Table 2 | **Novel mucosal adjuvants and delivery systems for the development of nasal vaccines**

Antigen	Adjuvant and delivery vehicle	T_H cells	Secretory IgA	Serum IgG	Protective immunity	References
Influenza HA	mCTA–nLTB	$T_H2 > T_H1$	+	+	+	93
PspA	mCT	$T_H2 > T_H1$	+	+	+	90
V3J1	rBCG	$T_H1 > T_H0$	–	+	+	43
HIV gp160	HVJ liposome	$T_H1 = T_H2$	+	+	+	76

gp160, glycoprotein 160; HA, haemagglutinin; HVJ, haemagglutinating virus of Japan; nLTB, B subunit of native form of heat-labile enterotoxin; mCT, mutant cholera toxin; mCTA, A subunit of MCT; PspA, pneumococcal surface adhesin A; rBCG, recombinant *Mycobacterium bovis* bacillus Calmette-Guérin; T_H, T helper cell; V3J1, peptide containing neutralizing epitope of HIV.

environmentally distinct locations. Targeting the CMIS would therefore seem to be a logical choice for the development of a second generation mucosal (nasal or oral) vaccine to induce antigen-specific immune responses — such as a combination of T_H1-cell or T_H2-cell responses, CTL responses, and IgA and IgG responses — in the common and/or selective regions of the mucosal compartments, as well as at systemic sites, through the use of the NALT- and Peyer's-patch-initiated mucosal immune responses (FIG. 1).

NALT-based vaccine development

As we have described, NALT is one of the key components of the organized lymphoid tissue, and it contains all of the immunocompetent cells that are required for the induction of antigen-specific immune responses. It is therefore likely to have a central role in the development of a 'nasal vaccine'. Nasal vaccination has proven to be an effective regimen for the stimulation of the respiratory immune system[1,2,41,45]. Furthermore, this route of mucosal immunization can elicit both humoral and cell-mediated antigen-specific immune responses[1,2,41,43–45]. Another attractive feature of nasal immunization is that it requires a much smaller dose of antigen than does oral vaccination for the induction of antigen-specific mucosal and systemic immune responses, because the antigens are not exposed to degradation by digestive enzymes. Because intranasal administration of vaccine antigen alone has failed to fully stimulate NALT, researchers are attempting to develop an effective NALT-targeted vaccine-antigen delivery system and to develop a safe and effective immune-enhancing molecule (or adjuvant) for intranasal administration with the vaccine antigen.

NALT-targeted vaccine delivery. Antigens are known to be more immunogenic in particulate form than in soluble form, but they are vulnerable to antigen-degrading enzymes and acids that are associated with the mucosal environment. To overcome these obstacles, much effort has been focused on the creation of novel non-toxic and non-immunogenic vaccine vehicles that can effectively deliver even the soluble form of antigen to the organized mucosal inductive tissue. Such vehicles need to protect vaccine components from degradation, enhance their uptake from mucosal surfaces and perhaps function as an adjuvant. Among the various candidates for mucosal antigen delivery, Sendai-virus-associated fusion protein seems particularly suited to function as a molecule that guides antigen to the mucosal epithelium, because the Sendai virus itself uses this fusion protein for the invasion of respiratory epithelial cells[74]. A novel hybrid antigen-delivery vehicle has also been devised using this envelope fusion glycoprotein of Sendai virus (or using the haemagglutinating virus of Japan, HVJ); the fusion protein is displayed on the surface of liposomes (either fusogenic liposomes or HVJ liposomes) that contain the antigen of interest. When this delivery vehicle was used, intranasally administered antigen that was conjugated to green-fluorescent protein successfully reached the antigen-sampling M cells that are located in the epithelium of NALT[75]. Fusogenic liposomes were also found to effectively deliver antigen to epithelial cells and macrophages in both NALT and the nasal passages[75]. Furthermore, it was shown that an intranasally administered HVJ liposome containing the HIV glycoprotein 160 antigen (gp160; also known as env) was a powerful tool for inducing gp160-specific serum IgG, and gp160-specific mucosal IgA was also detected in nasal wash, saliva, faecal extract and vaginal wash[76] (TABLE 2). These findings show that the novel hybrid antigen-delivery vehicle of fusogenic liposomes (or HVJ liposomes) effectively transports vaccine antigen to NALT for the initiation of antigen-specific IgA responses at distant mucosal effector sites. Furthermore, this immunization method can also induce antigen-specific immune responses (such as production of IgG) in the systemic compartment (TABLE 2).

Because antigen-sampling M cells are scattered throughout the NALT epithelium[77], it seems logical to develop an M-cell-targeted nasal vaccine. One promising approach has been to use a molecule that is involved in the normal course of invasion of an infectious agent. Reovirus, an enteric pathogen, is known to invade its host through M cells that are located in the epithelium of Peyer's patches[78]. The 45-kDa viral haemagglutinin σ1 protein of reovirus has a crucial role in its attachment to and entry into M cells[79]. The virus has been shown to recognize mouse M cells that are present in the airways[80], and the recombinant form of the σ1 protein can bind to M cells that are associated with NALT epithelium[80]. On the basis of these findings, attempts have been made to develop an M-cell-targeted DNA vaccine using the σ1 protein as a guiding molecule[81]. When conjugated to a eukaryotic expression vector that encodes luciferase (known as pCMVLuc) and administered intranasally, the σ1 protein can specifically bind to the apical surface of M cells that are situated in the follicular epithelium

of NALT; it then leads to the generation of luciferase-specific serum IgG and mucosal IgA responses[80]. A nasal vaccine assembled using the σ1 protein and gp160 resulted in gp160-specific CTL responses in various mucosa-associated and systemic immune compartments, including reproductive tissue and spleen respectively[81]. These findings further emphasize the efficacy of NALT-targeted immunization for the induction of humoral and/or cell-mediated antigen-specific immune responses in mucosal and systemic immune compartments.

Because it only infrequently causes serious complications, BCG, a commonly used vaccine for the control of tuberculosis, is considered to be a low-risk vaccine. The recombinant form of BCG is a useful vaccine-antigen delivery vehicle, because it has strong adjuvant activity that can induce both humoral and cell-mediated immune responses[82]. Indeed, systemic administration of rBCG that expresses HIV antigen has been shown to effectively induce cell-mediated immunity[43,83]. Our own studies have shown that intransal administration of rBCG that expresses V3J1, a neutralizing epitope of HIV, can induce V3-peptide-specific IgG that has neutralizing activity for more than 0.5–1 years in both normal and immunodeficient (interferon-γ-deficient or *Il-4*$^{-/-}$) mice[43] (TABLE 2). Furthermore, V3J1–rBCG-induced serum IgG has also been shown to effectively neutralize a homologous strain of HIV[43]. Accordingly, rBCG shows promise as an effective nasal-immunization vehicle for the induction of prolonged antigen-specific antibody responses.

Creation of safe toxin-based adjuvants. Both cholera toxin that is produced by *Vibrio cholerae* and the heat-labile enterotoxin of *Escherichia coli* function as adjuvants to enhance mucosal and serum antibody responses to co-administered protein antigens delivered by oral or nasal routes[2,84]. Unfortunately, despite their efficacy as mucosal adjuvants, the native forms of cholera toxin (nCT) and heat-labile enterotoxin (nLT) cause severe diarrhoea and so are unsuitable for use in humans. To overcome these hurdles, researchers have substituted a single amino acid to generate non-toxic mutant forms of cholera toxin (mCT) and heat-labile enterotoxin (mLT)[85–88]; these retain the adjuvanticity of the native forms but do not induce the ribosylation of ADP that is associated with toxic activity. Our efforts to devise a safe first generation toxin-based adjuvant have focused on mCT S61F (in which phenylalanine replaces serine at position 61) and mCT E112K (in which lysine replaces glutamic acid at position 112); these mutations were created by making a single amino-acid substitution in the active centre of the ADP-ribosyltransferase in the A subunit of cholera toxin[89]. The two mutant forms of cholera toxin have been shown to be safe by *in vitro* analyses of ADP-ribosylation activity and cyclic AMP formation, as well as by *in vivo* examination for diarrhoea-like symptoms. When pneumococcal surface-protein A (PspA) — a new candidate vaccine antigen for preventing infection with *Streptococcus pneumoniae* — was intranasally administered with mCT, antigen-specific mucosal IgA and systemic IgG responses were elicited[90]. Mice intranasally immunized with PspA and mCT were also protected against a lethal challenge with *S. pneumoniae*[90]. Interestingly, when the tetanus-toxoid vaccine (which is currently administered by injection) was intranasally administered with one of these two mCTs, it generated protective immunity against challenge with the toxin[91]. An independent study has also shown that mCT E112K is the safest and most effective of the currently available toxin-based mutant adjuvants[92]. Taken together, these findings support the idea that mCT is a strong candidate for an effective mucosal adjuvant to generate protective immunity by the nasal route of administration. Indeed, these findings indicate that the current preference for injection-type vaccines should be reconsidered, and in future, greater use should be made of spray-type vaccines that include mCT and other safe toxin-based adjuvants (BOX 1).

To further enhance the efficacy of the mCT mucosal adjuvant, a second-generation, chimeric-type adjuvant was constructed from the A subunit of mCT (mCTA) and the B subunit of nLT (nLTB); therefore, the adjuvant has the immunobiological properties of both cholera toxin and heat-labile enterotoxin[93]. Nasal immunization with influenza-virus haemagglutinin plus the newly created chimeric mucosal adjuvant mCTA–nLTB resulted in significant haemagglutinin-specific serum IgG and IgA responses[93] (TABLE 2). In addition, mice that were intranasally immunized with haemagglutinin and mCTA–nLTB showed high levels of haemagglutinin-specific IgA in nasal and lung washes and were protected from viral challenge[93]. These findings show that nasal vaccines containing mCT or mCTA–nLTB are effective for the induction of protective immunity. The goal of mucosal-vaccine development cannot be realized without the creation of such novel and safe mucosal adjuvants.

Box 1 | **Advantages and disadvantages of nasal vaccination**

Advantages

- Is the most effective route to elicit optimal protective immunity in both mucosal and systemic immune compartments.
- Can effectively induce antigen-specific immunity in the reproductive tract, as well as in the upper respiratory tract.
- Can generate cross-protective immunity in the gut through the common mucosal immune system.
- Can avoid degradation of vaccine antigen caused by digestive enzymes, so requires a smaller dose of antigen than oral immunization.
- Does not require injection, so is less painful.
- Does not require trained medical personnel for delivery.

Disadvantages

- Possible deposition of antigen in the central nervous system through the olfactory bulbs and olfactory nerves; this requires further investigation.
- Requires adjuvant safety to be clinically determined; clinical studies indicate that Bell's palsy is caused by influenza nasal vaccine that contains the native form of *Escherichia coli* heat-labile enterotoxin as a mucosal adjuvant.

BELL'S PALSY
Facial paralysis that is thought to be triggered by viral infection. The facial nerve is oedematous in patients suffering from this disease.

Recent progress in clinical application of nasal vaccination. Between the late 1950s and the early 1960s, the efficacy of immunization with an intranasally administered vaccine against infection with influenza virus was shown in a large clinical trial in Osaka, Japan, in which a nasal-spray vaccine containing live attenuated influenza virus was administered to more than 10,000 volunteers[94,95]. In recent years, two types of intranasally administered influenza vaccine, an inactivated form and a live attenuated form, were introduced in Switzerland and the United States respectively. Indeed, as early as 1997, an inactivated form of nasal vaccine containing a small amount of nLT as a mucosal adjuvant was introduced in Switzerland. However, this influenza vaccine was withdrawn from the market because of the development of BELL'S PALSY by some recipients after nasal vaccination. A causal relationship between the intranasally administered inactivated-influenza vaccine used in Switzerland and the incidence of Bell's palsy was formally established in a recent case–control study[96]. At this stage, the causes and pathogenesis of Bell's palsy remain unclear; however, because nLT has been shown to have pro-inflammatory properties and possible neurological toxicity[97], the co-formulated nLT that is present in the inactivated-influenza vaccine is suspected to be the causative agent[98]. These findings highlight that the development of a safe mucosal adjuvant is crucial if progress is to be made towards a safe and effective mucosal vaccine.

In the past year, on the basis of promising clinical trials showing the induction of protective immunity, an intranasally administered cold-adapted influenza vaccine, known as 'FluMist', has been made available to healthy Americans of ages 5 to 49 (REFS 99,100). It should be noted that the concept of cold-adapted influenza virus was reported in 1967 (REF. 101), so more than 35 years were required for this discovery to be translated into an intransal vaccine against infection with influenza virus. More recently, the concept of nasal immunization was adopted for the development of a vaccine against severe acute respiratory syndrome (SARS). An experimental nasally administered vaccine against SARS that consists of a recombinant attenuated parainfluenza virus expressing the envelope spike protein of the SARS coronavirus was able to induce protective immunity in African green monkeys, including SARS-coronavirus-specific neutralizing antibodies[102]. Although further experiments are essential before this finding can be applied to developing a vaccine against SARS for use in humans, the study emphasizes the usefulness of mucosal immunization for the immunoprophylaxis of infectious diseases.

Concluding remarks

The mucosal immune system is now recognized to be an important first line of defence against invading pathogens. NALT and Peyer's patches are important inductive sites for the initiation of antigen-specific mucosal IgA and serum IgG responses, as well as CTL immune responses, at both mucosal and systemic sites; in this way, both NALT and Peyer's patches function to maximize the two-tiered immunological barrier of the host. The respiratory mucosal immune system has several immunological characteristics that are distinct from those of the Peyer's-patch-centred intestinal mucosal immune system. Although the tissue-genesis programme for other secondary lymphoid tissues, including Peyer's patches, begins during embryonic life, the NALT-organogenesis programme is initiated only after birth. Lymphoid organogenesis of Peyer's patches requires cytokine-mediated programmed inflammation (through the LT-βR) and signalling through the IL-7R, whereas the initiation of NALT development seems to be independent of the IL-7R, LT-$\alpha_1\beta_2$ and the LT-βR. Although the inducer cells for both NALT and Peyer's patches have a common phenotype, that is, $CD3^-CD4^+CD45^+$, NALT-inducer cells seem to be regulated by ID2 alone, whereas Peyer's-patch-inducer cells depend on both ID2 and ROR-γ. Because the organogenesis programme of NALT is different from that of other secondary lymphoid tissues, such as Peyer's patches, efforts should now be aimed at elucidating the distinct molecular characteristics of the NALT-genesis programme and the functional consequences of this. Clearly, it is important to have a thorough understanding of the unique molecular and cellular properties of the NALT-centred mucosal immune system for the development of a successful nasal vaccine.

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Competing interests statement

The authors declare no competing financial interests.

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Probiotic Bifidobacteria Protect Mice from Lethal Infection with Shiga Toxin-Producing *Escherichia coli* O157:H7

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The anti-infectious activity of probiotic Bifidobacteria against Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 was examined in a fatal mouse STEC infection model. Stable colonization of the murine intestines was achieved by the oral administration of *Bifidobacterium breve* strain Yakult (naturally resistant to streptomycin sulfate) as long as the mice were treated with streptomycin in their drinking water (5 mg/ml). The pathogenicity of STEC infection, characterized by marked body weight loss and subsequent death, observed in the infected controls was dramatically inhibited in the *B. breve*-colonized group. Moreover, Stx production by STEC cells in the intestine was almost completely inhibited in the *B. breve*-colonized group. A comparison of anti-STEC activity among several *Bifidobacterium* strains with natural resistance to streptomycin revealed that strains such as *Bifidobacterium bifidum* ATCC 15696 and *Bifidobacterium catenulatum* ATCC 27539^T did not confer an anti-infectious activity, despite achieving high population levels similar to those of effective strains, such as *B. breve* strain Yakult and *Bifidobacterium pseudocatenulatum* DSM 20439. The effective strains produced a high concentration of acetic acid (56 mM) and lowered the pH of the intestine (to pH 6.75) compared to the infected control group (acetic acid concentration, 28 mM; pH, 7.15); these effects were thought to be related to the anti-infectious activity of these strains because the combination of a high concentration of acetic acid and a low pH was found to inhibit Stx production during STEC growth in vitro.

A complex intestinal microflora provides protection against colonization by many pathogenic infectious agents (for reviews, see references 8 and 40), and the term colonization resistance was first used by van der Waaij et al. in 1971 to indicate a resistance to colonization by exogenous, potentially pathogenic microorganisms (PPMOs) (39). Vollaard and Clasener concluded that the flora providing colonization resistance to exogenous microorganisms are identical to the flora limiting the concentration of indigenous PPMOs (40).

Shiga toxin-producing *Escherichia coli* (STEC) infection models in streptomycin (SM)-treated mice have been the most popular (15, 20, 22, 41). An increased susceptibility to STEC infection by treating mice with antibiotics can be explained by the disruption of colonization resistance. However, high dosages of inoculum (more than 10^6 CFU/body) are usually required to establish an STEC infection. These effects were not precisely examined (determination of viable STEC counts or quantification of Shiga-like toxins) in previous studies. We have developed an SM-treated murine STEC infection model in which 100% lethality was achieved after inoculation with only ~5×10^3 CFU of STEC, followed by multiple mitomycin C (MMC) treatments (29). Moreover, a periodic quantitative analysis of Stx production in the intestines showed that there was a transient but dramatic increase of Stxs (especially Stx2) in the lower intestines after multiple MMC treatments during the early stationary phase of STEC growth in the lower intestines.

Probiotics are viable cell preparations or foods containing viable bacterial cultures or components of bacterial cells that have beneficial effects on the health of the host (19). Many of these probiotics are lactic acid bacteria, and anaerobic bifidobacteria have been reported to be useful in the treatment of disturbed intestinal microflora and diarrheal diseases (for a review, see reference 18). Feeding probiotic bifidobacteria to experimental animals has been reported to prevent gram-negative bacterial infections (23, 30, 32). Some probiotic bifidobacterial strains have been reported to lessen the severity of oral STEC infection in murine experimental infection models. Most of these reports, however, utilized gnotobiotic animal models (2, 31, 37, 38), and definite data have not been obtained in studies with conventional animals. Moreover, the precise mechanism of protection has not yet been clarified. The main purpose of the present study was to test the hypothesis that intestinal colonization by probiotic bifidobacteria prevents antibiotic-induced disruptions in the intestinal environment and reduces the lethal toxicity of STEC by using our previously reported lethal murine STEC infection model (29).

MATERIALS AND METHODS

Animals. Specific-pathogen-free 6-week-old male BALB/c mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Groups of 7 or 8 mice were housed in polypropylene cages (CLEA Japan, Tokyo, Japan) with sterilized bedding under controlled lighting (12 h light, 12 h dark), temperature (24°C), and relative humidity (55%) conditions. The mice were maintained on an MF diet (Oriental Yeast, Tokyo, Japan) and sterilized water (126°C for 30 min) containing Cl_2 at a final concentration of 1.5 ppm (μg/ml), ad libitum. SM sulfate (Sigma Chemical, St. Louis, Mo.) was dissolved in the drinking water at a

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concentration of 5 mg/ml. The water bottles were exchanged with freshly prepared bottles every 3 days. All experimental procedures were performed according to the standards set forth in the *Guide for the Care and Use of Laboratory Animals* (24).

Bifidobacteria. *Bifidobacterium breve* strain Yakult, *Bifidobacterium pseudocatenulatum* DSM 20439, *Bifidobacterium bifidum* ATCC 15696, and *Bifidobacterium catenulatum* ATCC 27539^T were used after the selection of the strains had been confirmed by growth in PY broth (16) containing SM at a dose of 4 mg/ml. All bifidobacterial strains were identified by PCR assay with the corresponding species-specific primers for 16S rRNA (21). Each bifidobacterial strain was cultivated separately in GAM broth (Nissui Pharmaceutical, Tokyo, Japan) for 24 h at 37°C, washed with saline twice, and then suspended in saline at a concentration of 10^9 CFU/ml. Colonization by bifidobacteria was established by three consecutive daily administrations of the bacteria to separate groups of mice receiving SM in their drinking water. Periodic examinations of viable counts of *B. breve* in stools were performed in subsets of 6 mice from each group. Briefly, fresh stool specimens (1 to 2 pellets) were weighed and placed in an Eppendorf tube containing 1 ml of sterilized anaerobic buffer solution [KH_2PO_4, 0.0225% wt/vol; K_2HPO_4, 0.0225% wt/vol; NaCl, 0.045% wt/vol; $(NH_4)_2SO_4$, 0.0225% wt/vol; $CaCl_2$, 0.00225% wt/vol; $MgSO_4$, 0.00225% wt/vol; Na_2C0_3, 0.3% wt/vol; L-cysteine hydrochloride, 0.05% wt/vol; resazurin, 0.0001% wt/vol] and homogenized with a pestle. TOS agar (33) supplemented with 0.625 g of SM/ml and 1 μg of carbenicillin disodium salt (Sigma)/ml (T-CBPC agar) was used for the quantitation of the *B. breve* strain Yakult, and CPLX agar (42) supplemented with 0.625 g of SM/ml was used for the selective isolation of other *Bifidobacterium* strains. The media were cultured anaerobically in an atmosphere of 7% H_2 and 5% CO_2 in N_2 at 37°C for 72 h, and the colonies on the plates were counted.

STEC O157:H7 infection. A clinically isolated STEC O157:H7 strain 89020087, which produces both Stx1 and Stx2, was used throughout the study. Cells were grown overnight in Casamino Acids-yeast extract broth (14) at 37°C. A murine gastrointestinal infection model (29) was developed based on the methods of Wadolkowski et al. (41). Briefly, STEC cells were suspended at a concentration of 5×10^4 CFU/ml in saline, and a 100-μl portion of the suspension was administered orally to mice. MMC (0.25 mg/kg; Kyowa Hakko Kogyo, Tokyo, Japan) was administered intraperitoneally a total of three times, once each at 18, 21, and 24 h postinoculation, when the fecal excretion levels of STEC reached as much as 10^9 CFU/g of feces. To assess the viable STEC counts in the feces, intestinal contents, livers, and mesenteric lymph nodes, samples were removed aseptically from the mice and homogenized in 1 ml (5 ml for liver) of sterile saline solution by using a Teflon grinder. The number of viable STEC cells was determined by their growth on sorbitol-MacConkey agar (Nissui Seiyaku, Tokyo, Japan) supplemented with cefixime (2.5 mg/ml; Sigma) and potassium tellurite (0.05 mg/ml; Oxoid, Bashingstoke, Hampshire, United Kingdom) at 37°C for 24 h.

Stx assay. Stxs (Stx1 and Stx2) in the intestinal contents (both free and bacterium associated) were extracted as follows. Briefly, sections of the gastrointestinal tracts were prepared as described above. After homogenization, samples were sonicated at 28 kHz for 60 min in ice-cold water to completely disrupt the bacteria and then centrifuged at 30,000 × *g* for 10 min to remove undisrupted debris. The supernatants were then filtered through a 0.45-μm-pore-size membrane filter and then ultrafiltrated (molecular weight cutoff, 20,000; 5,000 × *g* for 60 min) to remove low-molecular-weight substances, such as SM sulfate, which can affect Stx quantification when the reversed passive latex agglutination (RPLA) test (Denka Seiken, Tokyo, Japan) is used. After centrifugation, the resulting fraction on the membrane in the tube was reconstituted in the original volume of phosphate-buffered saline and then serial twofold diluted with phosphate-buffered saline supplemented with 0.5% bovine serum albumin and 0.1% NaN_3. Both Stx1 and Stx2 were then quantified by the RPLA test. The Stx concentrations in the intestinal contents were then calculated relative to a standard curve of purified Stx1 or Stx2 and expressed as micrograms per tissue weight.

Histopathology. Mice were dissected on day 2 or 7 after STEC infection. The mesenteric lymph nodes, femur, thymus, lungs, bronchus, heart, small intestine, cecum, colon, liver, spleen, kidneys, suprarenal gland, and brain were divided longitudinally and fixed overnight in 10% neutral buffered formalin. Paraffin-embedded sections stained with hematoxylin and eosin were then examined by light microscopy.

Detection of organic acids in cecal contents. The cecal contents were homogenized in 1 ml of distilled water, and the homogenate was centrifuged at 13,000 × *g* at 4°C for 10 min. A mixture of 0.9 ml of the resulting supernatant and 0.1 ml of 1.5 mM perchloric acid was mixed well in a glass tube and allowed to stand at 4°C for 12 h. The suspension was then passed through a filter with a pore size of 0.45 μm (Millipore Japan, Tokyo, Japan). The organic acid content of the sample was analyzed by high-performance liquid chromatography as described in a previous report (18). The high-performance liquid chromatography was performed with a Waters system (Waters 432 Conductivity Detector; Waters, Milford, Mass.) equipped with two columns (Shodex Rspack KC-811; Showa Denko, Tokyo, Japan). The concentrations of organic acids were calculated by using external standards.

Combined effect of pH and AA on STEC growth and Stx production. The pH and concentration of acetic acid (AA) were adjusted in tryptic soy broth so that the conditions were the same as those found in the cecal contents of the STEC-infected control group (pH 7.15; AA concentration, 28 mM) or in the *B. breve*-colonized cecum (pH 6.75; AA concentration, 56 mM). Then, STEC in media at a concentration of 10^5 CFU/ml was added and cultivated anaerobically in an atmosphere of 100% N_2 at 37°C; MMC at a final concentration of 1 μg/ml was added after 8 h of cultivation. Viable bacterial counts were determined periodically after 0, 2, 4, 6, 8, 12, and 16 h of incubation. Stx concentrations were determined after 16 h of cultivation (8 h after the addition of MMC).

Statistical analysis. The average number of bacteria was analyzed by using the Dunnet test to determine significant differences between the treatment and control groups. Differences in survival ratios were determined by using Fisher's exact probability test followed by correction with the Bonferroni inequality equation. A significant difference was defined as a P value of <0.05.

RESULTS

Inhibition of lethal STEC O157:H7 intestinal infection by *B. breve* colonization of the intestines in SM-treated mice. The excretion levels of STEC in feces after the oral administration of 5×10^3 CFU suggested that STEC proliferated dramatically in the intestines within 24 h of the infection (Fig. 1A). *B. breve* strain Yakult, when administered daily for three consecutive days (10^8 CFU/mouse/day), aggressively proliferated in the intestine, reaching a population level of 10^9 CFU/g of cecal contents; this level of proliferation was maintained at the time of the STEC infection on day 3, after the last administration of *B. breve* (Fig. 1A). The stable colonization of the intestines by *B. breve* did not inhibit the STEC cells from proliferating logarithmically during the initial phase of the infection, but once the proliferation had reached a plateau, further colonization was significantly inhibited for 16 days (Fig. 1A).

In the STEC-infected control group, a dramatic decrease in body weight and subsequent death was observed in 12 of 14 mice in the group within 10 days after MMC treatment (Fig. 1B and C). On the other hand, body weight was maintained and none of the mice died in the *B. breve*-treated group throughout the observation period. Extraintestinal STEC translocation was not observed in either group (data not shown), suggesting that sepsis was not the cause of death in the STEC-infected control group.

In the next series of experiments, mice were dissected at various intervals after MMC treatment, and the Stx levels in the cecal contents were analyzed by RPLA. Transient but dramatic increases in the concentrations of both types of Stx were observed 3 to 9 h after the last MMC treatment in the STEC-infected control group (Fig. 2). The Stx2 titers were relatively higher than those of Stx1 throughout the experimental period (Fig. 2). No significant increases in the Stx titers were detected in the *B. breve*-treated group after MMC treatment, and the titers were less than 1/50 (Stx1) and 1/500 (Stx2) of those in the controls, respectively (Fig. 2). The Shiga toxins were produced mainly in the lower parts of the intestine, whereas *B. breve* markedly inhibited production of both types of the toxin (Table 1).

Histological analysis. In the STEC-infected control mice, mild damages appearing to be apoptotic were observed in

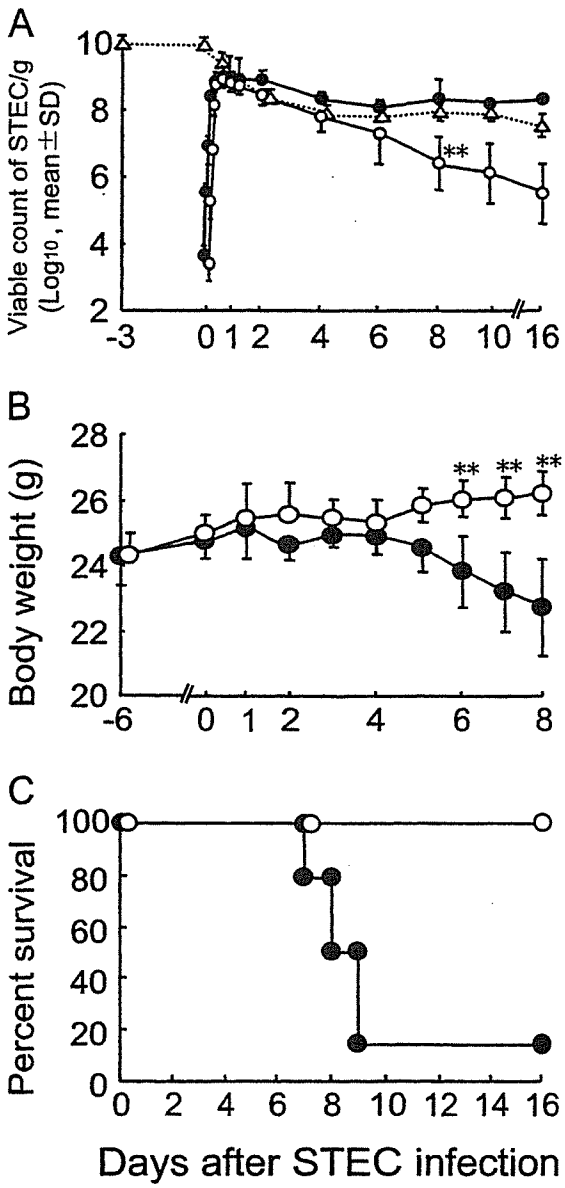

FIG. 1. Inhibition of lethal intestinal STEC infection by *B. breve* colonization in SM-treated mice. SM sulfate at a concentration of 5 mg/ml in drinking water was given to 28 mice from day −6 until day 16. *B. breve* strain Yakult (1×10^8 to 3×10^8 CFU/mouse/day) in 0.1 ml of saline was administered to half of the mice once a day from day −5 to −3, and the other half of the mice were administered saline on the same schedule as that for the *B. breve* treatment. Mice were infected orally with STEC (5×10^3 CFU) on day 0 and then treated with MMC at an inoculum dose of 0.25 mg/kg of body weight three times at 18, 21, and 24 h after the STEC infection. (A) Feces for bacteriological analysis were obtained from 6 randomly selected mice in each group on days 0 (at 3, 6, 9, 12, 15, and 18 h), 1, 3, 4, 7, 10, and 16 after the STEC infection, with the exception of the control group on days 10 to 16 ($n = 2$). Viable counts of STEC and *B. breve* were examined as described in the text. Symbols: ●, number of STEC organisms in the STEC-infected control mice; ○, number of STEC organisms in the *B. breve*-treated mice; △, number of *B. breve* organisms in *B. breve*-treated mice. (B) All 14 mice in each group were weighed every day until day 8. Symbols: ●, STEC-infected control; ○, *B. breve*-treated mice. (C) The STEC-infected control mice (●) and *B. breve*-treated mice (○) were observed for survival for 14 days after the challenge infection. **, a significant difference was observed between the *B. breve*-treated and the untreated control groups ($P < 0.01$).

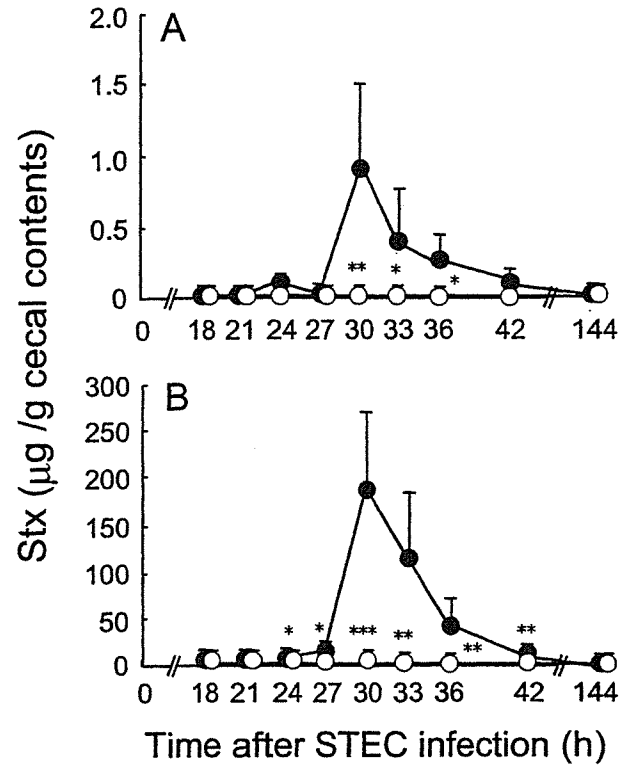

FIG. 2. Inhibition of MMC-induced production of Shiga toxins by *B. breve* colonization. Mice were infected with STEC and treated with MMC as described in the legend to Fig. 1 and then dissected at the indicated periods after STEC infection to examine Stx production. The concentrations of Stx1 (A) and Stx2 (B) in the intestinal contents were determined by RPLA test as described in the text. The results were expressed as the means and standard deviations of the results from 6 mice. Significant differences in Stx concentration were observed between the *B. breve*-treated and the untreated control groups (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

cryptic areas of the intestinal mucosa 30 h after STEC infection, when the concentrations of Stxs in the intestines peaked (Fig. 3A). Injuries in the hematopoietic organs, such as the bone marrow (erythroblastopenia) (Fig. 3C), mesenteric lymph nodes (apoptosis) (Fig. 3D), and toxic tubular necrosis accompanied by distention (Fig. 3B) were clearly observed on day 7 after STEC infection in the moribund STEC-infected control group. In the *B. breve*-treated group, however, no significant histopathologic disorders (Fig. 3E, F, G, and H) were observed, and erythroblast hematopoiesis was clearly observed in femur and spleen specimens. A hematological analysis showed no clear hemolytic-uremic syndrome signs, such as thrombocytopenia or hematolytic anemia, in either the STEC-infected control group or the *B. breve*-treated group. Significant increases in creatinine and blood urea nitrogen were detected in the infected control group but not in the *B. breve*-treated mice (creatinine, 0.6 ± 0.2 mg/dl for the control, 0.3 ± 0.0 mg/dl for the *B. breve*-treated mice, $P < 0.01$; blood urea nitrogen, 27.8 ± 2.2 mg/dl for the control, 23.4 ± 2.3 mg/dl for the *B. breve*-treated mice, $P < 0.01$). No distinct characteristics of lower leg paralysis or histopathological damage to the other organs, including the brain, were observed (data not shown). These results suggest that the intestinal mucosa injuries were

TABLE 1. Inhibition of MMC-induced production of Shiga toxins by *B. breve* colonization[c]

Intestinal part	Stx1 (μg/g of intestinal contents) in[a]:		Stx2 (μg/g of intestinal contents) in[a]:	
	Untreated control	*B. breve*-treated mice	Untreated control	*B. breve*-treated mice
Small intestine	ND[b]	ND	0.04 ± 0.02	ND
Cecum	0.9 ± 0.6	0.02 ± 0.03[d]	181.5 ± 85.6	0.4 ± 0.2[d]
Large intestine	0.2 ± 0.2	0.02 ± 0.01[e]	33.2 ± 33.8	0.3 ± 0.4[d]

[a] The concentrations of Stx1 and Stx2 in intestinal contents were determined by the RPLA test as described in the text, and the results are expressed as the means and standard deviations of the results for 6 mice.
[b] ND, not detected.
[c] Mice were infected with STEC and treated with MMC as described in the legend to Fig. 1 and dissected at 30 h after STEC infection for examination of Stx production. To examine localization of Stx in the intestines, each part of the intestine was resected at 30 h after infection.
[d] Significant difference between the *B. breve*-treated mice and the untreated control group ($P < 0.01$).
[e] Significant difference between the *B. breve*-treated mice and the untreated control group ($P < 0.001$).

caused by the transient but marked increase in Stx after MMC treatment.

Comparison of antitoxic activity among several strains of bifidobacteria with natural resistance to SM. The anti-infectious activities of four bifidobacterial strains belonging to four species and confirmed to exhibit a natural resistance to SM in vitro were assessed for their antitoxic activity against STEC in vivo. Although all the strains were colonized in the intestine at similarly high population levels, marked differences in antitoxic activity were observed among the strains (Table 2). Two of four strains that were tested showed potent antitoxic activity, but *B. bifidum* ATCC 15696 and *B. catenulatum* ATCC 27539^T did not exhibit an antitoxic activity. No correlations between antitoxic activity and colonization level were observed among the strains (Table 2). As shown in Fig. 4, the total organic acid concentration decreased and the pH increased in the cecum after SM treatment in the STEC-infected control group; these levels remained unchanged at 30 h after the STEC infection. The disruption in the balance of organic acid concentrations was characterized by a decrease in the acetate concentration and an increase in the succinate concentration when compared with the healthy controls (Fig. 4). Significantly lower pHs, higher concentrations of both total organic acids and AA, and lower concentrations of succinic acid were observed at 30 h after STEC infection in the *B. breve*- and *B. pseudocatenulatum*-treated groups than in the STEC-infected control group. No significant changes in these markers were observed in the groups treated with the ineffective strains, *B. bifidum* and *B. catenulatum*, when compared with the infected controls.

Effect of pH and acetate concentration on Stx production in vitro. The differences in pH and acetate concentration observed between the lower intestines of the infected control group and the *B. breve*-treated group, when reproduced in vitro, produced no differences in the STEC growth patterns, with or without the addition of MMC (Fig. 5A). As shown in Fig. 5B, the addition of MMC to media simulating the conditions observed in the infected control group markedly enhanced the production of Stx2 by the STEC cells. Stx2 production at levels as low as 1/30 of that produced under the control conditions were detected when the conditions in the *B. breve*-treated group were reproduced, and the inhibitory activity of the combined lower pH and higher acetate concentration conditions was exerted almost equally in cultures with or without the addition of MMC. Superinduction of Stx2 by the other agents such as ciprofloxacin hydrochloride and UV under the STEC-infected control condition was detected in a similar fashion as that induced by MMC, which was also markedly inhibited under the conditions in the *B. breve*-treated group (data not shown).

DISCUSSION

It was previously demonstrated that the injection of a lethal dose of 5-fluorouracil (400 mg/kg) into mice induced an extraordinary increase in the levels of indigenous *E. coli* in the intestine and the systemic translocation of these bacteria to the liver; *Bifidobacterium* was the only species whose intestinal population markedly decreased after treatment with this chemotherapeutic agent (25, 26). Moreover, the daily administration of fermented milk containing probiotic bifidobacteria prevented both the drug-induced intestinal outgrowth and the extraintestinal translocation of indigenous *E. coli* (3). Analysis of the organic acid concentrations in the intestinal contents suggested that the administered species compensated for a decrease in the production of organic acids by the disrupted indigenous microflora. More recently, it was demonstrated that the antibiotic-induced intestinal overgrowth and extraintestinal translocation of *Salmonella enterica* serovar Typhimurium was markedly inhibited by precolonization of the intestine by specific strains, such as the probiotic *B. breve* strain Yakult, and suggested that the pH-lowering and acid-producing effects of this strain appeared to be important for enabling this anti-infectious activity (4). These observations suggested that compensation for chemotherapy-induced disruptions in colonization resistance throughout the use of probiotic bifidobacteria may be effective for preventing intestinal infections by PPMOs and that not only the population level but also the metabolic activity of the intestinal colonizer is important for this anti-infectious activity. In the present study, we clarified that the stable colonization of intestines by specific strains of bifidobacteria, such as *B. breve* strain Yakult and *B. pseudocatenulatum* DSM 20439, results in the protection of mice from lethal STEC infections, possibly by inhibiting the production of Stxs in the intestines. To our knowledge, this is the first clear evidence that specific strains of bifidobacteria, including certain probiotics, have such a potent anti-infectious activity against lethal STEC O157:H7 infection in a lethal mouse infection model. The important features of the mechanism responsible for the anti-infectious activity of bifidobacteria are as follows: (i) the inhibition of Stx production but not of STEC growth and (ii)

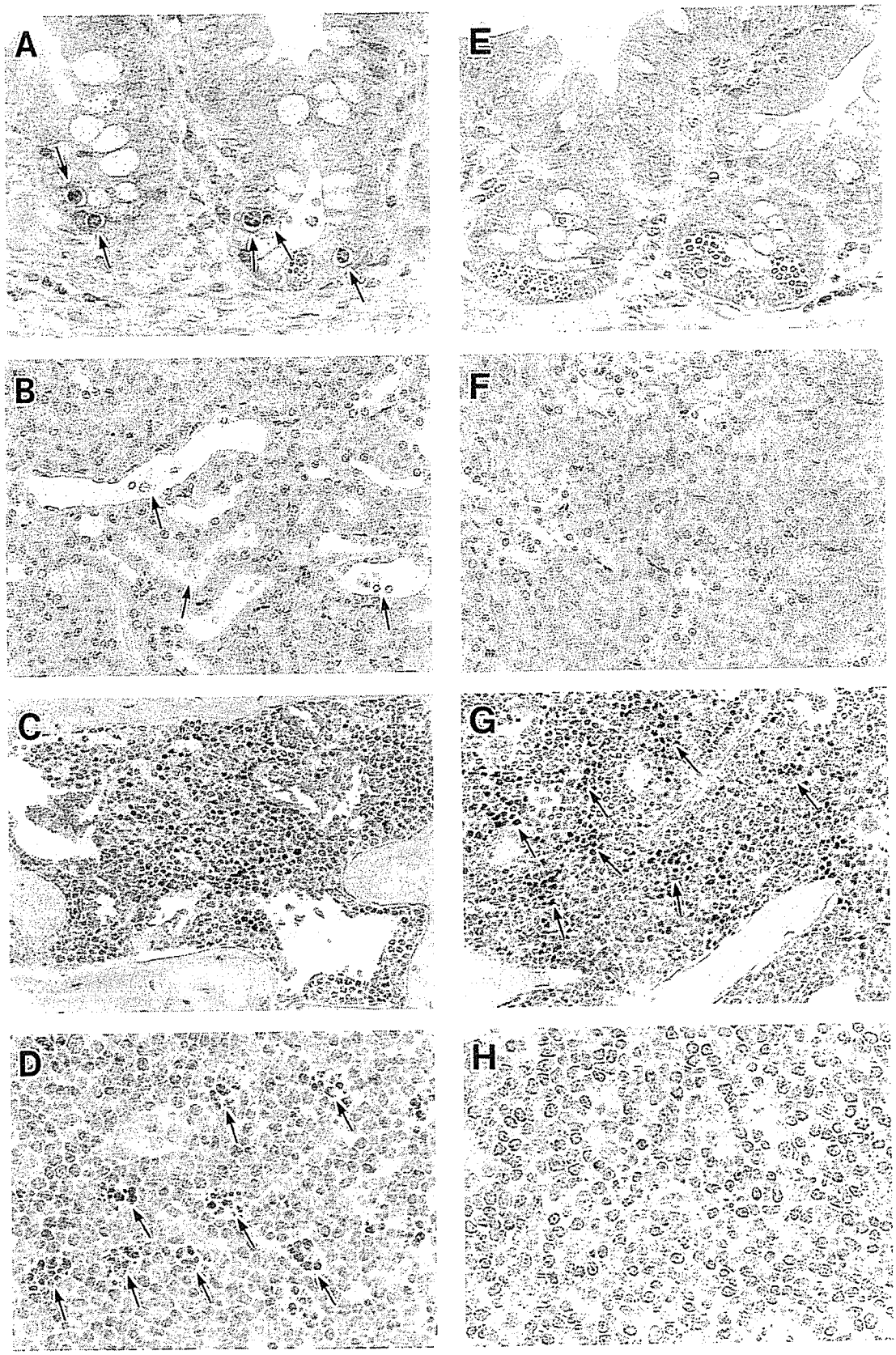

FIG. 3. Histopathological analysis. Hematoxylin and eosin staining of the ileum (A and E), kidney (B and F), bone marrow (C and G), and mesenteric lymph node (D and H) from a mouse in the STEC-infected control group (STEC inoculum, 3.8×10^3 CFU) (A to D) and a mouse in the *B. breve*-treated group (E to H). Organs were obtained on day 2 (A and E) or 7 (B to D and F to H) after STEC infection. Black arrows: panel A, changes suggestive of apoptosis; panel B, necrotic tubular endothelial cells with distention; panel D, changes suggestive of apoptotic bodies; panel G, erythroblasts. Magnifications in both groups: ileum, ×520; kidney, ×520; bone marrow, ×260; mesenteric lymph node, ×520.

TABLE 2. Comparison of antitoxic activity among several strains of bifidobacteria with natural resistance to SM sulfate[a]

Treatment	STEC viable counts/g of feces 18 h after infection (log_{10}, mean ± SD)	No. of deaths/total no. of mice (survival time [days, mean ± SD])	Concn in cecal contents of[b]:	
			Stx1	Stx2
None (untreated control)	9.2 ± 0.2	8/10 (8.0 ± 1.2)	0.7 ± 0.5	200.0 ± 162.1
B. breve strain Yakult	9.0 ± 0.3	0/10[c]	0.1 ± 0.1[c]	1.9 ± 1.2[d]
B. pseudocatenulatum DSM 20439	9.1 ± 0.3	0/10[c]	0.1 ± 0.1[c]	1.4 ± 1.4[d]
B. bifidum ATCC 15696	9.1 ± 0.2	8/10 (8.6 ± 1.4)	0.6 ± 0.6	141.3 ± 99.7
B. catenulatum ATCC 27539^T	9.0 ± 0.3	7/10 (8.6 ± 1.4)	0.8 ± 0.3	155.7 ± 128.6

[a] SM sulfate at a concentration of 5 mg/ml in drinking water was given to mice from day −6 to day 16. Bifidobacterial strains (1×10^8 to 4×10^8 CFU/mouse/day) at an inoculum size of 0.1 ml/mouse were administered to separate groups of mice (10 mice/group) once a day from day −5 to day −3. Population levels of bifidobacteria at the time of STEC infection (log_{10}; mean ± standard deviation) are as follows: *B. breve* strain Yakult, 9.7 ± 0.3; *B. pseudocatenulatum* DSM 20439, 9.7 ± 0.1; *B. bifidum* ATCC 15696, 9.6 ± 0.2; *B. catenulatum* ATCC 27539^T, 9.8 ± 0.3. Mice were orally infected with STEC at a dose of 8.1×10^3 CFU on day 6 after starting SM treatment. MMC (0.25 mg/kg of body weight) was administered a total of three times, once each at 18, 21, and 24 h postinfection. Mice were observed for survival for 16 days after STEC infection. Mice were sacrificed 6 h after the last MMC shot, and Stx concentrations in the cecal contents were determined by the RPLA test as described in the text.

[b] Results are expressed as mean Stx concentrations (micrograms per gram of cecal contents) ± standard deviations for the results from 6 mice.

[c] Significant difference between the *Bifidobacterium*-treated mice and the untreated control mice ($P < 0.05$).

[d] Significant difference between the *Bifidobacterium*-treated mice and the untreated control mice ($P < 0.01$).

the improvement of intestinal environmental factors, such as pH and acetate concentration.

Treatment of mice with SM depleted the facultative intestinal flora, which appeared to allow the explosive opportunistic proliferation of SM-resistant STEC cells (Fig. 1A), and the initial logarithmic phase of STEC proliferation in the intestines was not influenced by the *B. breve* cocolonization. Organic acids, such as AA, lactic acid, and citric acid, have been reported to possess a higher bactericidal activity than inorganic acids, such as hydrochloric acid; furthermore, the bactericidal activity of organic acids depends mainly on their undissociated form (6, 13). Undissociated organic acids can permeate the cell membrane by diffusion and release protons within the cell. The influx of protons is thought to induce cytoplasm acidification

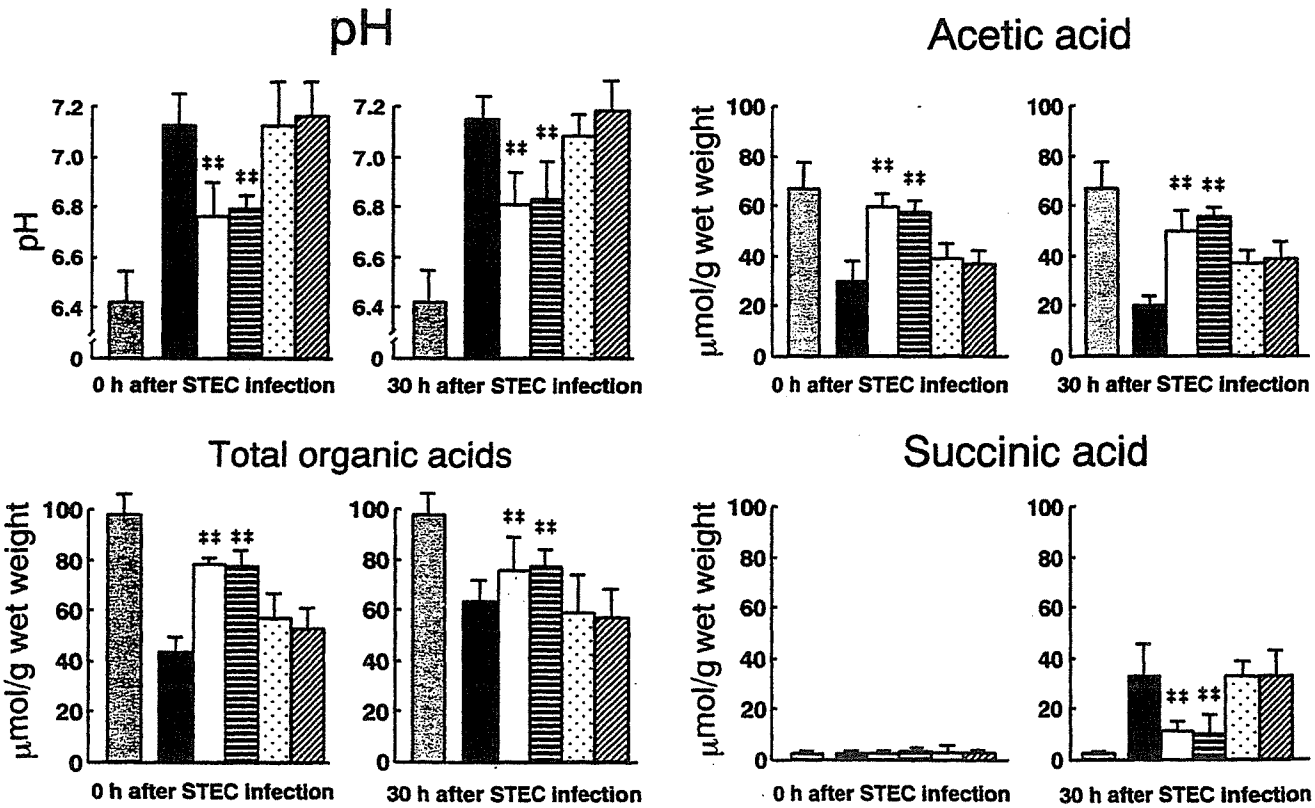

FIG. 4. Changes in intestinal pH and concentrations of organic acids after STEC infection in SM-treated mice. Mice were treated as shown in Table 2. Cecal contents were obtained from mice both at the time of STEC infection (0 h) and 30 h after STEC infection. pH and organic acid concentrations were determined as described in the text. Results are expressed as the means and standard deviations of the results from 6 mice. Columns: grey, nontreated healthy mice; black, SM-treated mice; white, *B. breve* strain Yakult-treated mice; hatched, *B. pseudocatenulatum* DSM20439-treated mice; slashed, *B. bifidum* ATCC 15696-treated mice; vertically lined, *B. catenulatum* ATCC 27539^T-treated mice. **, significant differences are shown for the *Bifidobacterium*-treated groups versus the untreated control group ($P < 0.01$).

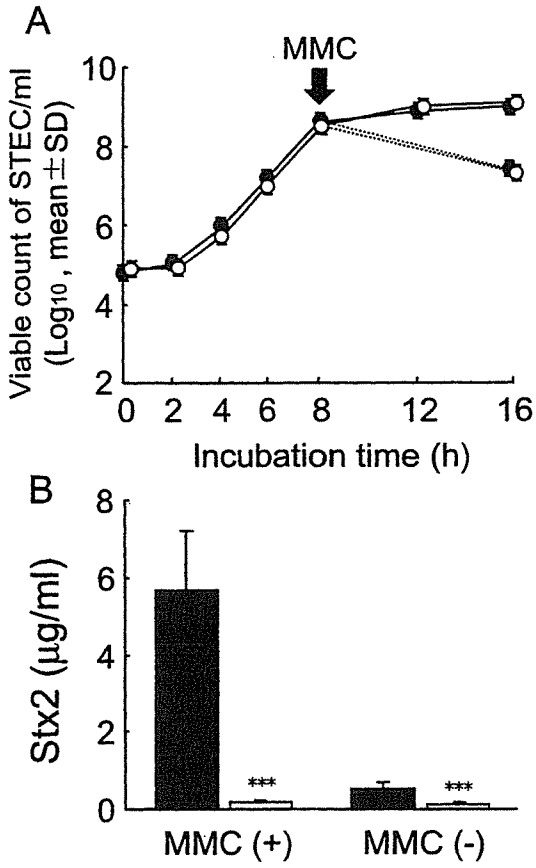

FIG. 5. Inhibition of Stx production but not STEC proliferation at higher AA concentrations and lower pH values in vitro. The pH and concentration of AA were adjusted in the growth medium so that the conditions were the same as those in the control cecum (●) (pH 7.15; AA concentration, 28 mM) or the *B. breve*-colonized cecum (○) (pH 6.75; AA concentration, 56 mM). STEC was added to each medium at a final concentration of 10^5 CFU/ml and cultivated at 37°C for 8 h. Cultures were then divided into two groups, and either 20 μl of fresh medium or MMC at a final concentration of 1 μg/ml in 20 μl of medium was added to each group, and the tubes were incubated for an additional 8 h. (A) Viable bacterial counts were determined at the indicated periods during incubation. The straight line and the dotted line show growth without (−) and with (+) MMC, respectively. (B) Stx2 concentrations were determined after incubation for 16 h. Columns: black, control, white, *B. breve*. Results are expressed as the means and standard deviations of the results from triplicate cultures. ***, significant differences are shown for growth under the *B. breve* colonization conditions versus growth under control conditions ($P < 0.001$).

and dissipate the membrane proton potential (6, 10, 13). This leads to the disruption of the proton motive force and the inhibition of substrate transport mechanisms, energy-yielding processes, and macromolecule synthesis (7, 12). In addition, anion accumulation is assumed to exert a bacterial toxicity (28). In a previous study, it was reported that the cytotoxic properties of undissociated lactic acid on STEC strain 89020087 in vitro was divided into two phases: a bacteriostatic phase (between 3.2 to 62 mM) and a bactericidal phase (over 62 mM) (27). Several investigators have noted the ability of STEC O157:H7 to survive in acidic conditions; a possible explanation for this survival ability could be an acid tolerance response (5, 9, 12). We analyzed the cytotoxic properties of undissociated AA against the STEC strain and found that an undissociated AA concentration of more than 20 mM was needed to exert cytotoxic or growth-inhibitory activity against the STEC strain in vitro (data not shown), and the higher acetate concentration and lower pH in the *B. breve*-colonized cecum, when reproduced in vitro, was not found to inhibit STEC growth (Fig. 5A). Taken together, these results may explain the reason why *B. breve* colonization did not inhibit STEC growth in vivo.

On the other hand, the higher concentration of AA and the lower pH in the *B. breve*-colonized intestines appear to play a somewhat important role in the inhibition of toxin production because the inhibitory effect of the combination of pH and acetate on Stx production was confirmed by in vitro experiments (Fig. 5B). The mechanism by which the acetate concentration and the pH of the intestines inhibit Stx production is not clear. Quorum sensing is a mechanism through which gene expression in bacteria is regulated by cell density (11). Recently, quorum-sensing systems have been reported to be involved in the expression of several pathogenic genes such as LEE, which encodes a component of a type III secretion system in STEC (17, 34, 36); the expression levels of such genes vary with the bacterial growth phases (1). Little is known about the regulatory mechanisms of Stx production (35), and both the host- and bacterium-related factors affecting Stx production remain to be elucidated. The present results suggest that environmental regulation via molecules in the intestine, such as AA, is an important regulator of Stx gene expression in intestinal colonies of STEC. Studies to determine the mechanism of Stx production in STEC at the gene expression level are in progress.

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Diverse CTX Phages among Toxigenic *Vibrio cholerae* O1 and O139 Strains Isolated between 1994 and 2002 in an Area Where Cholera is Endemic in Bangladesh

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PCR surveillance of the *rstR* genes of CTX phages in *Vibrio cholerae* O1 and O139 showed no relationship between the incidence of disease and changes in the *rstR* but showed variations in their presence in O1 and O139 strains and the occurrence of multiple types in a few strains.

Of the 209 currently recognized serogroups of *Vibrio cholerae*, only strains belonging to serogroups O1 and O139 can cause cholera. Two major virulence gene clusters are now known to carry key virulence genes that are essential for the pathogenicity of *V. cholerae* O1 and O139. These gene clusters include the CTXϕ prophage (14), which carries the *ctxA* and *ctxB* genes (the genes that encode cholera toxin [CT], which is responsible for severe diarrhea), and the toxin-coregulated pilus (TCP) pathogenicity island, which carries genes for the biosynthesis of the TCP, required for colonization of the small intestinal epithelium (7).

The approximately 7-kb CTXϕ genome consists of the core and the RS2 region. The core region encodes proteins needed for the assembly and secretion of viral particles (Psh, Cep, $pIII^{CTX}$, Ace, and Zot) and also encodes CT, which is not necessary for phage morphogenesis (3), while the RS2 region represents a site-specific recombination system that allows lysogenic phage to integrate at a specific site on the host chromosome (14). The RS2 region of CTX prophage encodes proteins required for replication (RstA), phage integration (RstB), and regulation (RstR) of the lysogeny of CTXϕ (14). An antirepressor, *rstC*, is carried by a satellite phage, RS1, often present adjacent to the CTX prophage in toxigenic *V. cholerae* O1 El Tor and O139 strains (1, 5).

Diversity of the CTX phage repressor *rstR* has been described previously, and this diversity constitutes heteroimmunity among diverse CTX phages (8, 2). The difference in the *rstR* gene is also the only known genetic difference between any two different CTX phage types. The existence of at least four different *rstR* genes carried by different CTX phages, namely, CTX^{ET}, CTX^{class}, CTX^{Calc}, and CTX^{Env}, has been recognized (8, 2, 10). The epidemiological significance of the diversity of CTX phages is not clearly known, but at least two periods of explosive resurgence of cholera have been associated with strains showing changes in the *rstR* type of CTX phages. The first was the resurgence of *V. cholerae* O139 in August 1996 in Calcutta, India, which continued for a year (8, 9, 13), and the second was the resurgence of strain O139 in March to April of 2002 in Dhaka, Bangladesh (6). On the basis of their *rstR* genes and other phenotypic traits, genetic hybrids of classical and El Tor biotypes that cause cholera have been shown to exist, and these hybrids have been designated the Matlab variants of *V. cholerae* (11). To further document the distribution and temporal changes in the CTX phage contents of epidemic strains, we conducted a surveillance of CTX phage types by analyzing the types of *rstR* genes carried by a large collection of toxigenic *V. cholerae* strains.

We selected every 10th consecutive strain of *V. cholerae* O1 or O139 isolated from cholera patients admitted to the Matlab hospital, 50 km south of Dhaka, Bangladesh, from 1994 to 2002. A total of 169 strains of *V. cholerae* O1 and 95 strains of *V. cholerae* O139 isolated between 1994 and 2002 (with the exception of the year 1999) were included in this study. The procedure for the selective isolation of *V. cholerae* from stool samples of patients with acute secretory diarrhea and subsequent identification has been described in detail previously (12).

The serogroup of the strains selected were confirmed by using polyclonal O1 and O139 antisera. PCR was performed

TABLE 1. Oligonucleotide primer sequences used in PCR assays[a]

Gene	Primer sequence (5′–3′)	Amplicon size (bp)
ctxA (forward)	5′-CTCAGACGGGATTTGTTAGGCACG-3′	
ctxA (reverse)	5′-TCTATCTCTGTAGCCCCTATTACG-3′	308
rstR1 (forward)	5′-CTTCTCATCAGCAAAGCCTCCATC-3′	500
rstR2 (forward)	5′-GCACCATGATTTAAGATGCTC-3′	500
rstR3 (forward)	5′-CTGTAAATCTCTTCAATCCTAGG-3′	~300
rstR4 (forward)	5′-GTTAACGCTTCAAGCCTG-3′	400
rstA3 (reverse)	5′-TCGAGTTGTAATTCATCAAGAGTG-3′	

[a] Primers were for the detection of *rstR* and *ctxA* genes in *V. cholerae* O1 and O139 strains isolated from hospitalized patients in Matlab, Bangladesh.

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TABLE 2. Occurrence of the various *rstR* genes examined in this study among *V. cholerae* O1 and O139 strains

rstR gene(s)	Original nomenclature (reference)	No. of positive isolates	
		V. cholerae O1	*V. cholerae* O139
$rstR_1$	$rstR^{class}$ (8)	9	0
$rstR_2$	$rstR^{ET}$ (8)	141[a]	83
$rstR_3$	$rstR^{Calc}$ (8)	0	0
$rstR_4$	$rstR^{Env}$ (10)	0	0
$rstR_1$ + $rstR_2$	Not reported	6	0
$rstR_2$ + $rstR_4$	Not reported	6	6
$rstR_2$ + $rstR_3$	Combination (8)	0	3
$rstR_1$ + $rstR_2$ + $rstR_3$	Not reported	0	1
None[b]		7	2
Total		169	95

[a] Two strains were negative for the *ctxA* gene.
[b] None, negative for all of the *rstR* genes tested.

according to a previously described procedure (11). The primer sequences are shown in Table 1. *V. cholerae* O1 isolates (classical 154) and *V. cholerae* O139 (AR-196318) and *V. cholerae* non-O1 non-O139 (environmental SCE-188) isolates (10) were used as standard reference strains. We also used an *rstC* probe as described previously (4) to examine whether CTX prophage-negative strains, which show an *rstR* amplicon, carried RS1. The PCR products from five representative isolates (MJ1347, MM1079, MM2071, MP1950, and MP2044) were purified with a Microcon centrifugal filter device (Millipore Corporation, Bedford, Mass.), and a cycle sequencing reaction was performed with the same primers. DNA sequencing was performed by using standard conditions in an ABI PRISM 310 automated sequencer (Perkin-Elmer–Applied Biosystems, Foster City, Calif.). DNA sequence editing and analysis were performed with DNASTAR package 5.06 software.

Table 2 shows the distribution of different types of *rstR* genes among 169 strains of *V. cholerae* O1 and 95 O139 strains, isolated between 1994 and 2002 from hospitalized patients in Matlab, Bangladesh. We propose to designate the *rstR* genes with subscript numbers ($rstR_1$, $rstR_2$, etc.) since we anticipate that the number of such *rstR* genes that will be discovered in the future is likely to increase and thus a number designation is more suitable. The nucleotide sequences of 10 *rstR* amplicons from five isolates of *V. cholerae* O1 and O139 were similar to those of canonical *rstR* genes, with minor differences, as shown in Table 3.

Of the 169 O1 strains and 95 O139 strains, 9 and 2 strains, respectively, did not carry the *ctxA* gene and were considered nontoxigenic. Two of the nontoxigenic strains of *V. cholerae* O1, however, carried the $rstR_2$ genes. We further examined all nine *V. cholerae* O1 and two *V. cholerae* O139 strains with a probe specific for *rstC* to search for the presence of the RS1 element, which would explain the presence of the $rstR_2$ gene in the nontoxigenic *V. cholerae* O1 strains. However, only one of the two nontoxigenic *V. cholerae* O1 strains hybridized with the *rstC* probe; the other strain did not hybridize with the probe.

Three isolates from the year 1997 are of special interest. One of the isolates, MM004, is like the CTX-negative isolates of previous years, in that it was negative for an *rstR* gene of any type, *ctxA*, and *rstC*. The isolate MM1079, however, was positive for $rstR_2$ but negative for the *ctxA* gene and positive for *rstC*. Yet another 1997 isolate, MM2644, was $rstR_2$ positive but negative for both the *ctxA* and *rstC* genes. These strains might have undergone deletion in part of the CTX prophage. Overall, PCR results for the incidence of different *rstR* types in O1 and O139 strains showed no relationship between the temporal incidence of the disease and changes in CTX. However, the data presented do indicate variation in the incidence of *rstR* types, their presence in O1 and O139 strains, and the infrequent but interesting occurrence of multiple types in some strains. The *rstR* gene offers a window to assess the evolution of the phage.

Nucleotide sequence accession numbers. The nucleotide sequence accession numbers of 10 *rstR* amplicons from five isolates of *V. cholerae* O1 and O139 have been submitted to GenBank under accession numbers AY704650 to AY704659.

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TABLE 3. Nucleotide sequences of various *rstR* genes of *V. cholerae* O1 and O139 isolates from Matlab, Bangladesh, in comparison with corresponding sequences in GenBank

Strain (gene)	Serogroup	GenBank accession no.	Mutation comparison (accession no.)
MJ1347 ($rstR_1$)	O1	AY704650	Identical to the *V. cholerae* 569B repressor *rstR* (AF055890)
MJ1347 ($rstR_2$)	O1	AY704651	Silent substitution of C to T at position 1938 compared to the *V. cholerae* O1 biovar El Tor N16961 transcriptional repressor *rstR* (AE004224)
MM1079 ($rstR_2$)	O1	AY704652	Silent substitution of C to T at position 1938 compared to the *V. cholerae* O1 biovar El Tor N16961 transcriptional repressor *rstR* (AE004224)
MM2071 ($rstR_2$)	O1	AY704653	Silent substitution of C to T at position 1938 compared to the *V. cholerae* O1 biovar El Tor N16961 transcriptional repressor *rstR* (AE004224)
MM2071 ($rstR_4$)	O1	AY704654	Silent substitution of A to G at position 375 and C to T at position 452 compared to the *Vibrio* phage CTX RSTR (*rstR*) (AY145127)
MP1950 ($rstR_1$)	O139	AY704655	Identical to the *V. cholerae* 569B repressor *rstR* (AF055890)
MP1950 ($rstR_2$)	O139	AY704656	Silent substitution of C to T at position 1938 compared to the *V. cholerae* O1 biovar El Tor N16961 transcriptional repressor *rstR* (AE004224)
MP1950 ($rstR_3$)	O139	AY704657	Identical to the *Vibrio* phage CTXϕ Calcutta *rstR* (AF133310)
MP2044 ($rstR_2$)	O139	AY704658	Silent substitution of C to T at position 1938 compared to the *V. cholerae* O1 biovar El Tor N16961 transcriptional repressor *rstR* (AE004224)
MP2044 ($rstR_3$)	O139	AY704659	Identical to the *Vibrio* phage CTXϕ Calcutta *rstR* (AF133310)

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Review

Defense Mechanisms against Influenza Virus Infection in the Respiratory Tract Mucosa

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SUMMARY: The respiratory tract mucosa is not only the site of infection for influenza viruses but also the site of defense against virus infection. Viruses are initially detected and destroyed non-specifically by innate immune mechanisms, but if the viruses escape the early defense mechanisms, they are detected and eliminated specifically by adaptive immune mechanisms. The major adaptive immune mechanisms are as follows. (i) Specific secretory-IgA (S-IgA) antibodies (Abs) and CTLs ($CD8^+$ cytotoxic T lymphocytes) are involved in the recovery from influenza following viral infection of naïve mice. (ii) Preexisting specific S-IgA and IgG Abs in the immunized animals are involved in viral elimination by forming virus–Ig complexes shortly after re-infection. By their polymeric nature, the S-IgA Abs, which are carried to the mucus by transepithelial transport used for dimeric IgA (dIgA) Abs, provide not only protection against homologous virus infection but also cross-protection against drift virus infection. The IgG Abs, which transude from the serum to the mucus by diffusion, provide protection against homologous virus infection. They are largely distributed on the alveolar epithelia to prevent influenza pneumonia. (iii) In the absence of Abs in the pre-immunized animals, the production of specific IgA and IgG Abs by B memory cells is accelerated after re-infection, and these antibodies play a role in viral elimination from day 3 onwards after re-infection. (iv) In epithelial cells of infected animals, specific dIgA Abs being trafficked through the epithelial cells may be involved in the prevention of viral assembly by binding to newly synthesized viral proteins. (v) In the pre-immunized animals, CTL production by memory T cells is also accelerated and these cells appear to participate in the killing of the host cells infected with different subtype viruses (within the same type) from day 3 onwards after re-infection. (vi) Similarly, memory Th1 cells that mediate an accelerated delayed-type hypersensitivity response are involved in blockade of virus replication by secreting IFN-γ in mice challenged with different subtype viruses. These defense mechanisms suggest that the development of a mucosal vaccine, capable of inducing S-IgA Abs, which provide cross-protection against variant viruses within the same subtype, serum IgG Abs to prevent lethal influenza pneumonia and CTLs, which provide broad cross-protection against different subtype viruses, is strategically important to control influenza.

1. Introduction

Influenza is a contagious, acute respiratory disease caused by an influenza virus infection, which attacks the host respiratory tract mucosa (1,2). Influenza viruses infect host epithelial cells by binding to receptors (sialic acid) on the cell surface via one of the major viral surface glycoproteins, hemagglutinin (HA). The viruses then replicate in the host infected cells. Several hours after infection, the newly synthesized viruses are released from the infected cells by the action of another major glycoprotein, neuraminidase (NA). Influenza viruses are divided into types A, B and C, based on the antigenic differences of the core proteins. Influenza A viruses are further subdivided into subtype viruses (H1N1, H3N2, etc.), according to marked antigenic changes in the HA and NA molecules. These subtype viruses arose from an exchange of gene segments between the avian influenza gene

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pool and human influenza genes (antigenic shift). The A subtype viruses and B viruses cause an antigenic drift every year, resulting in an annual epidemic or local outbreak of influenza. To recover from influenza or to prevent influenza, both innate and adaptive immune responses must be induced in the respiratory mucosa following viral infection. Thus, the respiratory tract mucosa is not only the site of infection by influenza viruses but also the site of defense against viral infection in the host.

Most influenza viruses are detected and destroyed within a few hours by the innate immune mechanisms, which are not antigen-specific and do not require a prolonged period of induction (2,3). Several components such as mucus, macrophages, interferon (IFN) α, β and other cytokines, fever, natural killer (NK) cells and complement are involved in the innate immune system. If influenza viruses can escape these early defense mechanisms, they are detected and eliminated by adaptive immune mechanisms, where T and B cells and their products function as antigen-specific effectors (cytotoxic T lymphocytes [CTLs] and antibodies [Abs]) to target the virus. Also, antigen-specific memory cells (T and B cells) are involved in the prevention of the subsequent viral infection. The effector cells and molecules involved in the defense mechanisms following influenza virus infection are shown in Fig. 1. This review summarizes recent findings on these defense mechanisms induced following influenza virus infection in the respiratory tract mucosa. Furthermore, this review discusses that the development of a mucosal vaccine which is capable of inducing both secretory-IgA (S-IgA) and serum IgG Abs will be strategically important to control influenza. Such a vaccine can provide broad cross-protection against variant viruses including viruses with pandemic potential by the S-IgA Abs and prevent lethal influenza pneumonia by the serum IgG Abs.

2. Innate Immunity against influenza

2-1. Effectors involved in innate immunity

Influenza virus infection triggers the innate immune responses where the following effector cells, molecules and factors are implicated in the restriction of viral spread. Some of these effectors work within a few days following infection, not only as helpers to keep infection under control, but as communicators (antigen-presenting cells [APCs]) or activators (cytokines) in the subsequent adaptive immune response. Examples of the action of these effectors are listed in the following text. i) Inhibitory factors in the mucus of the respiratory tract, that are similar to or identical to N-acetylneuraminic acid-containing receptors for HA molecules reduce markedly the ability of the viruses to infect host cells (4-6). ii) Nasal and pulmonary levels of IFN-α and -β rise rapidly after infection and correlate directly with the degree of viral replication in ferrets, mice and humans (2,7,8). Alveolar macrophages or lymphocytes from infected lungs have been shown to release interferons in vitro (8). iii) Macrophages secrete IL-1, IL-6, TNF-α and IL-12 which activates NK cells (9). Macrophages recovered from infected lungs mediate lysis of infected cells, probably through apoptosis-dependent phagocytosis (10,11). In addition, cytokines (IL-1, TNF-α and IL-6) produced by macrophages induce fever, and the magnitude of the febrile response correlates strongly with the level of virus shedding in humans and animals (2,12). iv) The NK cells that are detected in pulmonary lymphocytes 48 h after influenza virus infection produce IFN-γ and limit viral spread by virus-infected cell lysis, which is mediated probably by pore formation in the infected cells involving perforin (13-16). v) Complement mediates protection; this is highlighted by the increase in mortality in C5-deficient mice infected with a lethal dose of influenza virus (17,18).

2-2. Enhancement of innate immunity

Efforts to activate the effectors involved in innate immune responses would lead to the enhancement in the adoptive immune responses, because some of the effectors act in both systems as a bridge. For example, the intranasal administration of cholera toxin (CT) prior to infection with influenza viruses activates macrophages and NK cells via the binding of CT to the receptor (ganglioside GM1) on the cells and results in non-specific replication reduction of the infected viruses in the respiratory tract (19) (Fig. 2). The non-specific activation of macrophages (APCs) and the enhancement of cytokine production by CT cause the subsequent enhance-

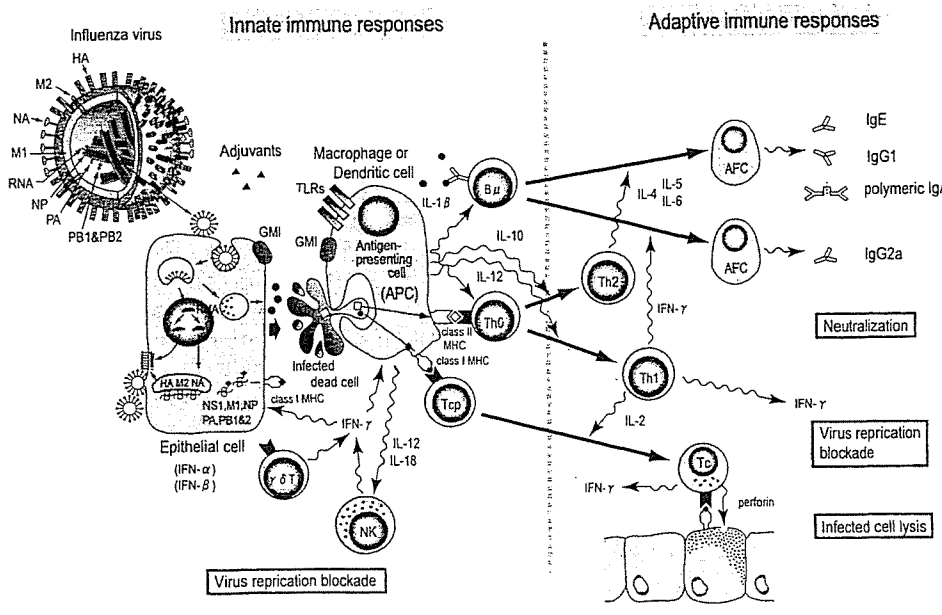

Fig. 1. Defense mechanisms induced by influenza virus infection.

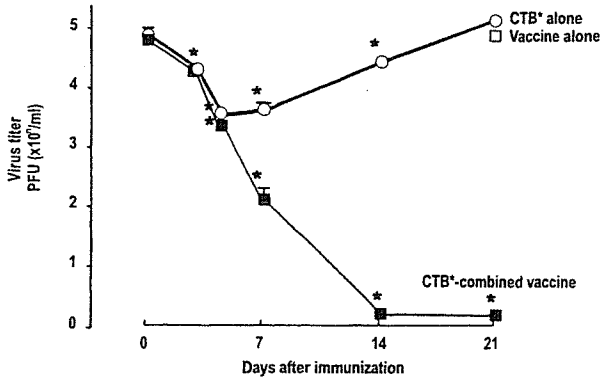

Fig. 2. Non-specific reduction of influenza viral replication by pre-treatment with cholera toxin adjuvant (CTB*). Mice were immunized intranasally with CTB* (1 μg) alone (○), A/PR8 vaccine alone (1 μg) (□) or, CTB*-combined vaccine (■), and then challenged 3, 5, 7, 14 and 21 days later with A/PR8 viruses. Three days after challenge, the virus titer in the nasal wash (PFU/ml) was measured. Each value represents the mean ± SD of the virus titer/ml in the nasal washes of the mice in each group of six mice. The asterisk represents a statistically significant difference between non-immunized and infected mice.

ment of the adaptive immune responses against influenza viruses, which results in complete protection against infection. Similarly, pretreatment of mice with *Propionibacterium acnes* (*Corynebacterium parvum*) or baculovirus before a lethal viral challenge results in lower lung-viral titers and lower mortality (20-22). In these cases, various constituents of the pathogens used for the pretreatment can activate macrophages and dendritic cells (DCs) via members of the Toll-like receptor (TLR) family on these cells to induce protection against infection (23-25). For example, bacterial DNA has stimulatory effects on mammalian immune cells by the presence of unmethylated CpG dinucreotides, which are far more common in bacterial DNA than in vertebrate DNA (26,27). Recognition of CpG DNA by mammalian immune cells is mediated by TLR9, which can distinguish bacterial DNA from self-DNA (23). Intranasal administration of CpG DNA can enhance mucosal Ab responses to co-administered inactivated influenza vaccines (28). Thus, various constituents of the pathogens can be used to enhance innate immune responses via various receptors, such as TLRs and GM1 gangliocide, on macrophages and DCs, so as to induce augmented mucosal immune responses in the respiratory tract (29).

3. Adaptive immunity against influenza

3-1. Roles of APCs in adaptive immunity

APCs (macrophages and DCs) are essential in the induction of the adaptive immune responses (25). Exogenous viral antigens, which comprise inactive viral particles, intact viruses and apoptotic, infected cells, are taken up by APCs through endocytosis and provide a potential source of peptides that could bind to MHC class I or II molecules in the APCs (30-34). In addition, influenza-infected macrophages can also act as APCs (35), and APCs secrete IL-12, which contributes to Th1-type helper cell development (9). APCs also secrete IL-1β, one of the most important cytokines in bridging between the innate and adaptive immune systems (19).

3-2. Roles of T and B cells in adaptive immunity

Virus-specific CD4$^+$ helper T cell precursors, from which Th1-type and Th2-type cells are produced, and CD8$^+$ precursor T cells for CTLs recognize MHC class II- and MHC class I-antigenic peptide complexes on APCs, respectively. These cells are subsequently activated by cytokines produced by APCs (36). Th1 cells secrete IFN-γ and IL-2, and help IgG2a Ab production by Ab-forming cells (AFCs) in mice, while Th2 cells secrete IL-4 and IL-5, and help IgA, IgG1 and IgE Ab production by AFCs (3). Th1 cells also enhance the proliferation of CD8$^+$ CTLs by secreting IL-2 (37,38). The Abs produced during these responses contribute to viral neutralization (NT) by binding with viral antigens (4,30). Th1 cells mediate delayed-type hypersensitivity (DTH) reaction by secreting IFN-γ, which results in the inhibition of virus replication (40-42). In turn, CTLs recognize MHC class I-antigenic peptide complexes on virus-infected epithelial cells and destruct the virus-infected cells mainly by exocytosis of granules containing perforin and granzymes (43-45). IFN-γ secreted by NK and CD8$^+$ T cells seems not to be essential for the target cell lysis (46,47).

4. Mucosal immune system in the respiratory tract

The mucosal immune system can be divided into two sites, inductive sites and effector sites. The inductive sites are mucosal-associated lymphoid tissues (MALT), where initial responses including antigen uptake by APCs and priming of T and B cells for IgA Ab production are induced and the effector sites are the mucosa that covers the internal surface of the whole body, where IgA AFCs are found and where S-IgA Abs play a protective role. Thus, specific IgA AFC precursor populations, induced by antigenic stimulation at one inductive site, migrate not only to the effector site near the original inductive site, but also to other mucosal sites via the homing pathways. This system is collectively referred to as the common mucosal immune system (48-54), and is responsible for the recovery from influenza and for influenza prevention in the respiratory tract.

4-1. Common mucosal immune system

The mucosal immune response in the upper respiratory tract is induced in the nasopharyngeal-associated lymphoid tissues (NALT) in rodents (52,53). The NALT seems to be functionally and anatomically different from Waldeyer's ring, which comprises the nasopharyngeal tonsil (adenoid), the paired tubal tonsils, the paired palantine tonsils and the linguial tonsil in humans (55). Since AFC responses are induced in NALT cell cultures from naïve mice after in vitro culture with influenza virus, it is evident that the NALT comprises inductive tissues from which mucosal AFC precursors originate (56). NALT is also a mucosal inductive site for virus-specific cellular immune responses (57). Exogenous antigens penetrate through highly pinocytotic and phagocytotic M cells present on the NALT and interact with resident T and B cells, resulting in a large number of IgA AFC precursors (47,48,51). The primed T and B cells then leave the NALT and enter the cervical lymph nodes and eventually the general circulation via the thoracic duct. The primed T and B cells then migrate to the lamina propria mucosae of the respiratory tract, intestinal tract, and other sites where IgA AFC precursors differentiate into specific IgA AFCs. Of relevance to this, intranasal immunization is superior to oral immunization in inducing S-IgA Abs in not only the respiratory tract but also the gastrointestinal tract (58). Thus, the NALT appear to be the inductive site that most effectively provides S-IgA Ab in the respiratory tract that is required for protection against

influenza virus infection. In addition, the intranasal route may be the most practical for other vaccines whose protective sites are mucosa, other than the respiratory tract, and that are sensitive to gastrointestinal conditions such as low pH and the presence of proteolytic enzymes (59).

The mucosal immune responses in the lower respiratory tract are induced in the bronchus-associated lymphoid tissue (BALT), which has been well characterized in rats and rabbits, but not in humans and mice (54). Since AFC responses are induced in lung cultures from naïve mice after in vitro culture with influenza viruses, it seems probable that the BALT or its equivalent tissue in mice works as the inductive site in the lung from which mucosal AFC precursors originate (60).

4-2. NALT

The NALT, which is composed of paired lymphoid cell aggregates in the noses of rodents, is the only well-organized MALT in the upper respiratory tract (52,53). It is situated in the mucosa of both lateral walls of the nasal cavity, near the nasal floor on the posterior side of the palate (Fig. 3). It can be isolated easily by peeling away the palate from the upper jaw. It consists of a reticular network filled with various types of lymphoid and non-lymphoid cells. The NALT is not only an inductive tissue from which precursors of mucosal AFCs and CTLs originate (56,57), but also an important site in lymphocyte re-circulation, since NALT lymphocytes migrate back to the NALT and cervical lymph nodes (CLNs) in far greater numbers than cells from Peyer's patches, and, reciprocally, cells from the CLNs migrate back more frequently to the NALT than to Peyer's patches (52).

4-3. Secretion of S-IgA Abs

AFCs that disseminate to the lamina propria underneath the mucosal epithelium via the general circulation cause S-IgA Ab production in the mucosal secretions. The S-IgA Abs are the J-chain-containing dimeric IgA (dIgA) Abs that are produced by IgA AFCs. The dIgA Abs bind to a polymeric Ig receptor (pIgR) on the basolateral surface of the epithelial cells and are carried to the apical surface. They are released as S-IgA Abs by combining with the secretory component (SC), which is the extracellular region of pIgR cleaved by a specific protease (50). Thus, anti-influenza HA-specific polymeric IgA (pIgA) Abs injected intravenously could be transported more efficiently into nasal secretions than monomeric IgA (mIgA) or IgG1 Abs (61). Blocking of the transcytosis of dIgA in pIgR-knockout mice immunized intranasally with an adjuvant-combined influenza vaccine resulted in a marked increase in serum IgA concentration and a decrease in the nasal IgA concentration (62).

5. Recovery from influenza following primary viral infection by adaptive immune responses

5-1. Primary Ab responses

5-1-1. Ab responses in humans experimentally infected with influenza virus

In naïve children experimentally infected with influenza A virus, infection with live, attenuated A viruses induces a high-level anti-HA IgA Ab response with lower levels of IgM and IgG Abs observed in the nasal wash within 2 weeks after infection (63). The nasal wash IgA and IgG Ab titers persist for 1 year in about half of the vaccinated subjects. Approximately 85% of seronegative adult volunteers who are infected with a live attenuated virus developed an IgA Ab response. In addition, there is evidence for active secretion of IgA Abs. In individuals infected with wild-type or attenuated viruses, the nasal wash IgA Abs are mainly polymeric and almost

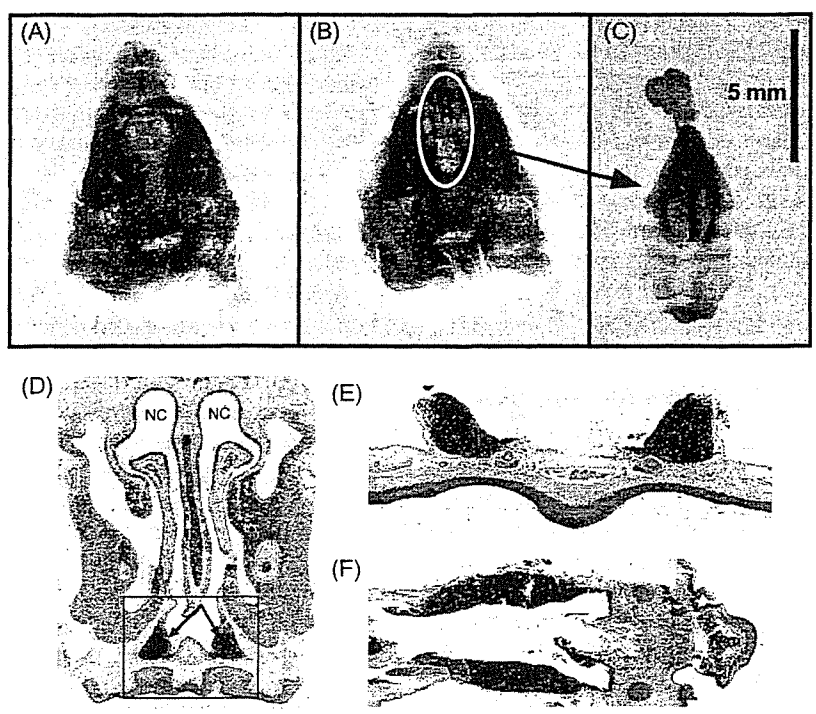

Fig. 3. Isolation method and histology of NALT of BALB/c mice. NALT was isolated from the rest of the nasal tissue by peeling away the palate from the upper jaw (A and B). A palate fragment isolated from the upper jaw, including a pair of NALT on the posterior surface (C). A frontal section of the upper jaw stained by hematoxylin and eosin showing that the NALT are situated in the mucosa of both lateral walls of the nasal cavity, near the nasal floor (D). A cross section of an isolated palate showing that the NALT is found beneath the columnar epithelium of the nasal cavity (E). A horizontal section of an isolated palate showing the longitudinal presence of NALT (F).