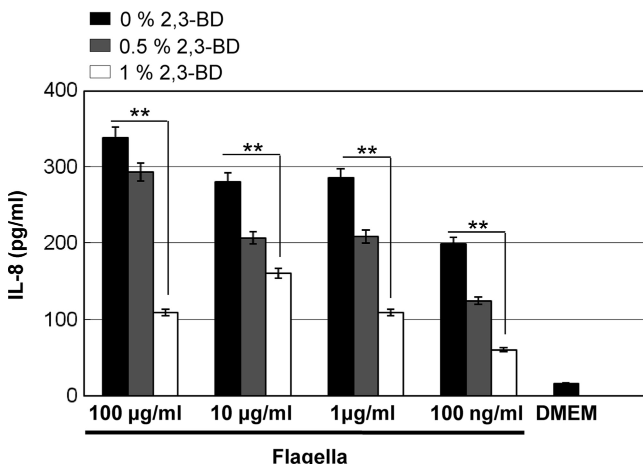

FIG. 5. IL-8 production in response to purified flagella was also downregulated by exogenously added 2,3-BD. Purified *V. cholerae* flagella at final concentrations of 100 μg/ml, 10 μg/ml, 1 μg/ml, and 100 ng/ml were used to stimulate HT29 cells. IL-8 levels in the HT29 cell culture supernatants were determined by ELISA, and values of means ± SDs ($n = 4$) are displayed in each bar. Aliquots of each flagellum preparation were mixed with 2,3-BD at final concentrations of 0.5% and 1% and used to treat HT29 cells. **, $P < 0.01$ for comparison between flagellum-only treatment and treatment with flagella plus 1% 2,3-BD.

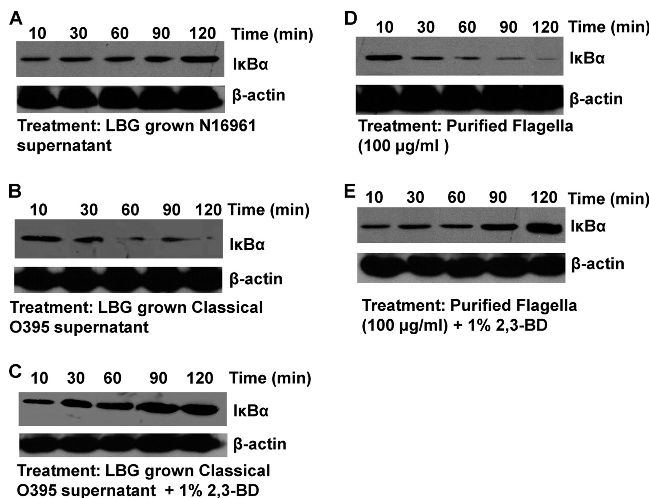

FIG. 6. Effect of extraneous 2,3-BD on the cellular levels of IκBα. (A) Anti-IκBα Western blot analysis of HT29 cell extracts. Before harvest, HT29 cells were treated with LBG-grown N16961 supernatant for 2 h. At the times indicated at the top, HT29 cells were harvested and processed for the Western blotting. The β-actin level was monitored as a loading control. (B) Anti-IκBα Western blot analysis of HT29 cells treated with LBG-grown O395 supernatant; (C) anti-IκBα Western blot analysis of HT29 cells treated with LBG-grown O395 supernatant plus 1% 2,3-BD; (D) anti-IκBα Western blot analysis of HT29 cells treated with purified flagella; (E) anti-IκBα Western blot analysis of HT29 cells treated with purified flagella plus 1% 2,3-BD.

2,3-BD-mediated downregulation of cytokine production occurred by inhibition of NF-κB signaling pathway. Next, we investigated whether the underlying mechanism of 2,3-BD-induced cytokine downregulation was targeting the transcription factor NF-κB, which is involved in the production of a number of proinflammatory cytokines, including IL-8 and TNF-α (45). To address this issue, we assessed the level of IκBα protein, an inhibitor of NF-κB activation (6, 45), by Western blotting. In all cases, β-actin was used as a loading control. As shown in Fig. 6A, an increased level of IκBα protein was detected with time after treatment with culture supernatants of the LBG-grown N16961. However, treatment with LBG-grown O395 supernatants, which induced a high level of production of IL-8 and TNF-α in HT29 cells, resulted in a time-dependent gradual decrease in the IκBα protein (Fig. 6B). When the same supernatant was reconstituted with extraneous 2,3-BD, a similar pattern for the elevated presence of IκBα was observed during the first 2 h of treatment (Fig. 6C). Likewise, when HT29 cells were treated with purified flagella, which also induced high-level IL-8 production (Fig. 5), a decreased level of IκBα protein was detected (Fig. 6D). Again, cotreatment with 1% 2,3-BD completely reversed the trend for the cellular levels of IκBα (Fig. 6E). Collectively, these results demonstrated that (i) IκBα degradation occurred in HT29 cells in response to the treatments that induced a high level of IL-8 production, and (ii) the sole addition of 2,3-BD is enough to inhibit the time-dependent degradation of the IκBα protein. Apparently, similar or comparable IκBα degradation patterns were observed in A549 cells in response to the identical set of treatments (see Fig. S7 in the supplemental material). We then examined whether the modulation of the IκBα level was in-

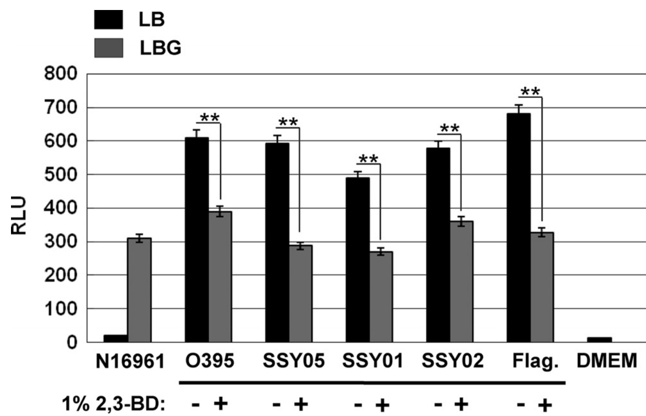

FIG. 7. Effect of extraneous 2,3-BD on NF-κB-responsive luciferase activity. A total of 3×10^5 cells per well cotransfected with pRL SV40 (simian virus 40) and 3× MHC (major histocompatibility complex)-*luc* firefly luciferase reporter plasmids were treated with the stimuli indicated at the bottom. Luciferase activity was determined by using a dual luciferase assay system. Experiments were performed in triplicate, and values of means ± SDs are displayed for each bar. NF-κB-responsive luciferase activity was expressed in RLU by normalizing firefly luciferase activity with constitutive *Renilla* luciferase activity. **, $P < 0.01$ for comparison between without and with extraneously added 2,3-BD.

deed reflected in the NF-κB-responsive promoter activity. To address this question, we measured the activity of firefly luciferase, production of which was regulated by activated NF-κB. Supernatants from the LBG-grown El Tor N16961 strain caused the weakest light production response of all treatments, as shown in Fig. 7. A much greater NF-κB-responsive promoter activity was observed upon treatments with the supernatants of LBG-grown O395, SSY05, SSY01, or SSY02, which lacked 2,3-BD. Similar luciferase activity was also detected in response to the purified flagella (Fig. 7). Importantly, a significantly decreased level ($P < 0.01$) of luciferase activity was observed when pretransfected HT29 cells were cotreated with exogenously added 2,3-BD (Fig. 7, gray bars). The light responses were comparable to those from the treatment with 2,3-BD-containing LBG-grown N16961 supernatant.

Finally, to further support the suggestion that 2,3-BD interferes specifically with the signaling cascade that involves NF-κB activation, we examined whether 2,3-BD also exerts any effect upon epithelial cells, which were stimulated by IFN-γ (300 U/ml) and TNF-α (100 ng/ml) to produce NO. When HT29 cells were stimulated with these two cytokines, they were reported to produce NO through a distinct pathway known as the inducible nitric oxide synthase (iNOS) pathway (26, 33). As shown in Fig. S8 in the supplemental material, similar levels of NO_2^- were produced in the IFN-γ- and TNF-α-primed HT29 cells, regardless of the presence or absence of 2,3-BD, indicating that the signaling cascade leading to the activation of the iNOS pathway was not affected by the addition of 2,3-BD. Taken together, these results further support the suggestion that the 2,3-BD-mediated cytokine downregulation is mediated by the selective inhibition of the NF-κB signaling pathway.

DISCUSSION

A unique event in cholera epidemiology was the displacement of the epidemic-causing Classical biotype of *V. cholerae* with the El Tor biotype. This occurred within a decade of the emergence of the El Tor biotype in 1961, marking the beginning of the seventh cholera pandemic (13). The genetic basis or environmental factors behind this population switch are still unknown, despite many new findings on the molecular-level differences between the El Tor and the Classical biotypes (3, 12, 31). A marked difference in the glucose metabolism between the El Tor and Classical biotype strains provided insight for solving this long-standing mystery (48). In the present study, we focused on the additional benefit provided to *V. cholerae* El Tor biotype strains by the El Tor biotype-specific metabolic end product 2,3-BD.

V. cholerae can stimulate secretion of proinflammatory cytokines and chemokines, which are important factors in disease pathology (40). Establishing mechanisms to suppress production of proinflammatory cytokines might be crucial for successful infection of the host by *V. cholerae*. We observed substantial differences in IL-8 and TNF-α production in two distinct epithelial cell lines in response to the cell-free culture supernatants of *V. cholerae* El Tor and Classical biotype strains. Because 2,3-BD is produced by the El Tor but not by the Classical biotype strain in glucose-amended cultures, we hypothesized that the significantly reduced production of IL-8 and TNF-α by the El Tor N16961 culture supernatant might be caused by the presence of 2,3-BD.

In *V. cholerae*, production of virulence factors is strongly influenced by environmental signals, such as pH and temperature (42). Recently, a PadR family-type transcriptional regulator, AphA, an activator of the *tcpPH* promoter, was shown to repress the expression of genes for 2,3-BD synthesis by directly binding to the transcriptional start site of *alsD* (VC1589), the first gene in the 2,3-BD pathway gene cluster. Expression of biosynthetic genes was derepressed by AlsR (VC1588), the regulator gene for the 2,3-BD pathway which is activated upon acidification (28). These findings suggest that AphA, coupled with AlsR, can reciprocally regulate virulence gene expression and 2,3-BD biosynthesis in response to the pH change. Thus, AphA-dependent gene regulation may be more active in Classical biotype strains, ultimately leading to greater suppression of the 2,3-BD synthesis pathway in Classical biotype strains than in El Tor biotype strains. Thus, elevated production of IL-8 and TNF-α in epithelial cells in response to the LBG-grown O395 culture supernatant could be caused by (i) a higher level of secretion of immunostimulatory virulence factors like cholera toxin (40) and/or (ii) the absence of 2,3-BD, which can downregulate proinflammatory cytokine production.

LB-grown culture supernatants of N16961 were cytotoxic for epithelial cells within 2 h of treatment. Other studies hypothesized that this phenomenon is more likely exerted by toxins such as hemolysin or other accessory toxins produced predominantly by El Tor strains (14, 15, 32). When the El Tor strains were grown with 1% added glucose, however, the cytotoxic effect was not observed or, if it was present, was observed only occasionally at a negligible level (<5% com-

pared to the untreated control). Although more experiments are necessary to precisely uncover the molecular basis behind this differential cytotoxicity, it is likely that a metabolic shift induced by the presence of glucose might also play a role in downregulating the expression of virulence-associated genes.

To provide insight into the possible role of 2,3-BD in modulating the immune response inside the human host, it would be desirable to know more about the production yield of 2,3-BD from glucose catabolism. Our results in Table 2 showed that the level of 2,3-BD produced from 1% glucose (56 mM) was ~28 mM, a value equivalent to 0.25%, and this result is in accordance with previous findings (48). During the study, we have used chemically synthesized 2,3-BD at levels up to 1%, a concentration 4-fold higher than the level of 2,3-BD produced biologically from 1% glucose. In fact, 1% 2,3-BD was required to downregulate the production of proinflammatory biomarkers like IL-8 or TNF-α. Therefore, the use of such a higher concentration of 2,3-BD to cotreat host cells requires justification. Cholera patients with severe dehydration have usually been administered standard ORS every 4 h, according to WHO recommendations (46); standard ORS contains 111 mM glucose, twice the concentration used in the present study. Thus, although this possibility is speculative, continuous consumption of ORS may have created a microenvironment in the stomach or intestine with a higher level of glucose that may have imposed a selective pressure on various *V. cholerae* strains. In this sense, there is a possibility that the level of 2,3-BD produced in the intestine is much greater than that produced by *in vitro* culture in the defined medium with a single dose of glucose.

To further characterize the effect of 2,3-BD on cytokine production, we used three 2,3-BD-defective N16961 mutant strains (48). Two of the three mutant strains, SSY01 and SSY02, were lacking two vital open reading frames, VC1589 (acetolactate synthase, *alS*) and VC1590 (acetolactate decarboxylase, *alD*), of the 2,3-BD pathway. The third mutant strain, SSY05, is defective in VC locus 2614, which codes for the global regulator cyclic AMP receptor protein (CRP). CRP is indirectly involved in glucose metabolism and in the regulation of 2,3-BD pathway genes (5, 27, 29, 31). These strains were found to exhibit reduced growth in the presence of added glucose, similar to the growth of O395, due to the medium acidification. Of note, cell-free culture supernatants of N16961-derived mutant strains SSY01, SSY02, and SSY05 consistently induced high levels of IL-8 and TNF-α that were almost comparable to the response of O395 to the culture supernatant (Fig. 3), suggesting that these mutants behave like O395, at least in terms of stimulating the production of proinflammatory cytokines in cultured epithelial cells. Again, extraneously added 2,3-BD clearly downregulated IL-8 and TNF-α production, as was observed for the LBG-grown N16961 supernatant containing 2,3-BD. Therefore, the presence of a functional 2,3-BD pathway is vital for the El Tor biotype strains to modulate the level of proinflammatory cytokine production *in vitro*.

Increasing evidence suggests that flagellin monomers are released into the culture medium (2, 19, 47). Since bacterial flagella are known activators of innate immunity (43), we examined whether 2,3-BD can still exert a similar inhibitory effect in HT29 or A549 cells in response to the purified *V. cholerae* flagella. We extracted flagella from the *V. cholerae* El Tor N16961 strain and used them as an inducer of IL-8. At 100 μg/ml to 100 ng/ml, the flagellum fraction indeed induced high levels of IL-8 in HT29 cells, and when they were cotreated with various concentrations of 2,3-BD, a dose-dependent decrease in IL-8 levels was observed, further suggesting a clear inhibitory effect of 2,3-BD on the induction of the proinflammatory cytokine IL-8.

Next, we investigated the mechanism by which 2,3-BD exerts suppressive action on cytokine release in cultured epithelial cells. IL-8 expression involves activation of the MAPKs, c-Jun N-terminal kinase (JNK), and extracellular-regulated kinase (ERK), as well as the transcription factors NF-κB and activator protein 1 (AP-1) (20, 35). Among these, the NF-κB pathway has long been known to be involved in the central regulation of proinflammatory responses (10). Therefore, we performed experiments to see if this particular pathway is affected by the presence of 2,3-BD. The IκB protein family contains inhibitors of NF-κB. IκBα binds NF-κB in the cytoplasm and prevents its translocation to the nucleus (11, 45). Thus, measuring the cytoplasmic level of IκBα provides an indirect but recognized method for monitoring the inhibition level of the NF-κB-mediated signaling cascade (1). We found that degradation of IκBα was apparent during the treatment and lasted for up to 2 h after host epithelial cells were treated with culture supernatants that lacked in 2,3-BD. In contrast, levels of IκBα increased and reached their highest level at between 1 and 2 h in treatments with culture supernatants of LBG-grown N16961 or flagella plus 1% 2,3-BD (Fig. 6). Because sustained IL-8 mRNA synthesis requires sustained binding of NF-κB (35), the detection of elevated levels of IκBα suggests that this protein is efficiently preventing the activation of the NF-κB signaling pathway and thus suppressing IL-8 gene transcription. Our NF-κB reporter assays further verified the reduction of NF-κB-responsive promoter activity in the presence of 2,3-BD. Therefore, these data together provide supporting evidence for our hypothesis that 2,3-BD targets the NF-κB signaling pathway at least as a part of the mechanism to downregulate proinflammatory cytokine production in host epithelial cells.

Our current research sheds light on the potential molecular basis behind the biotype switch of the deadly enteric pathogen *V. cholerae* from Classical to El Tor. Our findings suggest that 2,3-BD can suppress the activation of proinflammatory mediators induced by bacterial products like flagella by modulating the NF-κB signaling pathway. Thus, the presence of excess glucose in the human gut from ORS consumption or other means may have contributed to the extinction of the Classical biotype strains in two ways: (i) by decreasing the ambient pH to a lethal range and (ii) by inducing higher levels of proinflammatory response against bacterial antigens. In contrast, El Tor biotype strains might have acquired an evolutionary advantage by metabolizing glucose to 2,3-BD and have been a dominant pathogenic species in cholera pandemics. It was reported that cholera patient stools were alkaline ($pH > 7$) (25). However, no further details were revealed with regard to (i) the biotype of the causative strain, (ii) the levels and types of cytokines present in the intestinal mucus of the patients, and (iii) whether the patients that produced the

same tested stools had been treated with the ORS. Such information would collectively provide a better clue as to whether the biotype-specific glucose metabolism indeed impacts the differential host proinflammatory immune activation and, thus, survival of the two different biotype strains *in vivo*.

Given these findings, we next propose to observe the effect of 2,3-BD, a unique metabolite produced by *V. cholerae* El Tor biotype strains, on the innate immune activation in a valid animal model system, as the present study investigated the production profile of simple biomarkers for proinflammatory responses in cultured host epithelial cells. Further *in vivo* studies will unambiguously verify the immune-suppressive role of 2,3-BD. We also propose that purified 2,3-BD from bacterial fermentation products may have potential to be developed as an anti-inflammatory agent that could be used for therapy.

ACKNOWLEDGMENTS

We thank Yun Gyong Ahn (Korea Basic Science Institute, Seoul Center) for helpful discussion on the GS-MS data analysis. We are truly indebted to Joo-Chun Yoon and Jae Myun Lee (Yonsei University College of Medicine, South Korea) for helping us with NF-κB reporter assay.

This work was supported by a grant from the National Research Foundation of South Korea (NRF), funded by the South Korea Government (MEST), grant no. 2009-0087951 (to S.S.Y.). Y.-J.S. was supported by the Kyungwon University Research Fund in 2011.W.B. was supported by a Yonsei University College of Medicine Brain Korea 21 graduate student scholarship.

REFERENCES

1. **Baldwin, A. S.** 1996. The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu. Rev. Immunol. **14:**649–681.
2. **Bandyopadhaya, A., M. Sarkar, and K. Chaudhuri.** 2008. IL-1B expression in Int407 is induced by flagellin of *Vibrio cholerae* through TLR5 mediated pathway. Microb. Pathog. **44:**524–536.
3. **Beyhan, S., A. D. Tischler, A. Camilli, and F. H. Yildiz.** 2006. Differences in gene expression between the Classical and El Tor biotypes of *Vibrio cholerae* O1. Infect. Immun. **74:**3633–3642.
4. **Biswas, S. K., R. Chowdhury, and J. Das.** 1992. A 14-kilodalton inner membrane protein of *Vibrio cholerae* biotype El Tor confers resistance to group IV choleraphage infection to Classical vibrios. J. Bacteriol. **174:**6221–6229.
5. **Botsford, J. L., and J. G. Harman.** 1992. Cyclic AMP in prokaryotes. Microbiol. Rev. **56:**100–122.
6. **Brockman, J. A., et al.** 1995. Coupling of a signal response domain in I kappa B alpha to multiple pathways for NF-kappa B activation. Mol. Cell. Biol. **15:**2809–2818.
7. **Centers for Disease Control.** 1993. Imported cholera associated with a newly described toxigenic *Vibrio cholerae* O139 strain—California, 1993. MMWR Morb. Mortal. Wkly. Rep. **42:**501–503.
8. Reference deleted.
9. **Dinarello, C. A.** 1991. Interleukin-1 and interleukin-1 antagonism. Blood **77:**1627–1652.
10. **Dinarello, C. A.** 2000. Proinflammatory cytokines. Chest **118:**503–508.
11. **Duckett, C. S., et al.** 1993. Dimerization of NF-KB2 with RelA(p65) regulates DNA binding, transcriptional activation, and inhibition by an I kappa B-alpha (MAD-3). Mol. Cell. Biol. **13:**1315–1322.
12. **Dziejman, M., et al.** 2002. Comparative genomic analysis of *Vibrio cholerae*: genes that correlate with cholera endemic and pandemic disease. Proc. Natl. Acad. Sci. U. S. A. **99:**1556–1561.
13. **Faruque, S. M., M. J. Albert, and J. J. Mekalanos.** 1998. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol. Mol. Biol. Rev. **62:**1301–1314.
14. **Figueroa-Arredondo, P., et al.** 2001. Cell vacuolation caused by *Vibrio cholerae* hemolysin. Infect. Immun. **69:**1613–1624.
15. **Fullner, K. J., et al.** 2002. The contribution of accessory toxins of *Vibrio cholerae* O1 El Tor to the proinflammatory response in a murine pulmonary cholera model. J. Exp. Med. **195:**1455–1462.
16. **Guerrant, R. L., B. A. Carneiro-Filho, and R. A. Dillingham.** 2003. Cholera, diarrhea, and oral rehydration therapy: triumph and indictment. Clin. Infect. Dis. **37:**398–405.
17. **Haines, G. K., III, B. A. Sayed, M. S. Rohrer, V. Olivier, and K. J. Satchell.** 2005. Role of Toll-like receptor 4 in the proinflammatory response to *Vibrio cholerae* O1 El Tor strains deficient in production of cholera toxin and accessory toxins. Infect. Immun. **73:**6157–6164.
18. **Han, G. K., and T. S. Khie.** 1963. A new method for differentiation of *Vibrio comma* and *Vibrio* El Tor. Am. J. Hyg. **77:**184–186.
19. **Harrison, L. M., et al.** 2008. *Vibrio cholerae* flagellins induce Toll-like receptor 5-mediated interleukin-8 production through mitogen-activated protein kinase and NF-κB activation. Infect. Immun. **76:**5524–5534.
20. **Hoffmann, E., O. Dittrich-Breiholz, H. Holtmann, and M. Kracht.** 2002. Multiple control of interleukin-8 gene expression. J. Leukoc. Biol. **72:**847–855.
21. **Houdt, R. V., A. Aertsen, and C. W. Michiels.** 2007. Quorum-sensing-dependent switch to butanediol fermentation prevents lethal medium acidification in *Aeromonas hydrophila* AH-1N. Res. Microbiol. **158:**379–385.
22. **Hsieh, S. C., et al.** 2007. The bacterial metabolite 2,3-butanediol ameliorates endotoxin-induced acute lung injury in rats. Microbes Infect. **9:**1402–1409.
23. **Jonson, G., J. Sanchez, and A. M. Svennerholm.** 1989. Expression and detection of different biotype-associated cell-bound haemagglutinins of *Vibrio cholerae* O1. J. Gen. Microbiol. **135:**111–120.
24. **Kaper, J. B., J. G. Morris, Jr., and M. M. Levine.** 1995. Cholera. Clin. Microbiol. Rev. **8:**48–86.
25. **Khan, M. U., R. Eeckels, A. N. Alam, and N. Rahman.** 1988. Cholera, rotavirus and ETEC diarrhoea: some clinico-epidemiological features. Trans. R. Soc. Trop. Med. Hyg. **82:**485–488.
26. **Kolios, G., Z. Brown, R. L. Robson, D. A. F. Robertson, and J. Westwick.** 1995. Inducible nitric oxide synthase activity and expression in a human colonic epithelial cell line, HT-29. Br. J. Pharmacol. **116:**2866–2872.
27. **Kovacikova, G., and K. Skorupski.** 2001. Overlapping binding sites for the virulence gene regulators AphA, AphB and cAMP-CRP at the *Vibrio cholerae tcpPH* promoter. Mol. Microbiol. **41:**393–407.
28. **Kovacikova, G., W. Lin, and K. Skorupski.** 2005. Dual regulation of genes involved in acetoin biosynthesis and motility/biofilm formation by the virulence activator AphA and the acetate-responsive LysR-type regulator AlsR in *Vibrio cholerae*. Mol. Microbiol. **57:**420–433.
29. **Liang, W., A. Pascual-Montano, A. J. Silva, and J. A. Benitez.** 2007. The cyclic AMP receptor protein modulates quorum sensing, motility and multiple genes that affect intestinal colonization in *Vibrio cholerae*. Microbiology **153**(Pt 9)**:**2964–2975.
30. **Mallonee, D. H., and A. Speckman.** 1988. Development of a mutant strain of *Bacillus polymyxa* showing enhanced production of 2,3-butanediol. Appl. Environ. Microbiol. **54:**168–171.
31. **Matson, J. S., J. H. Withey, and V. J. DiRita.** 2007. Regulatory networks controlling *Vibrio cholerae* virulence gene expression. Infect. Immun. **75:**5542–5549.
32. **Mitra, R., et al.** 2000. Cell vacuolation a manifestation of the El Tor hemolysin of *Vibrio cholerae*. Infect. Immun. **68:**1928–1933.
33. **Moncada, S., and A. Higgs.** 1993. The L-arginine-nitric oxide pathway. N. Engl. J. Med. **329:**2002–2012.
34. **Montgomery, J. A., F. David, M. Garneau, and H. Brunengraber.** 1993. Metabolism of 2,3-butanediol stereoisomers in the perfused rat liver. J. Biol. Chem. **268:**20185–20190.
35. **Mukaida, N., S. Okamoto, Y. Ishikawa, and K. Matsushima.** 1994. Molecular mechanism of interleukin-8 gene expression. J. Leukoc. Biol. **56:**554–558.
36. **Pradhan, S., A. K. Baidya, A. Ghosh, K. Paul, and R. Chowdhury.** 2010. The El Tor biotype of *Vibrio cholerae* exhibits a growth advantage in the stationary phase in mixed cultures with the Classical biotype. Infect. Immun. **192:**955–963.
37. **Ramamurthy, T., et al.** 1993. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. Lancet **341:**703–704.
38. **Romano, P., et al.** 2000. Evaluation of stereoisomers of 2,3-butanediol and acetoin to differentiate *Saccharomyces cerevisiae* and *Kloeckera apiculata* wine strains. Biotechnol. Lett. **22:**1947–1951.
39. **Sack, D. A., R. B. Sack, G. B. Nair, and A. K. Siddique.** 2004. Cholera. Lancet **363:**223–233.
40. **Satchell, K. J. F.** 2003. Activation and suppression of the proinflammatory immune response by *Vibrio cholerae* toxins. Microb. Infect. **5:**1241–1247.
41. Reference deleted.
42. **Skorupski, K., and R. K. Taylor.** 1997. Control of the ToxR virulence regulon in *Vibrio cholerae* by environmental stimuli. Mol. Microbiol. **25:**1003–1009.
43. **Steiner, T. S.** 2007. How flagellin and Toll-like receptor 5 contribute to enteric infection. Infect. Immun. **75:**545–552.
44. **Syu, J.** 2001. Biological production of 2,3-butanediol. Appl. Microbiol. Biotechnol. **55:**10–18.
45. **Thanos, D., and T. Maniatis.** 1995. NF-kappa B: a lesson in family values. Cell **80:**529–532.
46. **UNICEF.** 2001. New formulation of oral rehydration salts (ORS) with re-

duced osmolarity. UNICEF technical bulletin no. 9. Report from a meeting of experts jointly organized by UNICEF and WHO. UNICEF and WHO, July 2001, New York, NY.
47. **Xicohtencatl-Cortés, J., et al.** 2006. Identification of proinflammatory flagellin proteins in supernatants of *Vibrio cholerae* O1 by proteomics analysis. Mol. Cell Proteomics **5:**2374–2383.
48. **Yoon, S. S., and J. J. Mekalanos.** 2006. 2,3-Butanediol synthesis and the emergence of the *Vibrio cholerae* El Tor biotype. Infect. Immun. **74:**6547–6556.
49. **Yoon, S. S., and J. J. Mekalanos.** 2008. Decreased potency of the *Vibrio cholerae* sheathed flagellum to trigger host innate immunity. Infect. Immun. **76:**1282–1288.

Editor: A. Camilli