INFECTION AND IMMUNITY, Jan. 2006, p. 694–702 Vol. 74, No. 1
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.1.694–702.2006

A Bacterial Flagellin, *Vibrio vulnificus* FlaB, Has a Strong Mucosal Adjuvant Activity To Induce Protective Immunity

Shee Eun Lee,[1,2] Soo Young Kim,[1,3,5] Byung Chul Jeong,[2] Young Ran Kim,[1,3] Soo Jang Bae,[1,3] Ouk Seon Ahn,[1] Je-Jung Lee,[4] Ho-Chun Song,[5] Jung Mogg Kim,[6] Hyon E. Choy,[1] Sun Sik Chung,[1] Mi-Na Kweon,[7] and Joon Haeng Rhee[1,3]*

Research Institute of Vibrio Infection and Genome Research Center for Enteropathogenic Bacteria[1] and National Research Laboratory of Molecular Microbial Pathogenesis and Department of Microbiology,[3] Chonnam National University Medical School, Gwangju 501-746, South Korea; Department of Dental Pharmacology, Chonnam Dental Research Institute, College of Dentistry, Chonnam National University, Gwangju 500-757, South Korea[2]; Departments of Internal Medicine[4] and Nuclear Medicine,[5] Chonnam National University Hwasun Hospital, 160 Ilsim-ri, Hwasoon, Chonnam 519-809, South Korea; Department of Microbiology, Hanyang University College of Medicine, Seoul 133-791, South Korea[6]; and Mucosal Immunology Section, International Vaccine Institute, Seoul 151-818, South Korea[7]

Received 2 November 2004/Returned for modification 30 December 2004/Accepted 5 October 2005

Flagellin, the structural component of flagellar filament in various locomotive bacteria, is the ligand for Toll-like receptor 5 (TLR5) of host cells. TLR stimulation by various pathogen-associated molecular patterns leads to activation of innate and subsequent adaptive immune responses. Therefore, TLR ligands are considered attractive adjuvant candidates in vaccine development. In this study, we show the highly potent mucosal adjuvant activity of a *Vibrio vulnificus* major flagellin (FlaB). Using an intranasal immunization mouse model, we observed that coadministration of the flagellin with tetanus toxoid (TT) induced significantly enhanced TT-specific immunoglobulin A (IgA) responses in both mucosal and systemic compartments and IgG responses in the systemic compartment. The mice immunized with TT plus FlaB were completely protected from systemic challenge with a 200× minimum lethal dose of tetanus toxin. Radiolabeled FlaB administered into the nasal cavity readily reached the cervical lymph nodes and systemic circulation. FlaB bound directly to human TLR5 expressed on cultured epithelial cells and consequently induced NF-κB and interleukin-8 activation. Intranasally administered FlaB colocalized with CD11c as patches in putative dendritic cells and caused an increase in the number of TLR5-expressing cells in cervical lymph nodes. These results indicate that flagellin would serve as an efficacious mucosal adjuvant inducing protective immune responses through TLR5 activation.

The mucosal immune response serves as the first line of defense against many bacterial and viral infections. Many efforts have been focused on developing effective mucosal vaccines. Mucosal vaccines have many advantages; multivalent vaccines can easily be administered via mucosal and oral administration. However, most protein antigens administered mucosally are, in general, less immunogenic than systemically vaccinated ones (39). The development of safe and effective adjuvants is a prerequisite to the practical use of mucosal vaccines (12, 16, 39). Cholera toxin (CT), heat-labile toxin of *Escherichia coli* (LT), and genetically manipulated nontoxic chimeric enterotoxins showed powerful adjuvant activities triggering significant antibody responses to coadministered antigens in animal models (4, 20, 38). However, to date, we do not have any commercially available mucosal adjuvants that have been proved to be safe and efficacious.

Vibrio vulnificus causes a fatal septicemia. *V. vulnificus*, taken orally with contaminated shellfish and reaching the intestine, invades the bloodstream across the intestinal mucosal barrier of the host (21). Recently, we found that mutants defective in motility were less adherent and less cytotoxic to cultured cells (19). The motility of bacterial cells is supposed to be important for pathogenic enteric bacteria in penetrating mucosa and gaining access to the underlying epithelial cells. *V. vulnificus* has a single polar flagellum. Flagellin is the structural component of flagellar filament and a self-assembling protein subunit arranged in a helix to form a hollow tube (31). *V. vulnificus* has a total of six flagellin structural genes (*flaA*, *flaB*, *flaF*, *flaC*, *flaD*, and *flaE*). The six genes are organized into two distinct genetic loci with three genes per locus (NCBI microbial genome database [http://www.ncbi.nlm.nih.gov/Genomes/lproks.cgi]). Among the six flagellins, FlaB appeared to be the most crucial building block of the flagellar shaft (unpublished data). During the experiment, we encountered some experimental results, suggesting mucosal adjuvant activity of FlaB. Briefly, immunization with FlaB protected mice only against oral, not subcutaneous or intraperitoneal, challenges and stimulated an immunoglobulin A (IgA) response.

In the present study, we investigated the adjuvant activity of FlaB to mucosally administered tetanus toxoid (TT) by using a mouse model. To disclose the underlying molecular immunological mechanism, we tested whether FlaB specifically interacts with Toll-like receptor 5 (TLR5) expressed on host cells and stimulates NF-κB-dependent responses. TLRs have been demonstrated to recognize conserved pathogen-associated molecular patterns (PAMPs). They play crucial roles in innate immunity and can contribute to the development of appropriate adaptive immune responses (2, 32). TLRs receive special

* Corresponding author. Mailing address: National Research Laboratory of Molecular Microbial Pathogenesis and Department of Microbiology, Chonnam National University Medical School, 5 Hak-Dong, Dong-Ku, Gwangju 501-746, South Korea. Phone: 82-62-220-4136. Fax: 82-62-228-7294. E-mail: jhrhee@chonnam.chonnam.ac.kr.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
E. coli strains		
ER2566	F$^-$ λ$^-$ *fhuA2* [*lon*] *ompT lacZ*::*T7 gene1 gal sulA11* Δ(*mcrC-mrr*)*114*::IS*10* R(*mcr-73*::miniTn*10*-TetS)*2* R(*zgb-210*::Tn*10*)(TetS) *endA1* [*dcm*]	New England Biolabs, Inc.
DH5α	F$^-$ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ$^-$ *thi-1 gryA96 relA1*	Laboratory Collection
Plasmids		
pGEM7Zf	Cloning vector; Ap^r	Promega
pTYB12	N-terminal fusion expression vector in which the N terminus of a target protein is a fused intein tag; Ap^r	New England Biolabs, Inc.
pCMM255	2.2-kb EcoRI fragment containing *flaB* and part of *flaA* cloned into pGEM7Zf	This study
pCMM250	1.5-kb EcoRI-PstI fragment containing ORF of *flaB* cloned into pTYB12	This study

[a] Ap^r, ampicillin resistance.

attention as potent adjuvant receptors (1). Mammalian TLR5, expressed on epithelial cells and phagocytes such as dendritic cells (DCs) and macrophages, recognizes flagellins of both gram-negative and gram-positive bacteria and subsequently activates the NF-κB pathway of host cells (14, 27, 30, 35).

MATERIALS AND METHODS

Cloning of the *flaB* gene from a *V. vulnificus* library and purification of recombinant FlaB. *Escherichia coli* strains and plasmids used in the present study are listed in Table 1. A genomic cosmid library of *V. vulnificus* type strain ATCC 29307 was constructed by using the pLAFR3 vector as described earlier (18). A 2.2-kb EcoRI fragment containing the open reading frame (ORF) of *flaB* and part of *flaA* was excised from a cosmid genomic DNA library clone and ligated into pGEM7, yielding pCMM255. A 1.5-kb EcoRI-PstI fragment containing the *flaB* ORF was ligated into pTYB12 (New England Biolabs, Inc., Beverly, MA), yielding pCMM250. The pCMM250 plasmid was transformed into *E. coli* ER2566 (New England Biolabs, Beverly, MA) by electroporation. An intein-FlaB fusion protein was induced by 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside). To prepare a bacterial lysate for affinity column chromatography, the pellet was resuspended in a lysis buffer (20 mM Tris-Cl [pH 7.5], 500 mM NaCl, 1 mM EDTA [pH 8.0], 0.1% Triton X-100, 0.1% Tween 20, 20 μM phenylmethylsulfonyl fluoride) and sonicated (Vibra Cell VCX500; Sonics & Materials, Inc., Newtown, CT) on an ice bed. After sonication, recombinant tag-free FlaB was purified by using a chitin column and 50 mM 1,4-dithiothreitol solution in accordance with the manufacturer's protocol, and the purity of the recombinant FlaB was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with the rabbit anti-FlaB antibody elicited by glutathione *S*-transferase (GST)–FlaB. Contaminating lipopolysaccharide (LPS) was removed from the recombinant protein by using the AffinityPak Detoxi Gel Endotoxin Removing gel (Pierce Biotechnology, Inc., Rockford, IL), and the residual LPS content of the protein was determined by using the gel-clotting Endosafe LAL kit (Charles River Endosafe, Charleston, SC). The LPS levels in flagellin preparation were 0.48 EU/ml of resulting 0.0096 EU per dose.

Mice immunization and sample preparation. All vaccination experiments were performed in the specific-pathogen-free facilities at the College of Dentistry, Chonnam National University. Seven-week-old female BALB/c mice were intranasally immunized three times with 20 μl of phosphate-buffered saline (PBS) containing 3 μg of TT manufactured for vaccine application (kindly provided by Yasushi Higashi, Osaka University, Biken Foundation, Osaka, Japan) alone or in combination with FlaB under anesthesia (intraperitoneal injection of 100 μl of PBS containing 2 mg of ketamine and 0.2 mg of xylazine) at 7-day intervals. Seven days after the last immunization, saliva, vaginal wash, and serum samples were collected from the immunized mice to assess TT-specific antibody responses. All animal procedures were conducted in accordance with the guidelines of the Animal Care and Use Committee of Chonnam National University.

Tetanus toxin challenge. In order to assess the protective immune responses against a lethal challenge, a 200× minimal lethal dose of tetanus toxin (kindly provided by Yasushi Higashi, Osaka University) was diluted in 0.2% gelatin PBS and administered subcutaneously to the test mice. The mice were observed for 7 days and monitored for paralysis and death.

Assay of antigen-specific immune responses by ELISA. TT-specific antibody titer was determined by enzyme-linked immunosorbent assay (ELISA). To measure the antibody responses, flat-bottom 96-well ELISA plates (Corning Laboratories, Corning, NY) were coated with 50 μl of 2 μg of TT (Dongshin Pharmaceuticals, Seoul, Korea)/ml per well and incubated at 4°C overnight. Plates were then incubated with a blocking buffer (0.05% Tween 20, 1 mM EDTA, and 0.5% bovine serum albumin in PBS) for 2 h, and 50 μl of serum or mucosal samples was subsequently applied as a twofold dilution series in the blocking buffer. After a wash with distilled water, each well was coated with 50 μl of anti-mouse immunoglobulin rabbit antibody-horseradish peroxidase conjugates (Sigma) diluted with the blocking buffer, and the plates were incubated for 1 h at 37°C. Color was developed with 50 μl of the substrate TMB (3,3′,5,5′-tetramethylbenzidine) solution (Sigma). The reaction was stopped by the addition of 50 μl of 1 N H_2SO_4 (Sigma). Absorbance was read by a microplate reader (Molecular Devices Corp., Menlo Park, CA) at 450 nm. The titers represented the reciprocal of the dilution that yielded an optical density at 450 nm of 0.1.

IL-8 ELISA. Caco-2 cells were maintained in the Dulbecco modified Eagle medium (Invitrogen/Life Technologies) supplemented with 10% fetal calf serum (Invitrogen/Life Technologies). The cells were seeded at 2×10^5/well in 24-well plates and were treated with different concentrations of FlaB for 5 h without fetal calf serum supplementation. Interleukin-8 (IL-8) in the supernatant was measured by an ELISA kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions.

Real-time RT-PCR analysis for IL-8. IL-8 expression in the Caco-2 cells treated with FlaB was analyzed by the real-time reverse transcription-PCR (RT-PCR) analysis. Total RNA was isolated from the FlaB-treated cells and reverse transcribed into cDNA by using random primers (Promega, Madison, WI). Gene expression levels were compared using the SYBR green dye and the LightCycler system (Roche Applied Science, Sandhofer Strasse, Germany). The primers used for PCR were as follows: IL-8 forward, 5′-ACT TCC AAG CTG GCC GT-3′; IL-8 reverse, 5′-CAG CCC TCT TCA AAA ACT TCT C-3′; β-actin forward, 5′-GAG ATG GCC ACG GCT GCT-3′; and β-actin reverse, 5′-TTC TGC ATC CTG TCG GCA-3′. Relative mRNA quantities in the FlaB-stimulated cells were calculated as described elsewhere (34).

Transient-transfection and luciferase reporter assays. Caco-2 cells seeded at 2×10^5/well in 24-well plates were transfected with the appropriate amount of expression plasmids, the reporter pIL-8-Luc or pNF-κB-Luc (17) and p3XFlag-hTLR5 (27) using Fugene 6 (Roche). The levels of luciferase activity were normalized to the *lacZ* expression levels using the control expression plasmid pCMV-β-Gal (BD Biosciences Clontech, Palo Alto, CA). Total amounts of expression vectors were kept constant by adding appropriate amounts of blank vector. At 24 h after transfection, the culture was replaced with the fresh medium containing different concentrations of FlaB and incubated for 24 h. Cells were treated with a lysis buffer (Promega) and the luciferase activity was assayed by a luminometer (MicroLumatPlus LB 96V; Berthold, Wilbad, Germany).

Coimmunoprecipitation. A coimmunoprecipitation assay was performed by using Caco-2 cells transfected with p3XFlag-hTLR5. The transfected cells were cultured in the absence or presence of 500 ng of FlaB/ml for 24 h. Whole-cell lysates (400 μg) were incubated with the rabbit anti-FlaB polyclonal antibody for 2 h at 4°C and incubated further for another 1 h after adding pre-equilibrated protein-A Sepharose beads (Amersham Pharmacia Biotechnology, Uppsala, Sweden). The anti-FlaB antibody was raised in a rabbit with a GST-FlaB fusion protein as the antigen. The Sepharose beads were then washed three times with

the same buffer at 4°C. Bound proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to the Western blot hybridization analysis with an anti-Flag antibody (Sigma). Reactive proteins were detected by using the enhanced chemiluminescence kit (Amersham Pharmacia).

Trafficking of radiolabeled FlaB in mice. To trace the fate of intranasally administered FlaB, the protein was labeled with iodine-131 (^{131}I). The radioiodination was performed according to the modified chloramine-T (*N*-monochloro-*p*-toluenesulfonamide) method of McConahey and Dixon (23). Briefly, 0.5 ml of FlaB (200 μg/ml) was mixed with 5 μl of chloramine T (12 mg/ml) and 2 mCi of ^{131}I (20 μl in 0.1 N NaOH). The reaction mixture was gently mixed and incubated for 2 min at room temperature. Thereafter, the reaction was stopped by adding 5 μl of sodium metabisulfite (24 mg/ml). Unincorporated ^{131}I was removed by using a 10DG Bio-Rad desalting column (Bio-Rad, Hercules, CA). The labeling yield obtained by this method was ca. 98%. Trafficking of the radioiodinated FlaB was carried out as described earlier (36). In brief, 6 μl of ^{131}I-labeled FlaB for each nostril was given to BALB/c mice. The distribution of the radiolabeled protein in nasopharynx-associated lymphoid tissue (NALT), cervical lymph node (CLN), spleen, and blood was assessed 1, 6, 24, 48, and 144 h after the administration. Radioactivity in the tissue samples was measured by the scintillation counter Atomlab950 (Biodex Medical Systems, New York, N.Y.).

Confocal microscopic observation. To examine the distribution of FlaB after intranasal administration, CLNs from FlaB-treated BALB/c mice were stained with CD11c-fluorescein isothiocyanate (FITC) (BD Biosciences/Pharmingen, San Diego, CA), and the polyclonal anti-FlaB antibody was monitored by using Texas Red-labeled anti-rabbit IgG antibody (Molecular Probes, Eugene, OR). TLR5 expression level was also examined by staining the sample with an anti-TLR5 antibody (Imgenex, San Diego, CA), followed by the addition of Texas Red-labeled anti-rabbit IgG antibody (Molecular Probes). The mice were given 50 μg of FlaB intranasally, and then CLNs were excised from the mice after 6 h. Excised CLNs were fixed with 4% formaldehyde. The specimen was embedded with the OCT compound (Sakura Finetek, Japan) and sectioned to the thickness of 5 μm by using a cryostat. After being washed with PBS, the specimens were blocked with PBS containing 10% FBS for 1 h at room temperature and then stained with appropriate antibodies. The specimen mounted with a ProLong Gold antifade reagent (Molecular Probes) was observed with a confocal laser scanning microscope (Bio-Rad Laser Scanning System; Radiance 2100, London, United Kingdom).

Detection of TLR5 expression by Western blot analysis and RT-PCR in mice. Western blot analysis and RT-PCR were performed to detect TLR5 expression in BALB/c mice. The mice were intranasally given 50 μg of FlaB, and then the spleens were excised from the mice after 6 h. To prepare the tissue lysate, we homogenized the spleen in a lysis buffer (150 mM NaCl, 50 mM Tris-Cl [pH 8.0], 5 mM EDTA [pH 8.0], 1% NP-40, 20 μM phenylmethylsulfonyl fluoride, and protein inhibitor cocktail [one tablet; Roche]) using a glass homogenizer on an ice bed. Protein concentrations of the lysates were determined by using the DC Protein Assay Kit (Bio-Rad). A total of 20 μg of the lysate was subjected to SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The membrane was probed with a 1:1,000 dilution of anti-TLR5 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The same membrane was stripped and reprobed with a 1:1,000 dilution of anti-β-actin antibody (Santa Cruz Biotechnology). In vivo mRNA expression of TLR5 mRNA was analyzed by the RT-PCR analysis. Total RNA was isolated from the spleen and reverse transcribed into cDNA using random primers (Promega). The primers used for PCR were as follows: mouse TLR5 forward, 5′-GAA CAT CAG AGA TCC TGA-3′; and mouse TLR5 reverse, 5′-ATG GCC TTA AGA GCA TT-3′. Relative gene expression levels were compared by the Scion image analysis software for Windows (Scion Corp., Frederick, MD).

Statistical analysis. Statistical differences were analyzed by using the Student *t* test. All experiments were repeated three or four times, and results from a representative experiment are shown.

Nucleotide sequence accession numbers. The DNA sequence of *V. vulnificus flaB* has been deposited in the GenBank database as a part of a *V. vulnificus* whole-genome sequence done by our group under accession numbers NC_004459 and NC_004460.

RESULTS

Flagellin has a strong mucosal adjuvant activity to induce protective immunity. While working on the pathogenic significance of the six flagellins of *V. vulnificus*, we observed the possibility that FlaB would potentiate mucosal immune responses. When injected into the back of a mouse, FlaB caused exaggerated granulation scars at the injection site, suggesting nonspecific activation of immunocytes (data not shown). Subcutaneous immunization with FlaB protected only from intragastric challenges with live *V. vulnificus* and not from intraperitoneal challenges (unpublished data). We tested whether FlaB could act as a mucosal adjuvant by using an experimental system described elsewhere (20). The purity of recombinant FlaB was assessed by SDS-PAGE and Western blot analysis with a rabbit anti-FlaB antibody. Coomassie blue staining and Western blotting confirmed the highly purified 42-kDa protein corresponding to FlaB (Fig. 1A). BALB/c mice were intranasally immunized with 3 μg of TT, along with 1, 5, or 15 μg of FlaB three times, and the mice were systemically challenged with a supralethal dose (200× minimum lethal dose) of tetanus toxin. All of the PBS sham-immunized control mice died within 24 h. However, all of the TT-plus-FlaB-immunized mice were completely protected from the toxin challenge, whereas the mice immunized by only TT showed 17% survival (Fig. 1B).

In order to assess the mucosal adjuvant activities of FlaB, TT-specific IgG and IgA responses were measured. Intranasal immunization with TT plus FlaB resulted in significantly enhanced TT-specific serum IgG antibody responses (Fig. 1C). The types of immune responses to TT plus FlaB were further analyzed by measuring isotype-specific antibody titers. The TT-specific IgG1 response in the TT-plus-FlaB immunization groups was significantly increased, whereas the IgG2a production profiles were similar to the control group (Fig. 1C). These results show that FlaB potentiated TT-specific systemic immune response in favor of Th2 immune responses. We also assessed IgA production, the hallmark of mucosal immune responsiveness, in serum and in various mucosal samples. Nasal administration of TT plus FlaB induced significantly higher levels of TT-specific IgA production in serum and in all of the mucosal samples tested (Fig. 1C). These results show that FlaB is an efficacious mucosal adjuvant for the induction of antigen-specific systemic IgG and mucosal IgA productions.

The adjuvant activity was FlaB-dependent specific responses. To exclude an interfering effect of any contaminating cellular components from the host *E. coli*, we tested the adjuvant activity of a GST protein preparation by using the same experimental system. Since the LPS contaminating the FlaB preparation could possibly induce adjuvant function through TLR4 activation, we made sure that the residual LPS concentration in the preparation remained below 0.5 EU/ml. However, there remained a possibility that such a small amount of LPS might contribute to the mucosal adjuvant activity of FlaB. To address this problem, we expressed GST proteins in *E. coli*, and residual LPS was removed by the same methodology used for the FlaB preparation. The residual LPS concentration in the GST preparation was 3.07 EU/ml. More LPS contamination in the GST preparation seemed to have resulted from the more rigorous cell disruption method using a lysozyme treatment. The sonication method without lysozyme treatment used for the FlaB preparation seems to release less LPS from the cell envelope. We observed no adjuvant activity with the GST preparation that had significantly higher residual LPS contamination (Fig. 1D). On the other hand, a GST-FlaB fusion protein prepared through the same purification procedure showed strong adjuvant activities comparable with the FlaB

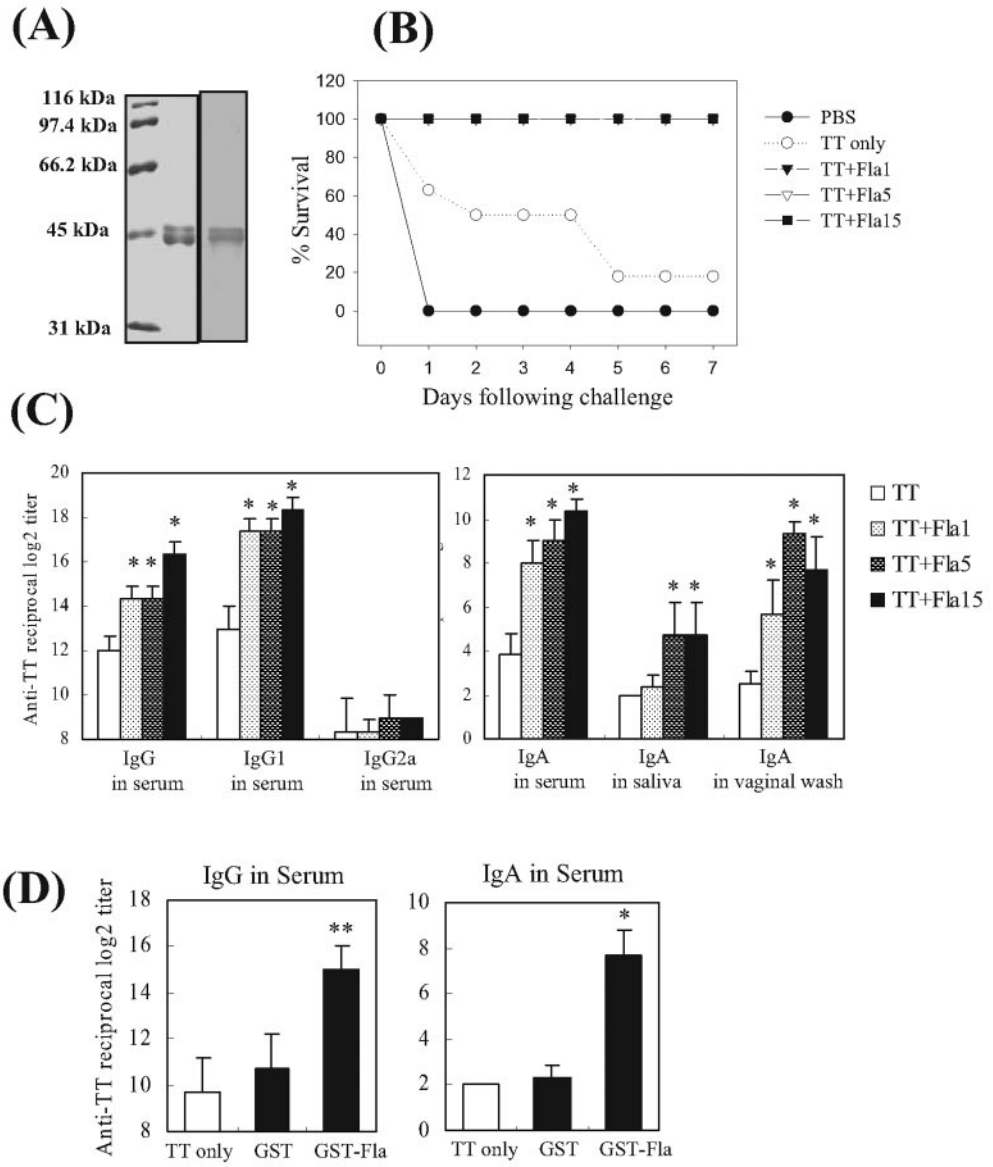

FIG. 1. FlaB had mucosal adjuvant activity to induce protective immunity. (A) Recombinant FlaB was analyzed by SDS-PAGE (left) and Western blot analysis (right) with an anti-FlaB antibody. (B) Female 7-week-old BALB/c mice ($n = 10$) were intranasally given PBS, 3 μg of TT alone, or TT in combination with 1, 5, and 15 μg of FlaB three times in 7-day intervals. Seven days after the last immunization, all of the mice were subcutaneously challenged with a 200× minimum lethal dose of tetanus toxin and observed for 7 days. (C) Seven days after the last immunization, blood and mucosal samples (saliva and vaginal wash) were collected from the mice ($n = 5$), and TT-specific antibody titers were measured by ELISA. (D) The BALB/c mice were intranasally given 3 μg of TT alone, TT in combination with 7 μg of GST, or 18 μg of GST-FlaB three times in 7-day intervals. Seven days after the last immunization, TT-specific IgG and IgA levels in sera were measured. Values represent the mean endpoint ($\log_2$) antibody titer ± the standard deviation in each group. The experiment was repeated three times with similar results. *, $P < 0.01$; **, $P < 0.05$ versus TT-only-immunized group.

preparation. This result suggests that the adjuvant activity was a FlaB–TLR5-specific phenomenon.

FlaB stimulated IL-8 production in epithelial cells. We determined whether FlaB could stimulate Caco-2 cells, which are known to express TLR5 constitutively, to produce IL-8 (33). Caco-2 cells were treated with different concentrations of FlaB. IL-8 secretion in the culture supernatant was determined by ELISA. The IL-8 mRNA transcript level in the Caco-2 cells was assessed by the real-time RT-PCR method. FlaB significantly stimulated both IL-8 mRNA expression and IL-8 secretion in dose-dependent manners (Fig. 2A and B).

Direct association of FlaB with TLR5 and subsequent transcriptional activation of NF-κB and IL-8. We examined whether the IL-8 production in the Caco-2 cells stimulated by FlaB was the result of a specific interaction between FlaB and TLR5 and of subsequent NF-κB activation. To assess the direct interaction between FlaB and TLR5, a coimmunoprecipitation analysis was performed. Caco-2 cells were transiently transfected with the TLR5 expression vector p3XFlag-hTLR5 and incubated with 500 ng of FlaB/ml. The cell lysates were immunoprecipitated with the anti-FlaB antibody. The precipitates were resolved by SDS-PAGE and subjected to the West-

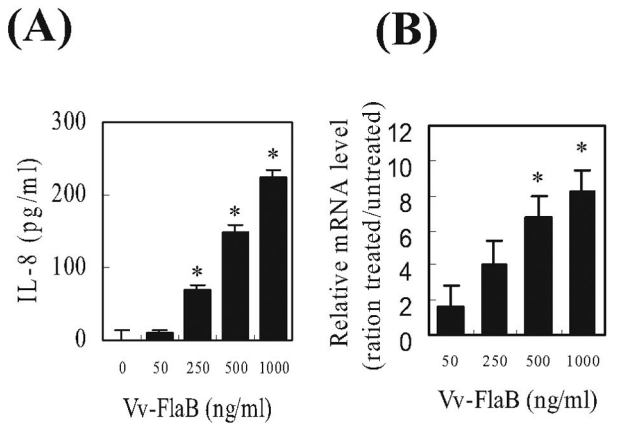

FIG. 2. FlaB stimulated IL-8 production in Caco-2 cells in a dose-dependent manner. Caco-2 cells were treated with indicated concentrations of FlaB for 5 h. (A) IL-8 concentrations in the supernatant were determined by ELISA. *, $P < 0.001$ compared to control cultures. (B) The level of IL-8 transcripts was assessed by the real-time RT-PCR with β-actin as an internal control. Values of mRNA expression were expressed as the relative increase of IL-8 mRNA compared to that in nontreated Caco-2 cells. Values represent the mean ± the standard deviation in each group. Vv-FlaB, *V. vulnificus* FlaB.

ern blot hybridization analysis with an anti-Flag antibody. TLR5 was precipitated, along with FlaB (Fig. 3A). Treatment of hTLR5-transfected Caco-2 cells with FlaB resulted in significant IL-8 and NF-κB transactivation in dose-dependent manners (Fig. 3B). These results suggest that FlaB directly interacted with TLR5 expressed on Caco-2 cells and that the binding of FlaB with TLR5 subsequently activated downstream signaling to produce IL-8.

Trafficking of FlaB. To elucidate the immunological mechanisms underlying the adjuvant activities of FlaB in both mucosal and systemic compartments, the distribution of ^{131}I-labeled FlaB in test mice was examined by a time course study (Fig. 4). Most of the protein seemed to come into systemic circulation and remained there up to 6 days (144 h). The ^{131}I-labeled FlaB level in blood peaked at 1 h and decreased continuously thereafter. Radioactivity in NALT also peaked at 1 h and decreased thereafter, rather abruptly reaching the background level at 48 h. The level of ^{131}I-labeled FlaB in spleen also peaked at 1 h and decreased to a plateau at 6 h that was maintained until 24 h. On the other hand, radioactivity in the CLNs reached their peak at 6 h and remained above the background level until 48 h. Background levels in the CLNs

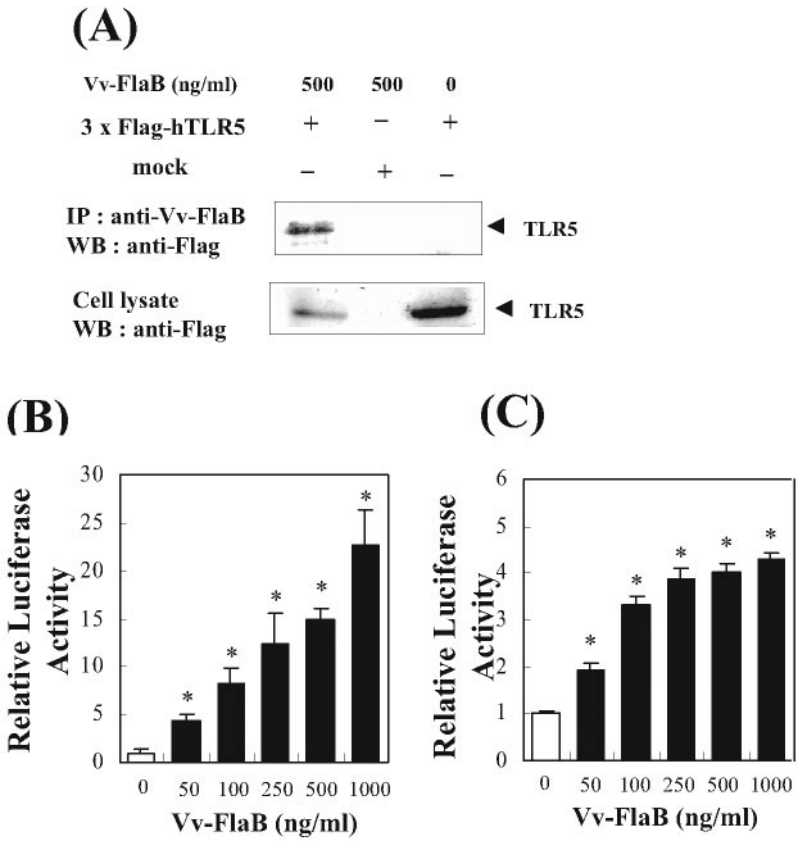

FIG. 3. FlaB directly interacted with TLR5 in epithelial cells and FlaB induced TLR5-mediated NF-κB and IL-8 transcription. (A) Caco-2 cells were transfected with a 3× Flag-TLR5-expressing plasmid and further treated with 500 ng of FlaB/ml for 24 h. Cell lysates were subjected to immunoprecipitation (IP) with an anti-FlaB antibody. The immunoprecipitated proteins were resolved by SDS-PAGE and analyzed by Western blotting (WB) with an anti-Flag antibody (top panel). Aliquots of cell lysates were subjected to anti-Flag Western blotting (bottom panel) to evaluate transfection efficiency. Caco-2 cells were transiently cotransfected with hTLR5 expression plasmid and pIL-8-Luc (B) or pNF-κB-Luc (C), along with increasing amounts of purified FlaB. Relative luciferase activities in cell extracts were analyzed by the dual-luciferase reporter assay system and normalized with pCMV-β-galactosidase as a control. All values represent the mean ± the standard error of at least three independent experiments. *, $P < 0.02$ compared to control cultures. Vv-FlaB, *V. vulnificus* FlaB.

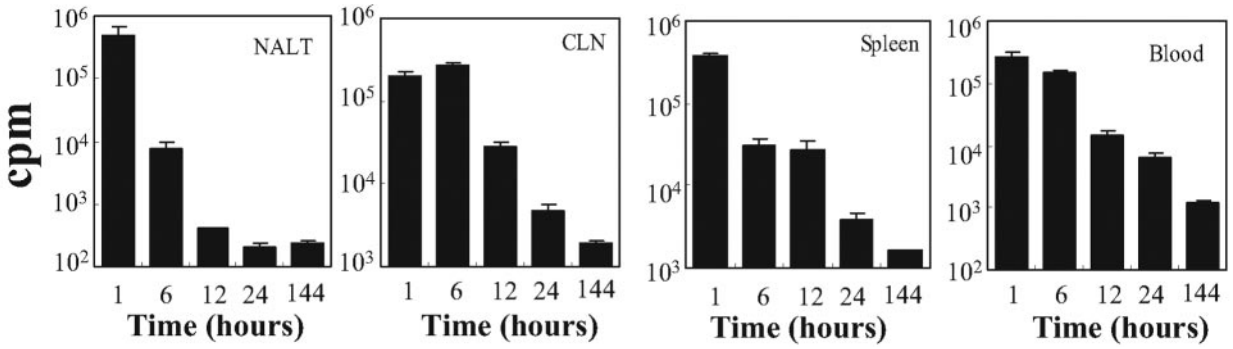

FIG. 4. Trafficking of radiolabeled FlaB in mouse. A total of 8×10^6 cpm of ^{131}I-labeled FlaB (6 μg) in 12 μl was given intranasally to BALB/c mice. In order to detect the distribution of the radiolabeled FlaB in lymphoid organs and blood, we prepared NALT, CLNs, spleens, and blood from the mice at the indicated times. The radioactivity of the sample was measured by using a scintillation counter. The data represent the average counts per minute of three mice ± the standard error in each group.

and spleen were reached in 6 days when blood still retained a detectable level of radioactivity. Taken together, intranasally administered FlaB readily reached systemic circulation, while the regional draining lymph nodes retained the adjuvant protein relatively longer than the spleen. This bimodal FlaB distribution in the systemic compartment and in the draining lymph nodes after the intranasal administration should, at least in part, be responsible for the experimental result that FlaB significantly potentiated TT-specific immune responses both in the mucosal and in the systemic compartments.

Examination of TLR5 expression in secondary lymphoid organs after intranasal administration of FlaB. To observe how the intranasally administered FlaB was distributed in the draining lymph nodes, CLNs were isolated 6 h after the administration and frozen for confocal microscopic examination. FlaB and DC marker CD11c were stained in the same slide. Interestingly, FlaB was stained only in the cells expressing CD11c and colocalized with CD11c molecules (Fig. 5).

The intranasal FlaB administration resulted in a significant increase in the TLR5 expression in both CLNs and spleen (Fig. 6). When the CLNs were stained for TLR5 and CD11c, the number of TLR5-positive DCs increased significantly. In the control mice, cells colocalizing TLR5 and CD11c were very scanty, whereas both the number of TLR5/CD11c double-positive cells and the TLR5 expression levels in each cell increased dramatically in the mice given FlaB (Fig. 6A). To quantitatively assess whether the intranasal FlaB administration affected TLR5 levels in secondary lymphoid organs, TLR5 expression in spleen was measured by Western blot analysis and quantitative RT-PCR. We could not use CLNs for that experiment since enough of the TLR5 protein or mRNA transcripts could not be obtained from a single test mouse. Pooling of CLNs from multiple test mice would complicate the comparison. From the trafficking experiments described above, we found that significant amounts of administered FlaB moved to the spleen. Moreover, we supposed that the spleen would reflect the immunological milieu in the CLNs. A significant increase of the TLR5 expression in spleen was clearly noted at both protein and mRNA levels (Fig. 6B and C).

DISCUSSION

In the present study, we demonstrated that one of the six flagellins produced by *V. vulnificus* acted as an efficacious mucosal adjuvant. We also tried to elucidate mechanisms underlying the strong adjuvant activity of FlaB. It has already been reported that flagellin is an effective adjuvant capable of en-

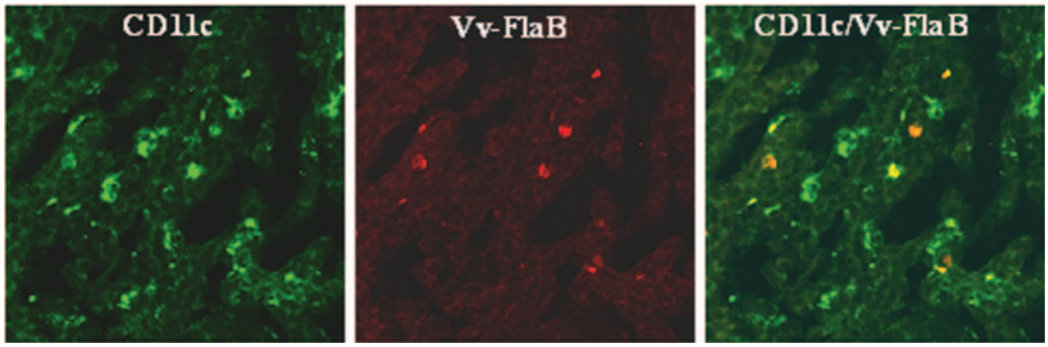

FIG. 5. FlaB was colocalized with CD11c$^+$ cells in mouse CLNs. BALB/c mice were intranasally administered with 50 μg of FlaB. After 6 h, CLNs were freshly isolated from the mice and were frozen for section. The samples were stained with CD11c-FITC (green) and anti-FlaB antibody, followed by the addition of Texas Red-labeled anti-rabbit IgG antibody (red). Localization of FlaB in the CLNs was determined by confocal microscopic observation. Vv-FlaB, *V. vulnificus* FlaB.

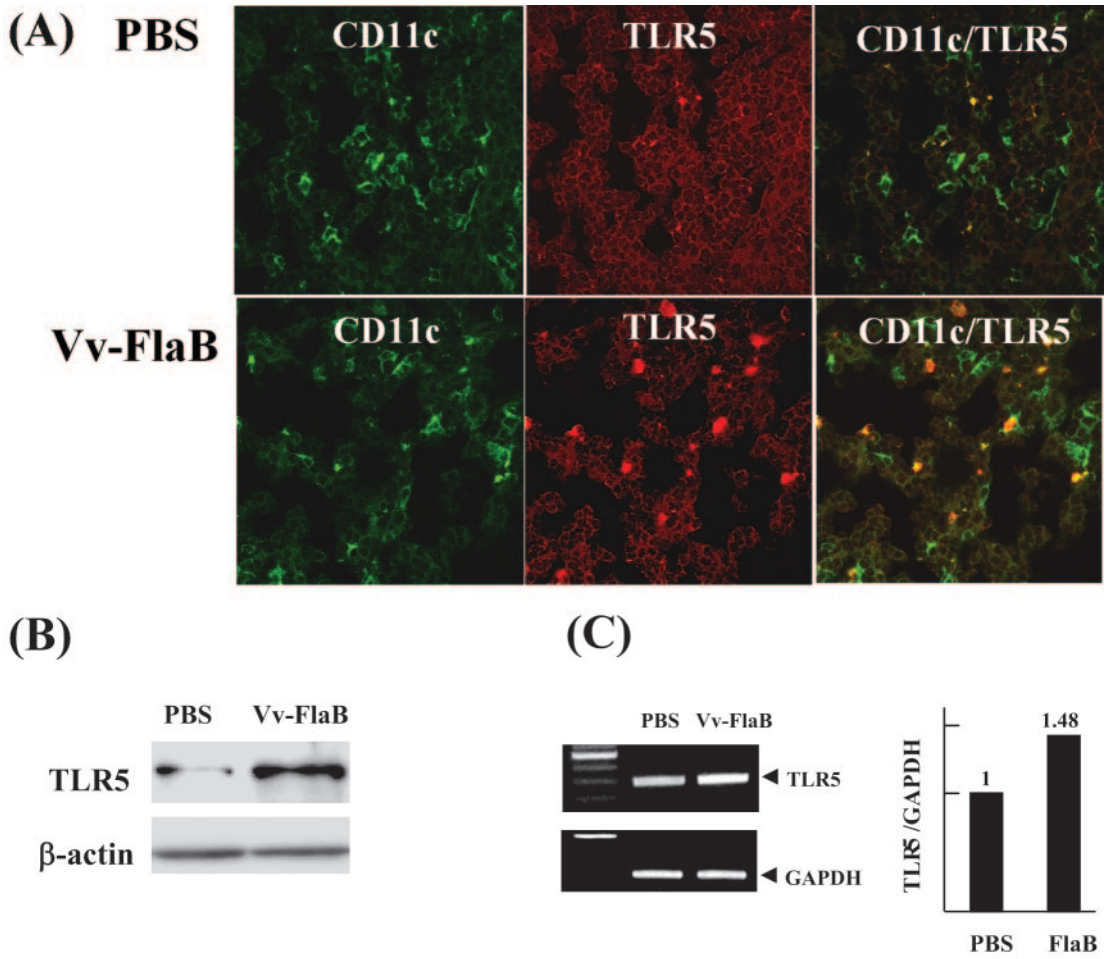

FIG. 6. FlaB treatment stimulated TLR5 expression in CLNs and spleen. BALB/c mice were intranasally given PBS or 50 μg of FlaB. After 6 h, CLNs and spleens were freshly isolated from the mice for the evaluation of TLR5 expression in vivo. (A) Frozen sections of CLNs were prepared and stained with CD11c-FITC (green) and anti-TLR5 antibody, followed by Texas Red-labeled anti-rabbit IgG antibody (red). Fluorescence images of cells were captured under a confocal microscope. (B) Tissue lysates were prepared from the spleen and Western blotting was carried out to detect TLR5 expression. The β-actin blot is shown as a loading control. (C) The mRNA expression of TLR5 mRNA in the spleen was analyzed by the RT-PCR analysis. One of three experiments with similar results is shown. Vv-FlaB, *V. vulnificus* FlaB.

hancing T-cell responses in vivo (11, 24). Other reports also suggested the possibility of flagellin as a systemic adjuvant. For example, epitopes inserted into the flagellin of live *Salmonella* could induce protective immunity (29, 37). Cuadros et al. recently showed that flagellin fusion proteins could induce maturation of antigen-presenting cells and raise specific T-cell responses in vitro (6). However, it is hard to find reports showing the adjuvant activity of flagellin in mucosal immune compartments. This is, to our knowledge, the first report addressing mucosal adjuvant activity of bacterial flagellins.

We presume that the mucosal adjuvant activity should have originated from FlaB-TLR5 interaction in nasal cavity, local draining lymph nodes, and the systemic immune compartments. First, FlaB might have stimulated epithelial cells and set local milieu for effective immune reaction. Epithelial cells react to specific components of the microorganisms by secreting antimicrobial molecules and chemokines (7, 9, 30, 34) that recruit phagocytic cells executing innate immune responses. Many epithelial cells constitutively express TLRs (5). Purified FlaB effectively stimulated IL-8 production in the intestinal epithelial Caco-2 cells constitutively expressing TLR5 (34). We claim that the effect of FlaB on epithelial cells resulted from a direct interaction with TLR5 by showing coimmunoprecipitation of the two molecules (Fig. 3A) and dose-dependent transactivation of NF-κB and IL-8 promoters in the cells stimulated by FlaB (Fig. 3B). Airway epithelial cells were reported to express TLR5 profusely (28). FlaB should have stimulated nasal epithelial cells to produce IL-8, which played a crucial role in recruiting immunocytes that subsequently fortified the immune responses in both mucosal and systemic compartments. This TLR5-mediated IL-8-inducing activity in epithelial cells seems to be a general property of bacterial flagellins. Different flagellins may have different adjuvant activities. The flagellin of *Helicobacter pylori* showed very poor activity as a TLR5 agonist (13). Flagellins from opportunistic pathogens such as *V. vulnificus* might have more potent TLR5 agonistic activity than those from organisms with a longer history of human association. The opportunists should have less chance for compromising flagellin reactivity with the human immune system throughout the evolution process. In that sense, we

suppose that FlaB could be a more potent adjuvant than flagellins of *Salmonella* or other gram-negative normal floras. FliC from *Salmonella enterica* serovar Typhimurium showed less activation of Caco-2 cells than FlaB (data not shown).

Second, longer trapping of FlaB in the local draining lymph nodes could have driven the mucosal immunity more efficiently. FlaB appeared to be trapped and accumulated in the local draining lymph nodes for a considerable duration. Whereas radioactivity in the blood and spleen peaked at 1 h after the administration, that in the locally draining CLNs increased until 6 h. Antigen-presenting cells in the nasal mucosa which captured FlaB should have migrated to the CLNs via the afferent lymphatics. A recent report showed that T cells located in the local lymph node underwent multiple short encounters with DCs for the first 8 h and thereafter formed long-lasting stable conjugates with DCs to secrete IL-2 and gamma interferon (26). Retention of FlaB-loaded DCs in the CLN for longer durations would provide more chance to activate homing T cells in the lymph nodes, which subsequently drive strong mucosal immune reactions.

Third, flagellin treatment seems to increase TLR5 expression in vivo. Under confocal microscopic examination, the number of TLR5-positive cells significantly increased in the FlaB-treated mice. Quantitative measurement of TLR5 expression in the systemic compartment (spleen) also showed a significant increase after the intranasal FlaB treatment. This might be a result of the homing of TLR5-expressing antigen-presenting cells to local draining lymph nodes or to the spleen. We could rule out this possibility since the TLR5 staining density of each cell was much higher in the FlaB-treated group (Fig. 6A).

Recent studies have demonstrated that TLR agonists such as CpG DNA, microbial lipoprotein, LPS, and flagellin from serovar Typhimurium (15, 25) could induce maturation of DCs. Sierro et al. (34) suggested that flagellin stimulation of intestinal epithelial cells triggers CCL20 expression, which is a chemoattracting factor for DCs, resulting in stimulation of immune responses. In our experimental system, FlaB might have stimulated secretion of IL-8 in epithelial cells and also induced the functional maturation of DCs (data not shown), which could have synergistically elicited strong adjuvant activity. There still remains some debate on whether flagellin could activate murine DCs or not. A recent report suggested that bacterial flagellin could directly stimulate human DC maturation but not murine DC maturation using an in vitro culture system (25). These authors also reported that TLR5 was not expressed in macrophages, splenic DCs, and bone marrow-derived DCs. If that is true, we might have to change our interpretation concerning the in vivo effects of FlaB on DCs. However, there are many contradictory reports showing TLR5 expression on murine DCs. Datta et al. (8) showed that mature bone marrow-derived mouse DCs expressed relatively low levels of TLR5 transcripts. McSorley et al. (24) demonstrated that bacterial flagellin administration increased the expression of costimulatory molecules on splenic DCs and elicited a CD4 T-cell response in vivo. Applequist et al. (3) showed that some mouse-derived DC and macrophage cell lines express low levels of TLR5. According to an organ-specific murine TLR5 expression pattern study (33), the mouse TLR5 gene was expressed in all tissues except kidney after long autoradiographic exposure. Madrazo et al. (22) reported that murine osteoblasts expressed TLR5, and this expression was upregulated after exposure to TLR5 agonist. Didierlaurent et al. proved TLR5 expression in splenic DCs by a real-time RT-PCR analysis normalizing TLR5 transcripts in DCs with those in whole splenic cells (10). They concluded that mouse DCs express TLR5 and are equipped to respond to flagellin. Our experimental data from the mouse model studies show a strong possibility that DCs, at least a small subset of them, should have expressed TLR5 in vivo and stimulated T cells in favor of mucosal immune responses.

In conclusion, we discovered a potent mucosal adjuvant, FlaB, that would accelerate the generalization of needless vaccines. FlaB effectively triggered specific immune responses in both mucosal and systemic compartments. FlaB, and presumably other flagellins, could become a crucial component in developing new paradigm mucosal vaccines against respiratory, gastrointestinal, and genitourinary tract infections.

ACKNOWLEDGMENTS

We thank Steven B. Mizel of Wake Forest University for kindly providing the p3XFlag-hTLR5 plasmid.

This study was supported by the 2001 National Research Laboratory grant from the Ministry of Science and Technology, and Korea Health 21 R&D project by the Ministry of Health and Welfare of the Republic of Korea (01-PJ10-PG6-01GM00-0002).

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Editor: V. J. DiRita