the genes that is responsible for the induction of these CD3⁻CD4⁺CD45⁺ inducer cells[30]. Not surprisingly, deletion of the *Id2* gene completely impaired the genesis of all secondary lymphoid tissues, including both NALT and Peyer's patches[5,30]. CD3⁻CD4⁺CD45⁺ inducer cells were shown to accumulate at the site of NALT formation after birth[5] (FIG. 2), thereby clarifying the role of these cells in the induction of NALT development. To directly show that CD3⁻CD4⁺CD45⁺ cells are responsible for the genesis of NALT, fetal liver cells were adoptively transferred from wild-type $Id2^{+/+}$ mice to newborn $Id2^{-/-}$ mice. Seven days after this transfer, CD3⁻CD4⁺ cells were observed to have migrated to the site of NALT formation, and 7 weeks after transfer, a NALT-like structure was detected[5]. These findings are the first to show directly *in vivo* that CD3⁻CD4⁺CD45⁺ inducer cells are essential for the initiation of organogenesis of secondary lymphoid tissues (such as NALT).

The transcriptional regulator ROR-γ has also been shown to be required for the development of CD3⁻CD4⁺CD45⁺ inducer cells[29,32]. Deletion of the gene that encodes ROR-γ suppressed Peyer's-patch and lymph-node organogenesis[29,33]. However, NALT development has been reported in ROR-γ-deficient mice[31]. This might indicate that although NALT and Peyer's patches have inducer cells of the same phenotype — that is, CD3⁻CD4⁺CD45⁺ — those inducer cells can be classified into two distinct groups on the basis of their dependence on ROR-γ and ID2 (FIG. 4). We think that a population of IL-7R-expressing CD3⁻CD4⁺CD45⁺ inducer cells that are essential for Peyer's-patch tissue genesis are regulated by both ROR-γ and ID2, whereas a subset of inducer cells that lack IL-7R expression and are required for NALT genesis are regulated by ID2 but not ROR-γ (FIG. 4) — although this has not been proven experimentally. So, the two CD3⁻CD4⁺CD45⁺ inducer-cell populations for NALT and Peyer's-patch organogenesis might be determined or programmed at the level of the transcriptional regulator ROR-γ (FIG. 4). In addition, it is also possible that a population of CD3⁻CD4⁺CD45⁺ inducer cells is absent in both $Id2^{-/-}$ mice and $Ror\text{-}\gamma^{-/-}$ mice, and instead, another as-yet-undefined cell population — which can substitute for the classical inducer cells during the formation of NALT — is present in $Ror\text{-}\gamma^{-/-}$ mice but not $Id2^{-/-}$ mice. Further studies are required to investigate these possibilities and others.

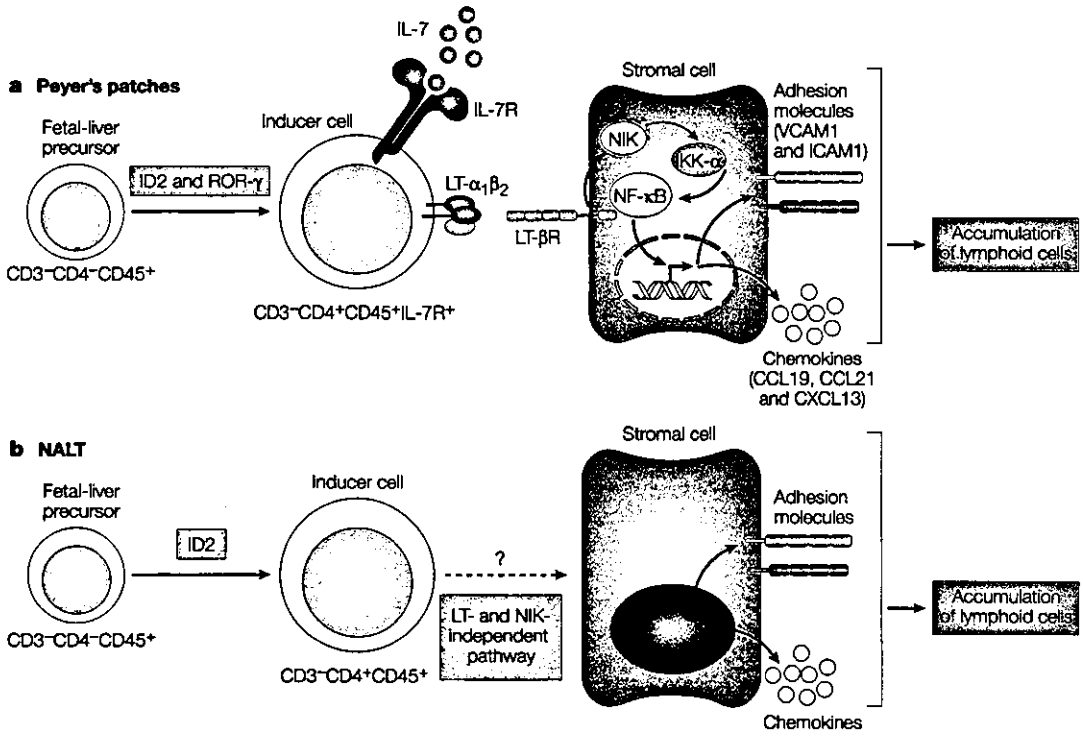


Figure 3 | **Comparison of the organogenesis programme of NALT and Peyer's patches.** CD3⁻CD4⁺CD45⁺ cells are considered to be the common inducers of secondary lymphoid tissue. ID2 (inhibitor of DNA binding 2) is indispensable for the induction and differentiation of these inducer cells from their fetal-liver precursors (which have the phenotype CD3⁻CD4⁻CD45⁺). **a** | For Peyer's patches, after activation through the interleukin-7 receptor (IL-7R) or TRANCE (tumour-necrosis-factor-related activation-induced cytokine), these CD3⁻CD4⁺CD45⁺ cells express the lymphotoxin-$\alpha_1\beta_2$ (LT-$\alpha_1\beta_2$) heterotrimer, which then binds to the LT-β receptor (LT-βR) displayed on stromal cells and induces signal transduction through NIK (nuclear factor-κB (NF-κB)-inducing kinase). In turn, NIK promotes the expression of adhesion molecules and/or chemokines. These homing molecules trigger the accumulation of lymphoid cells at the site of Peyer's patches. So, the IL-7R- and LT-βR-mediated signals are essential for the tissue genesis of Peyer's patches. **b** | The development of CD3⁻CD4⁺CD45⁺ cells in nasopharynx-associated lymphoid tissue (NALT) also requires ID2; however, the initiation of NALT organogenesis is independent of signalling that involves the IL-7R, LT-$\alpha_1\beta_2$–LT-βR interactions and NIK. CCL, CC-chemokine ligand; CXCL, CXC-chemokine ligand; ICAM1, intercellular adhesion molecule 1; IKK-α, inhibitor of NF-κB (IκB) kinase-α; ROR-γ, retinoic-acid-receptor-related orphan receptor-γ; VCAM1, vascular cell-adhesion molecule 1.

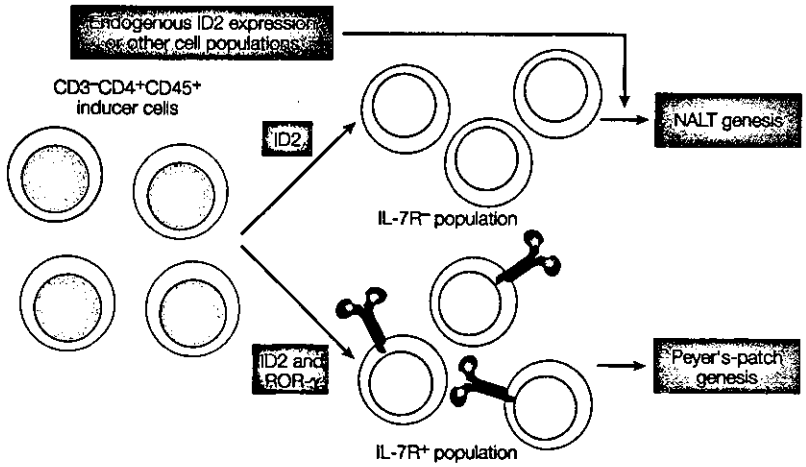


Figure 4 | **Model for the induction of organogenesis of NALT and Peyer's patches by two subsets of CD3⁻CD4⁺CD45⁺ inducer cells.** CD3⁻CD4⁺CD45⁺ cells differentiate from fetal-liver progenitors. We propose that both ROR-γ (retinoic-acid-receptor-related orphan receptor-γ) and ID2 (inhibitor of DNA binding 2) are essential for the generation of interleukin-7 receptor (IL-7R)-expressing CD3⁻CD4⁺CD45⁺ cells for the induction of Peyer's-patch organogenesis. By contrast, the generation of the IL-7R⁻CD3⁻CD4⁺CD45⁺ inducer cells that are involved in nasopharynx-associated lymphoid tissue (NALT) organogenesis is regulated by ID2 but not ROR-γ. However, this model remains to be tested experimentally, and other possibilities exist (see main text). NALT genesis in ID2-deficient mice can be initiated by the adoptive transfer of CD3⁻CD4⁺CD45⁺ cells from wild-type mice, but the maturation of NALT formation is incomplete. This indicates that other cell populations, or the endogenous expression of ID2 at the site of NALT, might be required for the full maturation of NALT.

WALDEYER'S RING
Human nasopharynx-associated lymphoid tissues, including the palatine tonsils and adenoids, which are considered to have an important role in the induction and modulation of mucosal immunity in the upper respiratory tract.

MIDDLE CONCHA
Bony plate that extends from the central section of the lateral wall of the nasal cavity.

$T_H0$ CELLS
Precursors of T helper 1 ($T_H1$) cells and $T_H2$ cells, which produce both interferon-γ and interleukin-4. This T-cell population has the capacity to become $T_H1$- and/or $T_H2$ cells.

CLASS-SWITCH RECOMBINATION
Molecular alteration of the constant-region gene of the immunoglobulin heavy chain ($C_H$) that leads to a switch in expression from the Cμ (or Cδ) region to one of the other $C_H$ genes. This leads to a switch in the class of the immunoglobulin that is displayed on the cell-surface of the B cell (and that subsequently differentiating plasma cells produce) — from IgM (or IgD) to IgG, IgA or IgE — without altering the specificity of the immunoglobulin.

## Immunological features of NALT

In rodents, NALT is found on both sides of the nasopharyngeal duct, dorsal to the cartilaginous soft palate, and it is considered analogous to WALDEYER'S RING in humans[34,35]. Also, in a recent study, a NALT-like structure of lymphocyte aggregates that form follicles was identified in human nasal mucosa, particularly in the MIDDLE CONCHA of children less than two years of age[36], indicating that an equivalent to mouse NALT can develop in humans. NALT consists of follicle-associated epithelium (FAE), HEVs, and T-cell- and B-cell-enriched areas. Antigen-sampling M cells are present in the epithelium of NALT, which is specialized for antigen uptake similar to the FAE of Peyer's patches[7,37]. Antigen-presenting cells, including dendritic cells (DCs) and macrophages, are also found in NALT[38]. So, NALT contains all of the lymphoid cells that are required for the induction and regulation of mucosal immune responses to antigens that are delivered to the nasal cavity. For example, the intranasal administration of reovirus resulted in the formation of germinal centres in NALT, leading to the clonal expansion of antigen-induced IgA⁺ B cells and the subsequent generation of reovirus-specific IgA in the respiratory and intestinal tracts[39]. Moreover, reovirus-specific CTLs were also induced in NALT with a high frequency. These findings show that NALT can be a potent inductive site for the mucosal immune system. In addition to the induction of positive immune responses, the nasal deposition of antigen has been shown to be effective for the induction of systemic unresponsiveness — a form of mucosally induced tolerance[40]. So, NALT has been shown to be involved in the generation of positive- and negative-regulatory signals for the induction of antigen-specific immunity and tolerance respectively. The cellular and molecular contributions of the immunocompetent cells present in NALT to the generation of tolerance to mucosally exposed antigens are unknown. Because little is known about the induction of nasally induced tolerance, we focus here on the role of NALT in the induction of protective immunity.

***$T_H0$ environment.*** Characterization of the mRNA that encodes $T_H1$ and $T_H2$ cytokines in CD4⁺ T cells isolated from mouse NALT revealed a dominant cytokine profile of $T_H0$ CELLS, indicating that these T cells are capable of becoming $T_H1$ or $T_H2$ cells immediately after antigen exposure of the nasal tract[41–43]. CD4⁺ T cells isolated from NALT of naive wild-type mice are $T_H0$ cells[42], so they can become either $T_H1$ or $T_H2$ cells depending on the identity of the nasally administered antigen. Nasal delivery of protein antigens (such as bacterial cell-wall components or virus-associated antigens) together with cholera toxin as a mucosal adjuvant induces antigen-specific $T_H2$-type responses that promote the generation of antigen-specific IgA-producing B cells, both in the nasal passages and at distant mucosal effector sites, including the genito-urinary, respiratory and intestinal tracts[41,44,45]. By contrast, intranasal vaccination with antigen-expressing recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (rBCG) results in $T_H1$-cell-mediated immunity[43].

***IgA class switching.*** Peyer's patches have long been thought to be the sites for the initiation of CLASS-SWITCH RECOMBINATION (CSR) of μ- to α-gene expression in the gastrointestinal tract, because they contain all of the cellular and microarchitectural elements that are required for the generation of IgA-committed B cells, including germinal-centre-containing B-cell follicles, a FOLLICULAR DC network and an interfollicular T-cell area[1,46,47]. The germinal-centre region contains a high frequency of IgM⁺B220⁺ B cells that express activation-induced cytidine deaminase (AID), which is essential for μ-to α-gene conversion[48]. In an early study, it was shown that incubation of IgM⁺IgA⁻ B cells isolated from Peyer's patches in the presence of the cytokine transforming growth factor-β (TGF-β) resulted in the generation of IgM⁻IgA⁺ B cells[49–51]. These post-switch IgA-committed B cells then migrated to mucosal effector tissues (such as the intestinal lamina propria), a process mediated by a group of homing and chemokine receptors and their ligands (such as MADCAM1–$\alpha_4\beta_7$-integrin and CCL25–CCR9 interactions)[52,53] (discussed later). In the intestinal lamina propria, these cells became IgA⁺ plasma cells in the presence of the IgA-enhancing cytokines IL-5, IL-6 and IL-10 (REFS 50,54–58). So, it was generally accepted that organized lymphoid structures of MALT, such as Peyer's patches, function as the inductive sites for generating IgA-committed B cells through μ- to α-gene CSR, whereas the diffuse tissues of the intestinal lamina propria function as effector sites for the production of IgA[1,2] (FIG. 1). However, the finding that IgA class switching can occur in the intestinal lamina propria without involvement of

the Peyer's patches[59] cast these assumptions into doubt. Stromal-cell-derived TGF-β present in the intestinal lamina propria was shown to trigger IgM⁺B220⁺ cells to switch to IgA⁺ B cells[59]. Mice with a deficiency in the programmed-inflammation-associated cytokine LT-α do not form Peyer's patches, so the levels of IgA responses are reduced compared with those of wild-type mice[60]. However, reconstitution of *Lt-α*$^{-/-}$ mice with LT-expressing bone-marrow cells, or transplantation of an intestinal segment from recombination-activating gene (*Rag*)$^{-/-}$ mice to *Lt-α*$^{-/-}$ mice, resulted in the recovery of IgA responses[60]. These findings imply that at least some IgA-committed B cells can develop, even in the absence of Peyer's patches. *Lt-α*$^{-/-}$ mice have also been shown to be capable of inducing antigen-specific IgA responses to orally administered *Salmonella typhimurium*, despite the absence of organized lymphoid tissues associated with the mucosal compartment[61]. Although these findings provide supporting evidence that the environment of the diffuse lamina propria region is self-sufficient for the μ- to α-gene CSR that leads to the generation of IgA-committed B cells, it is also possible that unidentified, programmed-inflammation-independent miniature lymphoid aggregates, and recently characterized isolated lymphoid follicles[62], have a role in mounting IgA responses in cases of Peyer's-patch deficiency.

The finding that IgA-specific CSR can occur in diffuse mucosal effector tissues indicates that organized mucosal tissue is not essential for the generation of IgA-committed B cells in the digestive tract[59], although this new view is still controversial[63,64]. Because it has been shown that intestinal IgA is produced by two groups of cells, B1 cells and B2 CELLS[63,65,66], an interesting possibility is that IgA-specific CSR of B1 cells does not require organized lymphoid structures, whereas these structures are essential for IgA-isotype switching in B2 cells. In support of this view, we have shown that most B cells in gut-associated lymphoid tissues are B2 cells, whereas B1 cells are located preferentially in the intestinal lamina-propria region[66]. So, we think that the initial antigenic stimulation for the triggering of IgA-isotype switching in B2 cells might be provided by antigen sampling through M cells that are located in the dome epithelium (or FAE) of Peyer's patches. By contrast, the IgA-specific CSR process that occurs for B1 cells might be triggered by antigens sampled through newly identified villous M cells that are located adjacent to lamina-propria regions that do not contain observable lymphoid-like structures[67].

Because NALT is of similar importance for the initiation of IgA⁺ B-cell responses as Peyer's patches, we have investigated whether IgA-isotype switching can also occur in the diffuse tissue of the nasal passage. IgM⁺B220⁺ B cells, which are a prerequisite for CSR, were found in the organized inductive sites (NALT) but were mostly absent from the diffuse effector tissues (nasal passage) of the respiratory mucosal immune system[68]. Similarly, IgM⁺B220⁺ B cells were observed in the organized Peyer's patches of the intestinal tract but not in the intestinal lamina propria. So, in this study, IgM⁺B220⁺ B cells that are preconditioned to undergo IgA class switching are selectively located in the organized mucosa-associated inductive tissues of NALT and Peyer's patches[68]. This finding was confirmed by the molecular analysis of IgA CSR-associated mRNA specific for AID, Iα-Cμ CIRCULAR TRANSCRIPTS and Iμ-Cα TRANSCRIPTS. Because the expression of AID and the Iα-Cμ circular transcript are upregulated preferentially during μ- to α-gene conversion and then quickly down-regulated, these molecular events are considered to be a hallmark of B cells that are undergoing IgA class switching[69]. The expression of Iμ-Cα transcripts indicates the completion of IgA-specific CSR[59]. This analysis showed that the expression of AID-, Iα-Cμ circular transcript- and Iμ-Cα transcript-specific mRNA was restricted to the organized mucosal inductive tissues of NALT and Peyer's patches but was not found in the diffuse effector tissues of the nasal passage and intestinal lamina propria[68]. Furthermore, these organized mucosal lymphoid tissues are known to be associated with B2 cells[66]. So, these findings indicate that the IgA class switching, at least for B2 cells, requires the organized lymphoid structures of NALT and Peyer's patches in the aero-digestive tract. NALT was also recently shown to be an important site for the generation of memory B cells, which produce high-affinity IgA[70]. Taken together, these findings show that NALT contains all of the immunocompetent cells that are required for the induction and regulation of antigen-specific $T_H1$- or $T_H2$-cell-mediated responses and B-cell immune responses.

***Differences between NALT- and Peyer's-patch-initiated immune responses.*** NALT and Peyer's patches are thought to have similar immunological characteristics and biological functions, as well as to contain the same types of resident immunocompetent cell. So, similar to oral immunization, nasal immunization can stimulate antigen-specific $T_H1$- or $T_H2$-cell-mediated responses and IgA responses in distant mucosal effector tissues[1,2,41,43–45]. However, in general, NALT-targeted immunization effectively induces antigen-specific immunity in the respiratory and reproductive tissues, whereas Peyer's-patch-targeted immunization promotes the generation of protective immunity in the gastrointestinal-tract tissues[1,2]. Further support for a compartmentalized CMIS was provided recently when it was shown that nasal immunization induces the expression of high levels of CCR10 and $\alpha_4\beta_1$-integrin by IgA-committed B cells, allowing them to efficiently traffic to the respiratory and genito-urinary tracts, which express the corresponding ligands, CCL28 and VCAM1 (REFS 71,72). By contrast, orally induced IgA-committed B cells express CCR9 and CCR10, as well as $\alpha_4\beta_7$- and $\alpha_4\beta_1$-integrins, so they migrate to sites such as the small intestine, which express CCL25 and/or CCL28 together with MADCAM1 and/or VCAM1 (REF. 73).

So, despite NALT and Peyer's patches both belonging to the mucosal immune system, the subtle differences that we have discussed indicate that the tissue genesis and biological functions of NALT and Peyer's patches might differ because of their anatomically and

FOLLICULAR DC (FDC). Cell with a dendritic morphology that is present in lymph nodes. These cells display on their surface intact antigens that are held in immune complexes, and B cells present in the lymph node can interact with these antigens. FDCs are of non-haematopoietic origin and are not related to dendritic cells.

B2 CELLS IgM$^{low}$IgD$^{hi}$MAC1$^{-}$B220$^{hi}$CD23$^{+}$ cells that originate from bone marrow and are distributed to mucosal and systemic immune compartments for the continuous secretion of antibodies with high affinity and fine specificity.

Iα-Cμ CIRCULAR TRANSCRIPTS Circular DNA molecules that are present in activated B cells and consist of Iα (intervening region-α) and Cμ genes. They are a hallmark of B cells that are in the process of IgA class switching.

Iμ-Cα TRANSCRIPTS Germline transcripts that can be detected in IgA-committed B cells after class switching.

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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## Acknowledgements

Our work is supported by grants from the Core Research for Evolutional Science and Technology of Japan Science and Technology Corporation, the Ministry of Education, Science, Sports and Culture of Japan, the Ministry of Health, Labor, and Welfare of Japan, and the Uehara Memorial Foundation (Japan). We express appreciation of our present and former colleagues at the University of Tokyo, (Tokyo, Japan), Osaka University (Osaka, Japan) and the University of Alabama at Birmingham (United States) for their help and contributions to the generation of the results summarized in this review. Finally, our appreciation extends to K. K. McGhee and J. R. McGhee for editorial help and careful reading of the manuscript, respectively.


## Competing interests statement

The authors declare no competing financial interests.

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INFECTION AND IMMUNITY, Apr. 2004, p. 2240–2247 Vol. 72, No. 4
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.4.2240–2247.2004


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

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Received 11 August 2003/Returned for modification 14 October 2003/Accepted 17 December 2003

**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 $5 \times 10^4$ 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 $5 \times 10^3$ 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

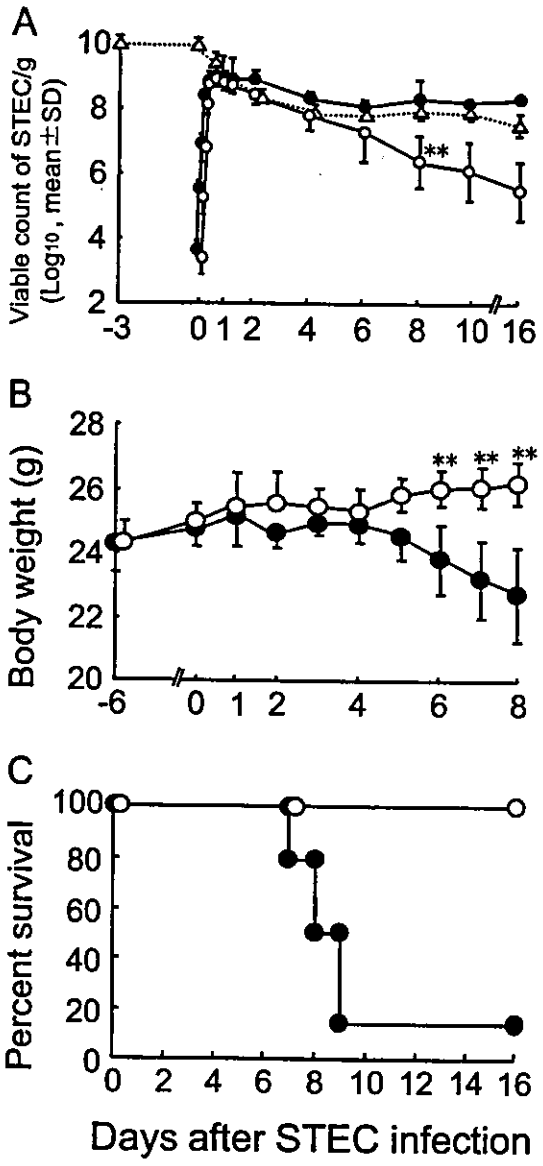


FIG. 1. Inhibition of lethal intestinal STEC infection by *B. breve* colonization in SM-treated mice. SM sulfate at a concentration of 5 mg/ml in drinking water was given to 28 mice from day −6 until day 16. *B. breve* strain Yakult ($1 \times 10^8$ to $3 \times 10^8$ CFU/mouse/day) in 0.1 ml of saline 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 ($5 \times 10^3$ 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 ($P < 0.01$).

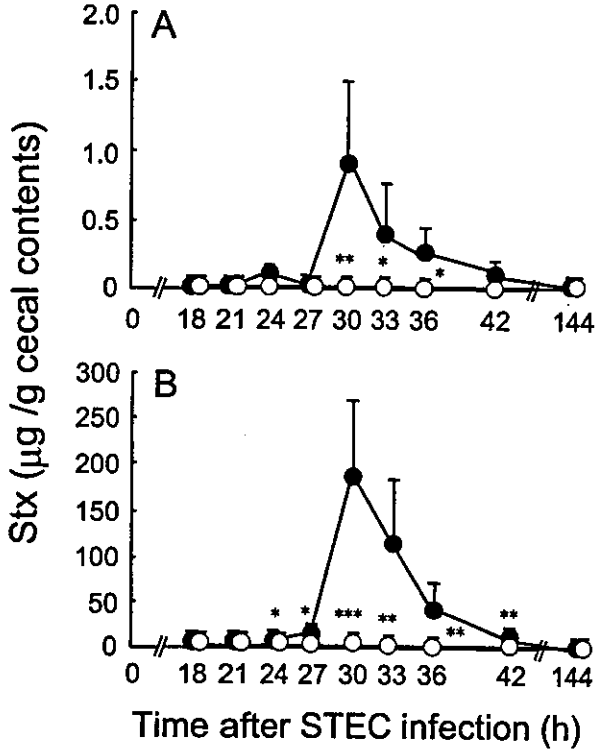


FIG. 2. Inhibition of MMC-induced production of Shiga toxins by *B. breve* colonization. Mice were infected with STEC and treated with MMC as described in the legend to Fig. 1 and then dissected at the indicated periods after STEC infection to examine Stx production. The concentrations of Stx1 (A) and 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)

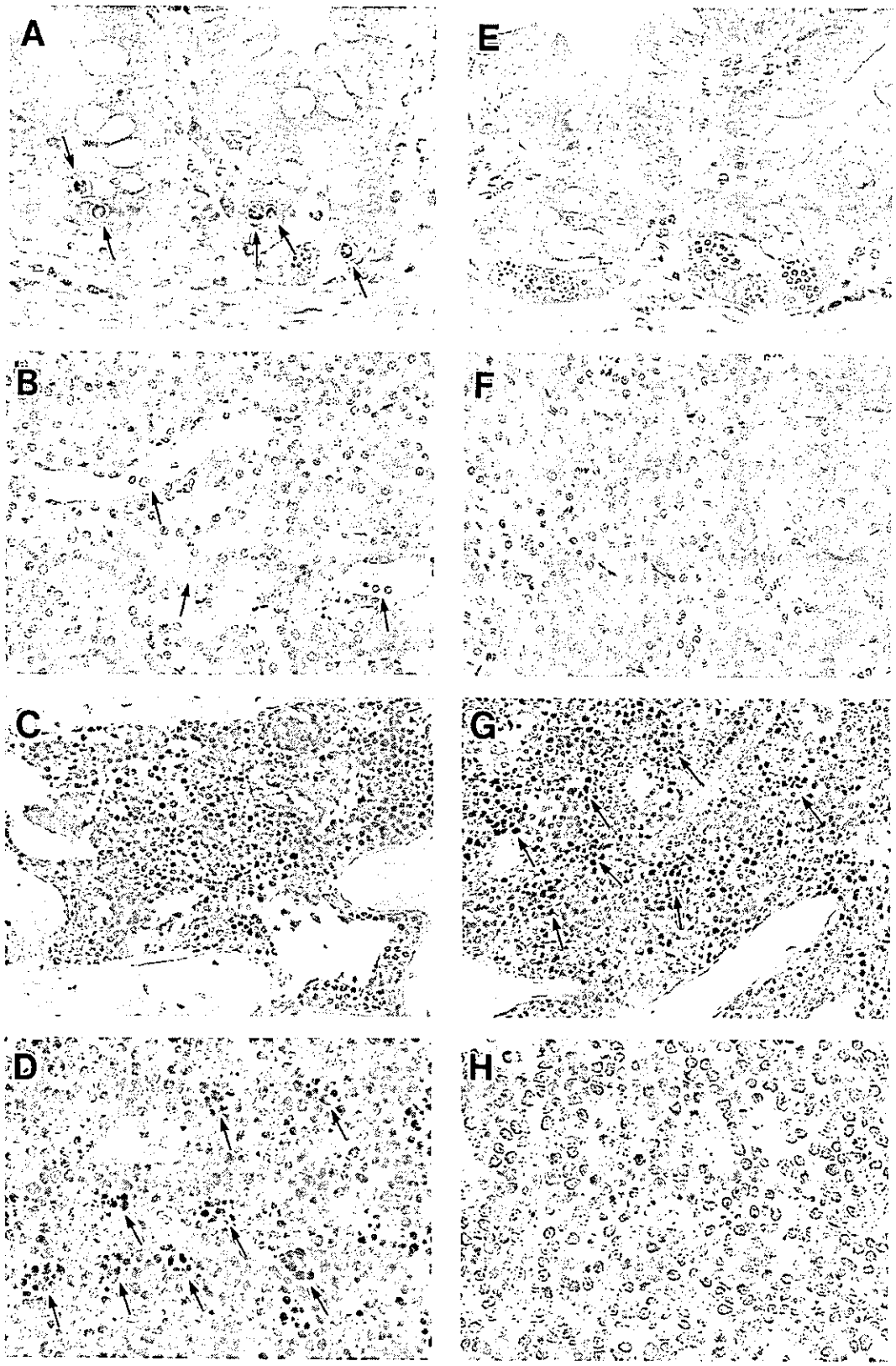


FIG. 3. Histopathological analysis. Hematoxylin and eosin staining of the ileum (A and E), kidney (B and F), bone marrow (C and G), and mesenteric lymph node (D and H) from a mouse in the STEC-infected control group (STEC inoculum, $3.8 \times 10^3$ CFU) (A to D) and a mouse in the *B. breve*-treated 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 ($1 \times 10^8$ to $4 \times 10^8$ 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 $8.1 \times 10^3$ 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

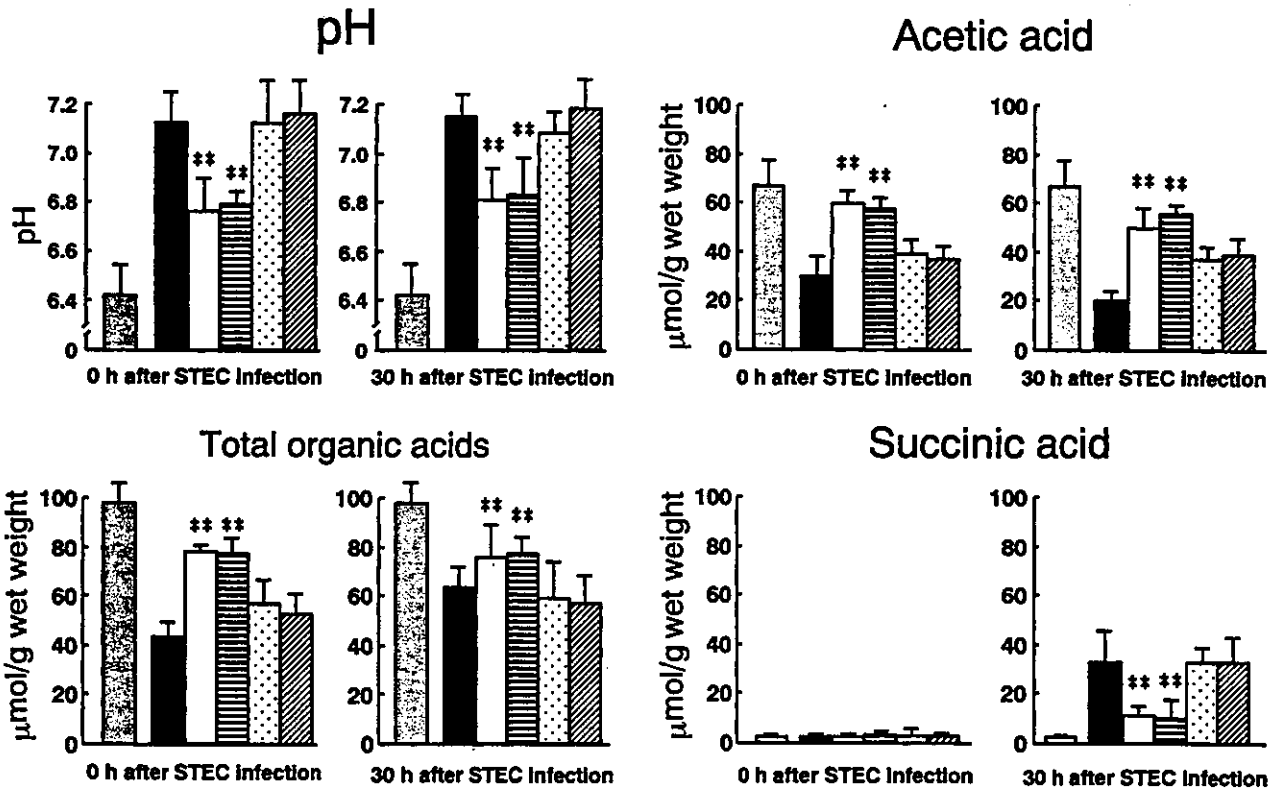


FIG. 4. Changes in intestinal pH and concentrations of organic acids after STEC infection in SM-treated mice. Mice were treated as shown in Table 2. Cecal contents were obtained from mice both at the time of STEC infection (0 h) and 30 h after STEC infection. pH and organic acid concentrations were determined 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 ($P < 0.01$).

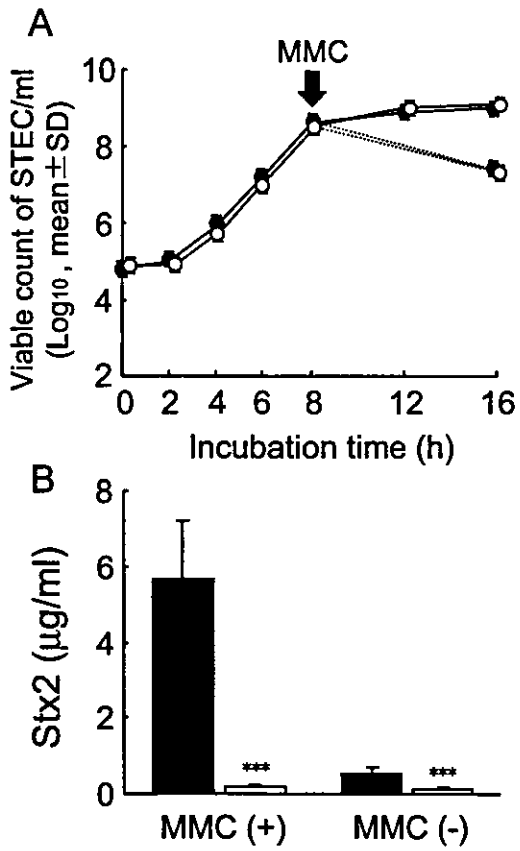


FIG. 5. Inhibition of Stx production but not STEC proliferation at higher AA concentrations and lower pH values in vitro. The pH and concentration of AA were adjusted in the growth medium so that the conditions were the same as those in the control cecum (●) (pH 7.15; AA concentration, 28 mM) or the *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.

## ACKNOWLEDGMENTS

We thank Kazumi Uchida and Kana Hashimoto for performing the histopathological analyses.

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*Editor:* A. D. O'Brien

JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2004, p. 5854–5856 Vol. 42, No. 12
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.12.5854–5856.2004


# 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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Received 3 May 2004/Returned for modification 28 June 2004/Accepted 30 August 2004

**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.

This research was supported by funds from the United States Japan Cooperative Medical Science Program, Asian Region Collaboration

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) |

Research Project 2002, and other core donors of the Centre who share its concern for the health problems of developing countries.

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Jpn. J. Infect. Dis., 57, 236-247, 2004

**Review**

# Defense Mechanisms against Influenza Virus Infection in the Respiratory Tract Mucosa

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(Received June 16, 2004. Accepted September 17, 2004)

**CONTENTS:**



**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-$\gamma$ 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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