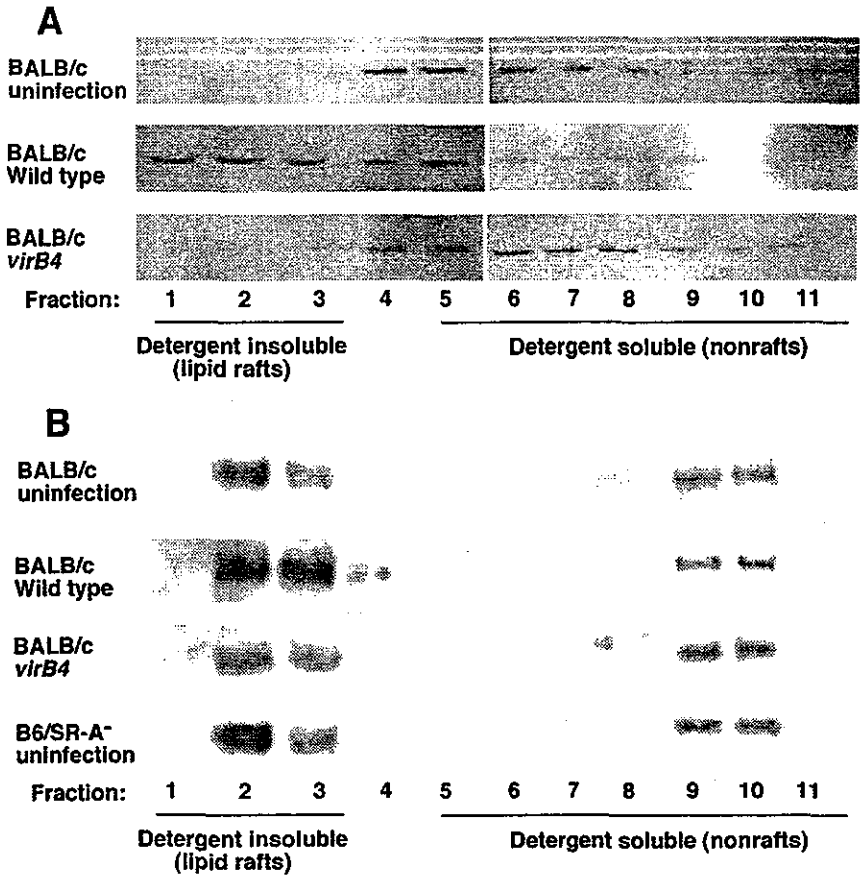

Fig. 5. Association between SR-A and lipid rafts in *B. abortus* internalization. Macrophages infected with or without the wild type or *virB4* mutant were lysed in 1% Triton X-100 lysis buffer, and the whole lysates were fractionated by sucrose density gradient ultracentrifugation. Individual fractions, indicated at the bottom, were analyzed for the presence of SR-A (A) or raft-associated GPI-anchored protein CD48 (B) by immunoblotting.

in the signaling pathway in *B. abortus* infection into macrophages.

3. Discussion

In this study, we showed that SR-A interacts with cell surface components of *B. abortus*, such as LPS, which was mediated by lipid raft microdomains on macrophage plasma membrane, and that the interaction contributes to establishing *B. abortus* infection in mice. Hsp60, a member of the GroEL family of chaperonins, of *B. abortus* can interact with the cellular prion protein (PrP^C) on mouse bone marrow-derived macrophages [18]. PrP^C tail-like formation aggregates during bacterial internalization into the macrophages and PrP^C is selectively included in macropinosomes containing *B. abortus*. PrP^C deficiency inhibits intracellular replication of *B. abortus*, but not bacterial internalization [18]. These results suggested to us that signal transduction induced by interaction between bacterial Hsp60 and PrP^C on macrophages contributes to form replicative phagosomes and that other receptors for bacterial internalization are on the macrophage surface. The Niemann–Pick type C1 gene (NPC1) regulates internalization and intracellular replication of *B. abortus* and also contributes to bacterial proliferation in mice [21]. Macrophages from NPC1-deficient mice do not support internalization and intracellular replication of *B. abortus*. In NPC1-deficient mice macrophages, lipid raft-associated molecules, such as cholesterol, GM1 ganglioside, and GPI-anchored proteins, accumulate only in intracellular vesicles [21]. In contrast, these molecules are present in both the plasma membrane and intracellular vesicles of macrophages from PrP^C-deficient mice as well as macrophages from parent mice (*unpublished data*). Therefore, lipid raft-associated molecules on the plasma membrane are essential for internalization of *B. abortus* and we examined if SR-A has a role as a receptor of *B. abortus*.

Macrophage scavenger receptors are implicated in the deposition of cholesterol in arterial walls during atherogenesis through receptor-mediated endocytosis of chemically modified low density lipoproteins (LDL) [22–25]. These receptors have a wide spectrum of biological roles in not only atherogenesis, but also host defense against pathogens and the removal and clearance of various arrays of negatively charged macromolecules, because of the broad ligand-binding capacity. The scavenger receptors are classified into class A (type I and type II

macrophage receptors (SR-A), and macrophage receptor with collagenous structure (MARCO)); class B (CD36 and SR-BI); class C (dSR-CI); class D (CD68/marcosialin); class E (lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1)); class F (scavenger receptor expressed by endothelial cells (SREC)); and Fc receptor (FcγRII-B2) (for reviews see Refs. [26,27]). SR-A is a homotrimeric membrane protein of mononuclear phagocytes [28] that mediate phagocytosis of apoptotic thymocytes [17], endocytosis of modified lipoproteins [28], and adhesion of macrophages to surfaces coated with serum proteins [16], glucose-modified basement membrane proteins [29], and β-amyloid fibrils [30]. The fact that macrophage scavenger receptors bind bacterial cell wall components, such as LPS from Gram-negative bacteria [14] or lipoteichoic acid from Gram-positive bacteria [31] and that SR-A-deficient mice show increased susceptibility to infection with *Listeria monocytogenes* [13,32], *Staphylococcus aureus* [33] and LPS-mediated shock [34], suggests that these receptors function in the absence of serum opsonins in host defense against bacterial infections. In contrast, SR-A-deficient mice in this study showed a decreased susceptibility to infection with *B. abortus*. The decreased susceptibility should be caused by the inhibition of bacterial internalization and intracellular replication in macrophages. Although SR-A is a pattern recognition molecule, which recognizes conserved motifs on pathogen surfaces directly [26] and which contributes to innate immunity, *B. abortus* uses these host defense mechanisms to survive in macrophages.

The soluble lipid A moiety of LPS has been implicated previously as an SR-A ligand and is the best candidate molecule to recognize SR-A [14]. In this study, the internalization of *B. abortus* was inhibited by LPS extracted from smooth and rough *B. abortus*, suggesting that lipid A of *B. abortus* may be candidate molecules for SR-A recognition. As LPS of *Brucella suis* has an important role in the lipid raft-associated infection pathway in macrophages [15], LPS of *B. abortus* should be important factor in their infection in macrophages. Our results also showed that internalization of *B. abortus* was inhibited by LPS from *S. enterica* serover Typhimurium. Although we do not know if SR-A contributes to the infection of *S. enterica* serover Typhimurium, LPS of *B. abortus* and *S. enterica* serover Typhimurium should share binding sites for SR-A. SR-A deficiency inhibits internalization of *Neisseria meningitidis* into bone marrow-derived macrophages [35]. Interestingly, LPS is not the ligand for SR-A on *N. meningitidis* [35]. Therefore, other ligands may exist on the *B. abortus* surface.

We next investigated if interaction between *B. abortus* and SR-A initiates signal transduction into macrophages for replicative phagosome formation. As lipid rafts regulate bacterial internalization and intracellular replication in macrophages [7], we expected that SR-A would be contained in lipid rafts on macrophages infected with the wild type strain. We used DRMs from macrophages infected with the wild type strain or the *virB4* mutant to clarify the hypothesis, because DRMs do not artificially create domains from previous homogeneous bilayers or recruit non-raft proteins and lipids into rafts during DRM isolation, and DRMs reflect at least a subset of raft-sorting properties in the membrane [19]. As expected, SR-A was contained in lipid rafts on macrophages infected with the wild type strain, but not in lipid rafts on macrophages infected with *virB4* or uninfected macrophages. These results were consistent with our previous results that internalization of the *virB4* mutant shows non-raft pathways in macrophages [7,18]. Thus, SR-A contributes to internalization of both the wild type strain and the *virB4* mutant. However, signal transduction to form replicative phagosomes was induced by only internalization of the wild type strain, which was mediated by lipid rafts.

Signal transduction mediated by SR-A remains unknown. But a signal transduction molecule mediated by SR-A, the Src family protein-tyrosine kinase Lyn, was reported recently [36,37]. The Lyn molecule is associated physically or functionally with SR-A and CD40 [37,38], which have important roles in the development of atherosclerosis [13,39], but their roles in bacterial infection are still unclear. As Lyn is activated in lipid rafts through cell surface receptors and induces signal transduction [40], signal transduction to form replicative phagosomes of *B. abortus* through SR-A may need Lyn and an environment of lipid rafts. Lipid rafts are increasingly being recognized as a gateway for intracellular pathogens [41]. Our study showed that lipid rafts and the type IV secretion system of *B. abortus* regulates downstream of signals from the cell surface receptor, which decides the intracellular fate of *B. abortus*.

4. Materials and methods

4.1. Bacterial strains and mice

All *B. abortus* derivatives were from 544 (ATCC23448) smooth virulent *B. abrotus* biovar 1 strains. Ba598 (544Δ*virB4*), complemented strain Ba603 (Ba598 *virB4* +) and rough *B. abortus* strain 45/20 were used in this study [42]. BALB/c mice, C57BL/6J, and SR-A knockout mice were described previously [13].

4.2. Cell culture

Bone marrow-derived macrophages from female BALB/c, C57BL/6J, and SR-A knockout mice were prepared by the method described previously [43]. After culturing in L-cell conditioned medium, the macrophages were replated for use by lifting cells in phosphate-buffered saline (PBS) on ice for 5–10 min, were harvested by centrifugation, and were resuspended in RPMI

1640 containing 10% fetal bovine serum (FBS). The macrophages were seeded ($2-3 \times 10^5$ or $1-2 \times 10^6$) in 24-well ($2-3 \times 10^5$ in each well) or 6-well ($1-2 \times 10^6$ in each well) tissue culture plates for all assays.

4.3. Isolation of LPS

The crude smooth or rough LPS were prepared by the method described previously [44]. To isolate the crude smooth LPS from wild type strain, the bacteria were grown in 500 ml of Brucella broth at 37 °C for 18 h. Bacteria were collected by centrifugation, were dried with acetone and were extracted with hot phenol–water. To isolate the crude rough LPS of strain 45/20, bacteria were grown in 500 ml of brucella broth at 37 °C for 18 h. Bacteria were collected by centrifugation, were dried with acetone and protein was extracted with 2.5% NaCl at 4 °C with stirring for 3 days [45]. The supernatant fluids were separated from the bacterial cells by centrifugation at 8000*g* for 30 min at 4 °C. The bacterial cells were then washed with PBS, were dried in acetone, and then were extracted with chloroform–petroleum ether–phenol. All smooth and rough LPS samples were treated with nuclease and pronase (Sigma), and were purified further by dialysis as described previously [44]. The amount of protein contamination of the smooth and rough LPS samples was determined by the Lowry method [46], and was less than 3%. Nucleic acids were estimated by the ratio of absorbance at 280/260 nm after hydrolysis of the material with 0.001N NaOH [44], and was less than 1%. The concentration of LPS was estimated by the method described previously [47,48]. The LPS of *E. coli* serotype O111:B4 and *S. enterica* serovar Typhimurium was obtained from Sigma.

4.4. Determination of efficiency of bacterial internalization and intracellular growth in macrophages

Bacterial internalization and intracellular growth assay in macrophages was done by using the method described previously [49]. Briefly, *B. abortus* strains were deposited onto mouse bone marrow-derived macrophages grown on 24-well tissue culture plates filled with RPMI 1640 and 10% FBS at a multiplicity of infection of 20, and were centrifuged at 150*g* for 10 min at room temperature. To analyze the bacterial uptake efficiency, macrophages were washed once with medium after 30 min incubation at 37 °C and then were incubated with RPMI 1640 and gentamicin (30 μg/ml) for 30 min. The macrophages were then washed three times with PBS and then were lysed with distilled water. Colony forming units (CFU) were measured by serial dilutions on brucella agar plates. For intracellular growth efficiency, macrophages were incubated at 37 °C for 30 min, were washed once with medium, were incubated with RPMI 1640 and gentamicin (30 μg/ml), and then were incubated for 2, 24, and 48 h. The macrophages were then washed three times with PBS and then were lysed with distilled water. The CFU were measured by serial dilutions on Brucella agar plates. The percentage protection was calculated by dividing the number of bacteria surviving the assay by the number of bacteria in the infectious inoculum, by viable counts. Macrophages were preincubated for 30 min with antibodies for SR-A as described [13]; 10 μg/ml of 2F8, 100 μg/ml of polyinosinic acid (polyI) or polycytidylic acid (polyC) (Sigma), and 100 μg/ml of LPS from smooth wild type *B. abortus* and rough strain 45/20, *E. coli* O111:B4, and *S. enterica* serovar Typhimurium, respectively. After the treatments, the macrophages were washed with PBS and were measured for viability by trypan blue staining. The viability of macrophages was not affected by these treatments.

4.5. Opsonization

Bacteria ($2-3 \times 10^7$/ml) were opsonized by incubation in PBS containing a 1:1000 dilution of anti-*B. abortus* polyclonal rabbit serum for 30 min at room temperature. Bacteria were washed twice with PBS before addition to macrophages. Opsonized bacteria were deposited onto macrophages at a multiplicity of infection of two. This opsonization procedure resulted in a greater than 10-fold enhancement in the amount of internalized bacteria [7].

4.6. Isolation of lipid rafts

Lipid rafts were isolated by modified sucrose density gradient ultracentrifugation as described [40]. Briefly, macrophages (2×10^7) were infected with *B. abortus* strains as described in Section 4.5, and were incubated at 37 °C for 15 min [7]. Infected macrophages were harvested, and were lysed with 1 ml of the cold lysis buffer (1% Triton X-100, 20 mM Tris–HCl, pH 8.0, 140 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, 0.5 U/ml aprotinin and 0.5 U/ml leupeptin) on ice. After 15 min, the lysate was homogenized by passing it 10 times through a 27-gauge needle and was adjusted to 40% (wt/vol) sucrose by adding an equal amount of 80% sucrose. Sucrose solutions were prepared by mixing the appropriate amount of the gradient buffer (25 mM Tris–HCl, pH 7.5, 125 mM NaCl, 2 mM EDTA) and 80% sucrose. The gradient was formed by adding of 1 ml of 80% sucrose to the bottom of the tube, and then by 2 ml of 40% sucrose (containing the cell lysate), 6 ml of 30% sucrose and 2.5 ml of 5% sucrose. The solutions were then centrifuged at 200,000*g* for 12 h at 4 °C by using a SW41 Ti rotor (Beckman Instruments). Eleven fractions were collected from the top of the gradient and equivalent portions of each fraction were analyzed by SDS-PAGE and immunoblotting. Fractions 1–3 (15–30% sucrose) contained detergent-insoluble lipid rafts, which were confirmed by immunoblotting with antibody for raft-associated protein CD48 [7].

4.7. Virulence in mice

The virulence was measured by quantifying the survival of the strains in the spleen after 10 or 20 days. Groups of five mice were infected intraperitoneally with approximately 10^4 CFU of brucellae in 0.1 ml saline. At 10 or 20 days after infection, their spleens were removed and were homogenized in saline. Tissue homogenates were serially diluted with PBS and were plated on Brucella agar to count the number of CFU in each spleen.

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Brucella abortus nicotinamidase (PncA) contributes to its intracellular replication and infectivity in mice

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Abstract

Brucella spp. are facultative intracellular pathogens that have the ability to survive and multiply in professional and non-professional phagocytes, and cause abortion in domestic animals and undulant fever in humans. The mechanism and factors of virulence are not fully understood. Nicotinamidase/pyrazinamidase mutant (*pncA* mutant) of *Brucella abortus* failed to replicate in HeLa cells, and showed a lower rate of intracellular replication than that of wild-type strain in macrophages. Addition of nicotinic acid, but not nicotinamide, into medium supported intracellular replication of *pncA* mutant in HeLa cells and macrophages. The *pncA* mutant was not co-localizing with either late endosomes or lysosomes. The *B. abortus virB4* mutant was completely cleared from the spleens of mice after 4 weeks, while the *pncA* mutant showed a 1.5-log reduction of the number of bacteria isolated from spleens after 10 weeks. Although *pncA* mutant showed reduced virulence in mice and defective intracellular replication, its ability to confer protection against the virulent *B. abortus* strain 544 was fully retained. These results suggest that PncA does not contribute to intracellular trafficking of *B. abortus*, but contributes to utilization of nutrients required for intracellular growth. Our results indicate that detailed characterizations of the *pncA* mutant may help the improvement of currently available live vaccines.

Keywords: Brucella; Nicotiamidase; Macrophages

1. Introduction

Brucellosis is a major bacterial zoonosis that causes a serious debilitating disease in humans and abortion and sterility in domestic animals. The etiologic agents of brucellosis are *Brucella* spp., small gram-negative and facultative intracellular pathogens that can multiply within professional and non-professional phagocytes [1,2]. In contrast to other intracellular pathogens, *Brucella* species do not produce exotoxins, antiphagocytic capsules or thick cell walls, resistant forms or fimbriae and do not show antigenic variation [3]. A key aspect of the virulence of *Brucella* is its ability to proliferate within professional and non-professional phagocytic host cells, thereby successfully bypasses the bactericidal effects of phagocytes, and their virulence and chronic infections are thought to be due to their ability to avoid the killing mechanisms within host cells [4,5]. The molecular mechanisms and genetic basis for intracellular survival and replication, however, are not understood completely. Some studies with non-professional phagocytes have shown that *Brucella* invades host cells and is contained within early endosome-like vacuoles. These vacuoles rapidly fuse with early autophagosomes that acquire vacuolar H^+-ATPase and lysosome-associated membrane proteins (LAMP), mature into a late auto-

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phagosome, inhibit fusion with lysosome and finally become a replicating vacuole normally associated with the endoplasmic reticulum [4,6–8]. The genetic basis of *Brucella* virulence is still poorly understood. The VirB type IV secretion system of *Brucella* has been identified recently [9]. This operon is composed of 13 open reading frames (ORFs) that share homology with other bacterial type IV secretion systems in the intracellular trafficking of pathogens. Bacteria with deletion or polar and non-polar mutations of these ORFs were not able to replicate and survive within phagocytes [10,11]. Thus, the VirB proteins of *B. abortus* are thought to be constituents of the secretion apparatus.

A preliminary characterization of *Brucella abortus pncA* (nicotinamidase) mutant showed that it had reduced intracellular growth within HeLa cells [12], suggesting that this nicotinamidase might be a virulence-associated factor. In this study, we examined the virulence of *B. abortus pncA* mutant in mice and their intracellular replication in macrophages. The protection induced in mice by a *B. abortus pncA* mutant against a challenge with the virulent strain *B. abortus* 544 was also evaluated.

2. Materials and methods

2.1. Bacterial strains and media

All bacterial strains employed in this study are the derivatives from *B. abortus* 544 (ATCC23448), a smooth virulent *B. abortus* biovar 1 strain. Isogenic mutants of *B. abortus* 544 *pncA*::Tn *5*Km2 and Δ*virB4* were described previously [11,12]. All these strains were maintained as frozen glycerol stocks and were cultured in Brucella broth (Becton Dickinson, Sparks, MD) or Brucella broth containing 1.5% agar. Kanamycin (30 μg/ml) and ampicillin (100 μg/ml) were used when necessary.

pSK *pncA* (*pncA*$^+$) was constructed by cloning a PCR fragment into *Kpn*I/*Sac*I-cleaved pBBR1MCS-4 [13]. The 1195-bp *Kpn*I–*Sac*I PCR fragment spanned a site located 393 nucleotides upstream of the 5′ end of *pncA* to a position 48 nucleotides downstream from the 3′-end [14] and was amplified using the primers 5′-GGTACCGCATCTGCGGCACCTGCAAGG-3′ (*Kpn*I site underlined) and 5′-GAGCTCAACCCGAACTGGCAGAACGAG-3′ (*Sac*I site underlined).

2.2. Cell culture

HeLa cells were grown at 37 °C in a 5% CO_2 atmosphere in eagle minimum essential medium (MEM) (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS). Bone marrow-derived macrophages from female BALB/c mice were prepared by the method described previously [15]. After culture in L-cell conditioned medium, macrophages were harvested and resuspended in RPMI 1640 (Sigma) containing 10% FBS. The HeLa cells or macrophages were seeded (2–3×10^5 per well) in 24-well tissue culture plates one day before infection for all assays.

2.3. Comparative growth of the mutants in minimal medium

Growth of the *pncA* mutant in minimal media, RPMI 1640, which contains no nicotinic acid and 1 μg/ml nicotinamide, was assessed according to the modified method described previously [16,17]. Briefly, $2–3 \times 10^{10}$ colony forming units (CFU) of *B. abortus* wild-type, *pncA* mutant and complemented strain were pelleted at 8000 rpm for 15 min and resuspended to $2–3 \times 10^7$ CFU/ml in RPMI 1640 with 0.3 mM nicotinic acid or 0.3 mM nicotinamide, and then were incubated at 37 °C for 0, 6, 24 and 48 h with shaking. To measure the CFU of brucellae, equal portions were withdrawn from each culture, serially diluted into PBS, and spread on the surface of brucella plates to determine the number of viable cells at intervals of up to 48 h.

2.4. Intracellular growth of the mutants in cultured HeLa cells and macrophages

Bacterial infection and intracellular survival assay were done by using the modified method described previously [12]. Briefly, HeLa cells or mouse bone marrow-derived macrophages were grown on 24-well microtiter plates in MEM or RPMI 1640 with 10% FBS. Cells were infected with *B. abortus* strains at a multiplicity of infection (MOI) of 20 by centrifugation at 150*g* for 10 min at room temperature. To determine the effect of disruption of nicotinamidase in vivo, HeLa cells or macrophages were preincubated for 30 min with the nicotinic acid (0.1 mM) and nicotinamide (0.1 mM) [16], and infected with bacteria as above.

After centrifugation, infected cells were incubated at 37 °C in a 5% CO_2 for 30 min, washed twice with 0.5 ml of sterile PBS and cultured in the media containing gentamicin (30 μg/ml) in the presence or absence of 0.1 mM nicotinic acid or nicotinamide for 2, 24 and 48 h. Serial dilutions of the infected cells lysed with distilled water were plated onto Brucella plates for determination of CFU.

2.5. LAMP-1 staining

LAMP-1 staining was performed as described previously [15]. Briefly, infected macrophages were fixed in 4% periodate–lysine–paraformaldehyde (PLP)–sucrose for 1 h at 37 °C. All antibody-probing steps were for 1 h at 37 °C. Samples were washed three times in PBS for 5 min and then were permeabilized at −20 °C in methanol for 10 s. After incubating three times for 5 min with a blocking buffer (2% goat serum in PBS), the samples were stained

with anti-LAMP-1 rat monoclonal antibody 1D4B, which was obtained from the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine and the Department of Biology, University of Iowa, diluted 1:100 in blocking buffer. After washing three times for 5 min in blocking buffer, the samples were stained with Texas red-goat anti-rat IgG (Molecular Probes, Inc.). Then the samples were stained with anti-*B. abortus* polyclonal rabbit serum [11] and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Molecular Probes, Inc.) in blocking buffer to identify the bacteria, and then they were placed in mounting medium and were visualized by fluorescence microscopy. One hundred bacteria within macrophages were selected randomly and the LAMP-1-positive bacteria were counted.

2.6. Virulence determination in mice

Six-week-old female BALB/c mice were infected intraperitoneally with approximately 10^4 CFU of brucellae in 0.1 ml saline. Groups of five mice were infected with each strain. At each week post-infection, mice were sacrificed by decapitation and their spleens were removed, weighed and homogenized in saline. Tissue homogenates were serially diluted with PBS and were plated on Brucella agar to count the number of CFU in each spleen to assess the virulence of each strain.

2.7. Vaccination against challenge infection with a virulent strain

Six-week-old BALB/c mice were vaccinated with 10^4 CFU of *B. abortus* S19 or *pncA* mutant. Each group consisted of five mice. At 8 weeks postvaccination, mice were challenged intraperitoneally with approximately 10^4 CFU of a virulent strain, *B. abortus* 544. Two weeks after the challenge, the numbers of viable *B. abortus* 544 in the spleens and the weights of the spleens were determined as described [11].

3. Results

3.1. Effect of disruption of pncA in vitro and in vivo

Brucella wild-type and complemented strain showed almost the same growth yield but *pncA* mutant showed a different growth comparing to that of wild-type in RPMI 1640, RPMI 1640 with nicotinic acid or nicotinamide (Fig. 1A, B and C). In the media with nicotinamide, two strains with intact *pncA* showed almost the same growth yield but *pncA* mutant did not grow (Fig. 1A and C). In the media with nicotinic acid, growth of *pncA* mutant was restored (Fig. 1B).

As survival and multiplication in professional and non-professional phagocytic host cells is an important

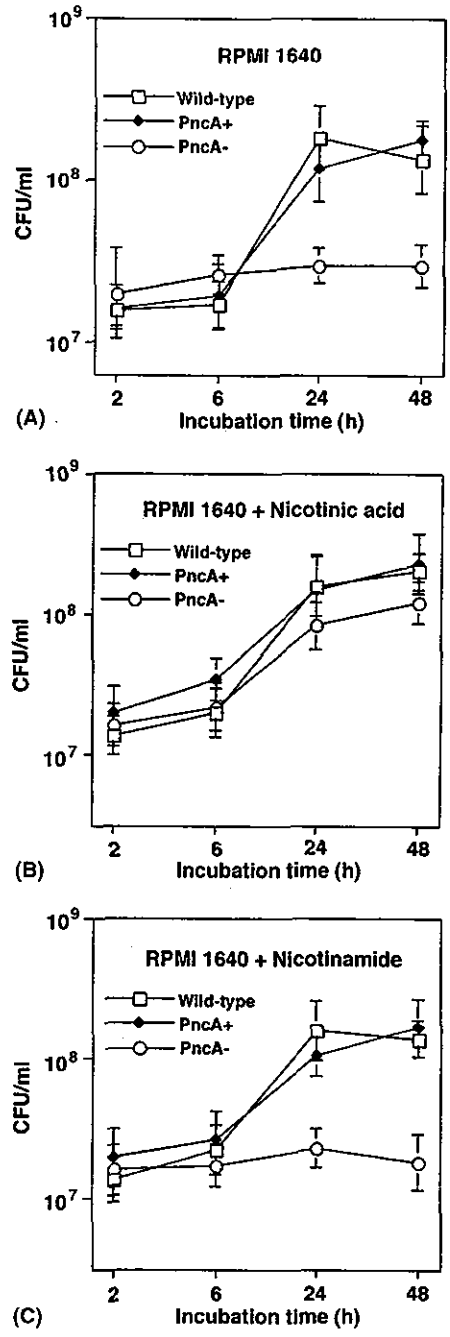

Fig. 1. Bacterial growth of wild-type and *pncA* mutants under minimal medium condition. Wild-type *B. abortus*, *pncA* mutant (*pncA*−) and complemented strain (*pncA*+) were grown in RPMI 1640 (A), RPMI 1640 with nicotinic acid (B) or nicotinamide (C) as described in Section 2. The numbers of viable bacteria at each time point were determined by making serial dilutions in PBS and plating on Brucella plates. Data points and error bars represent the mean CFU of triplicate samples from a typical experiment (performed at least four times) and their standard deviation.

virulence mechanism of *Brucella*, we examined the intracellular replication of *Brucella* strains in HeLa cells and mouse bone marrow-derived macrophages. The *pncA* mutant failed to replicate in HeLa cells, and displayed a lower rate of intracellular replication in

macrophages than the wild-type strain and complemented strain (Fig. 2A and B). To further analyze the effect of disruption of nicotinamidase, cells were preincubated in the media supplemented with nicotinic acid or nicotinamide and then infected with *Brucella* strains. In the presence of nicotinic acid, *pncA* mutant was able to replicate inside the cells as well as wild-type (Fig. 2C and D). In the medium with nicotinamide, the *pncA* mutant, which was unable to convert nicotinamide to nicotinic acid, showed a reduced ratio of intracellular replication in HeLa cells and macrophages as compared to that in other two strains (Fig. 2E and F). Nicotinamide deamidase is known to be released into medium from HeLa cells and macrophages and converts nicotinamide into

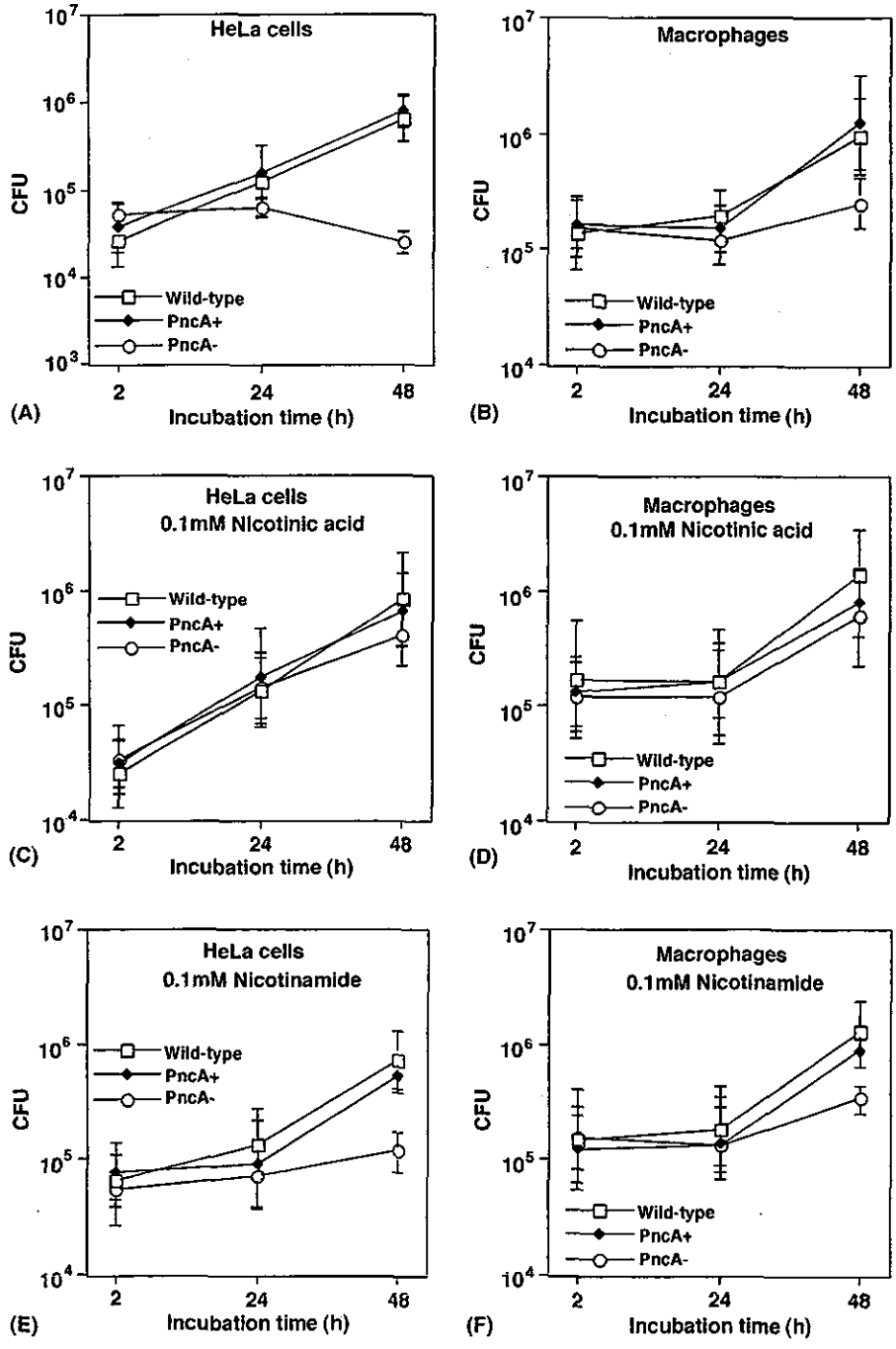

Fig. 2. Intracellular replication of wild-type and *pncA* mutants within HeLa cells and mouse bone marrow-derived macrophages. HeLa cells (A), macrophages (B), nicotinic acid (C and D) or nicotinamide (E and F) added HeLa cells or macrophages were infected with wild-type *B. abortus*, *pncA* mutant (*pncA*−) and complemented strain (*pncA*+) as described in Section 2. At different times of incubation, the cells were lysed, and the numbers of viable intracellular bacteria were determined. Data points and error bars represent the mean CFU of triplicate samples from a typical experiment (performed at least four times) and their standard deviation.

nicotinic acid for NAD synthesis [18,19]. Therefore, it is likely that by internalizing little amounts of nicotinic acid, *pncA* mutant was able to replicate to some extent in medium with nicotinamide.

Phagosomes containing virulent *B. abortus* are reluctant to fuse with lysosomes, whereas dead *B. abortus* phagosomes co-localize with endocytic compartments in the early stage of infection in macrophages [20]. To test the ability of *B. abortus* to target properly within macrophages early in infection, interaction of the mutants with the endocytic pathway was quantified by immunofluorescence localization of LAMP-1, a membrane protein of late endosomes and lysosomes [21]. As expected, most phagosomes containing the wild-type and complemented strain did not co-localize with the LAMP-1 (15.2 ± 2.3% or 13.8 ± 4.2% positive). Interestingly, *pncA* mutation did not affect LAMP-1 acquisition (17.2 ± 4.8% positive) (Fig. 3). It suggests that both wild-type strain and *pncA* mutant are not co-localizing with either late endosomes or lysosomes.

3.2. B. abortus pncA *mutant has reduced virulence in mice*

To assess whether the reduced in vitro virulence of *pncA* mutant as determined by the replication within HeLa cells and macrophags correlates with a virulence in the host, mice were infected with wild-type *B. abortus*, *pncA* and *virB4* mutant. As bacterial growth in spleen and liver are same level [22,23], only bacterial growth in spleen

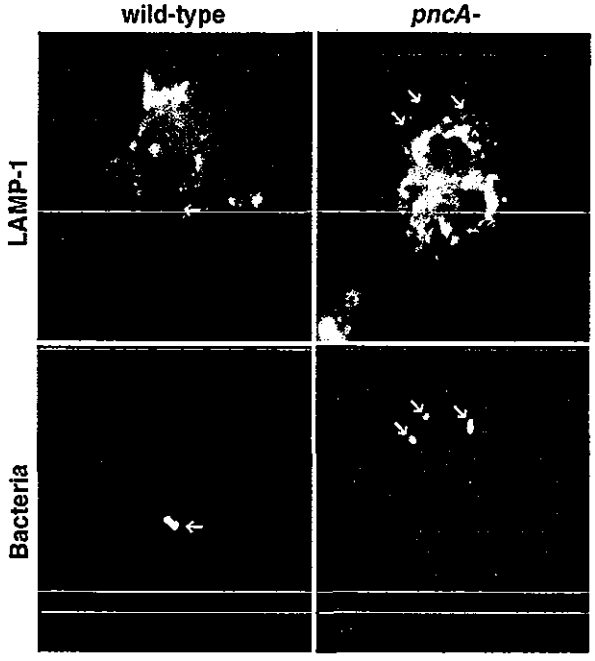

Fig. 3. Localization of *pncA* mutant and late endosomal and lysosomal marker LAMP-1 in bone marrow-derived macrophages by immunofluorescence microscopy. Macrophages were infected with wild-type or *pncA* mutant for 1 h, fixed and stained for LAMP-1 and intracellular bacteria.

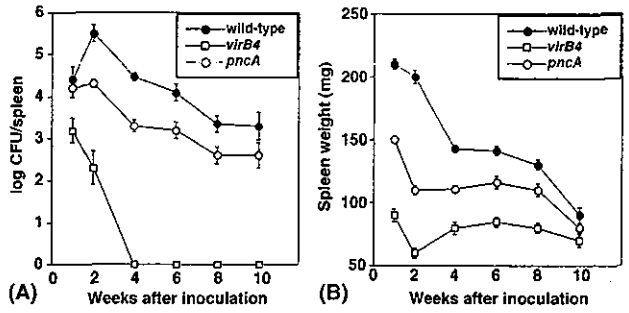

Fig. 4. Kinetics of bacterial growth in infected mice. Mice were infected intraperitoneally with wild-type *B. abortus*, *pncA* or *virB4* mutant (10^4 CFU/0.1 ml). Recovery of viable bacteria from the spleen (A) and the weights of spleens (B) of infected mice at 1–10 weeks postinfection are shown. Error bars indicate standard deviation.

was checked in this study. Two weeks after infection, many bacteria were recovered from the spleen of mice infected with the wild-type strain (6.7×10^5 CFU/spleen), but fewer bacteria were recovered from mice infected with *pncA* mutant (4.2×10^4 CFU/spleen) (Fig. 4A). Bacteria were still recovered from the spleen of mice infected with wild-type strain at 10 weeks after infection (6.8×10^3 CFU/spleen), while fewer bacteria were recovered from mice infected with *pncA* mutant (7.8×10^2 CFU/spleen). In contrast, *virB4* mutant was cleared to the undetectable level in the spleen at four weeks after infection (Fig. 4A).

Wild-type strain induced splenomegaly as a consequence of host inflammatory response, but *pncA* mutant induced a reduced response compared to that of the wild-type strain (Fig. 4B). Splenomegaly was not observed at all in mice infected with *virB4* mutant.

3.3. *Effect of vaccination with* B. abortus pncA *mutant*

One of the drawbacks of the vaccine strain *B. abortus* S19 is that it displays some degree of virulence causing abortion in pregnant cows [24]. In order to determine if the less pathogenic *pncA* mutant remains immunogenic,

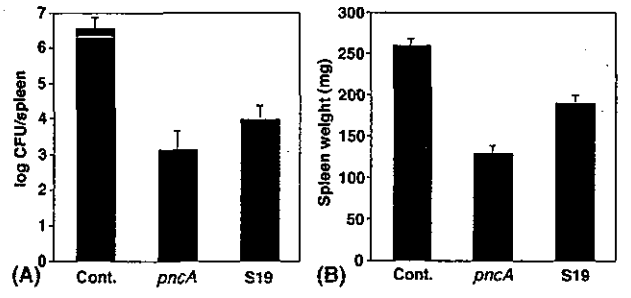

Fig. 5. Protection against *B. abortus* 544 in mice vaccinated with *pncA* mutant or *B. abortus* S19. Mice were inoculated intraperitoneally with 10^4 CFU of *pncA* mutant or strain S19. Eight weeks postvaccination, mice were challenged with *B. abortus* 544. Two weeks later, mice were killed and the numbers of viable *Brucella* recovered from the spleens (A) and the weights of the spleens (B) were determined as described in Section 2. Error bars indicate standard deviation.

experiment of protection was carried out in mice. It was shown that vaccination with *B. abortus pncA* mutant protected mice against a challenge with the virulent strain *B. abortus* 544 to the same extent as *B. abortus* S19 (Fig. 5). This indicated that *pncA* mutant with a reduced virulence and an impaired ability for intracellular multiplication completely retained the ability to induce the protective immunity in mice.

4. Discussion

The *pncA* gene encoding nicotinamidase/pyrazinamidase is an enzyme involved in the production of NAD. *Salmonella emterica* serovar Typhimurium and *Escherichia coli* use PncA in the preferred exogenous pathway for NAD synthesis [25]. In the less preferred endogenous pathway, NadB, NadA, NadC catalyse the conversion of aspartate to nicotinate D-ribonucleotide. The genome of *Brucella melitensis* and *Brucella suis* contains PncA and PncB, NadD and NadE, but not NadB, NadA or NadC [14]. Likewise, *B. abortus* may rely upon a single pathway for NAD synthesis. *B. abortus* lacking *pncA* was able to survive and grow in vitro because Brucella broth contains nicotinamide, nicotinic acid, NAD and NADP. One or more of these compounds may permit the growth of *pncA* mutant at the same rate as wild-type strain. It is also possible that PncA participates in other pathways required for survival and growth in vivo. As a recent study reported that plasmid-encoded PncA contributed to infectivity of *Borrelia burgdorferi* in mice [16], PncA may have important roles for virulence of intracellular pathogens. Our results in this study indicate that both wild-type strain and *pncA* mutant prevent phagosome–lysosome fusion after uptake by macrophages. Thus, PncA does not contribute to intracellular trafficking of *B. abortus*, but contribute to utilization of nutrients required for intracellular growth.

PncA is involved in the conversion of pyrazinamide to pyrazinoic acid. Pyrazinamide is an important antituberculosis drug and mutation of *pncA* is a major mechanism of pyrazinamide resistance in *Mycobacterium tuberculosis* [26]. Unlike most antibacterial agents, pyrazinamide, despite its remarkable in vivo activity, has no activity against *M. tuberculosis* in in vitro except at an acidic pH [27]. Presumably, pyrazinamidase would act under acidic environment. Vacuole acidification in phagocytic cells to pHs between 4.0 and 4.5 has been shown to be essential for intracellular survival during early infection by *B. suis* [28]. Pyrazinamidase may participate in the resistance of *Brucella* spp. to the acidic condition of the phagosome.

B. abortus virB mutant was cleared from the infected mice faster than wild-type strain. On the other hand, *pncA* mutant did not replicate in mouse spleen, but it was not cleared from the infected mice soon. These results indicate that *virB* genes have important roles in resistance to clearance by host immunity. Our results showed that the *pncA* mutant protected mice against a challenge with the pathogenic *B. abortus* strain 544 to the same extent as attained by the *B. abortus* vaccine strain S19. The decreased virulence with retention of the capacity to confer immunity suggests that detailed characterizations of the *pncA* mutant may improve current live vaccines.

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Gangliosides Act as Co-receptors for *Salmonella enteritidis* FliC and Promote FliC Induction of Human β-Defensin-2 Expression in Caco-2 Cells*

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Antimicrobial peptides such as defensins are crucial for host defense at mucosal surfaces. We reported previously that *Salmonella enteritidis* flagellin (FliC) induced human β-defensin-2 (hBD-2) mRNA expression in Caco-2 cells via NF-κB activation (Ogushi, K., Wada, A., Niidome, T., Mori, N., Oishi, K., Nagatake, T., Takahashi, A., Asakura, H., Makino, S., Hojo, H., Nakahara, Y., Ohsaki, M., Hatakeyama, T., Aoyagi, H., Kurazono, H., Moss, J., and Hirayama, T. (2001) *J. Biol. Chem.* 276, 30521–30526). In this study, we examined the role of ganglioside as co-receptors with Toll-like receptor 5 (TLR5) on FliC induction of hBD-2 expression in Caco-2 cells. Exogenous gangliosides suppressed FliC induction of hBD-2 promoter activity and binding of FliC to Caco-2 cells. Incorporation of exogenous ganglioside GD1a into Caco-2 cell membranes increased the effect of FliC on hBD-2 promoter activity. In support of a role for endogenous gangliosides, incubation of Caco-2 cells with DL-*threo*-2-hexadecanoylamino-3-morpholino-1-phenylpropanol, a glucosylceramide synthase inhibitor, reduced FliC induction of hBD-2 promoter activity. GD1a-loaded CHO-K1-expressing TLR5 cells had a higher potential for hBD-2 induction following FliC stimulation than GD1a-loaded CHO-K1 cells not expressing TLR5. FliC increased phosphorylation of mitogen-activated protein kinase, p38, and ERK1/2. Exogenous gangliosides GD1a, GD1b, and GT1b each suppressed FliC induction of p38 and ERK1/2 phosphorylation. Furthermore, FliC did not enhance luciferase activity in Caco-2 cells transfected with a plasmid containing a mutated activator protein 1-binding site. These results suggest that gangliosides act as co-receptors with TLR5 for FliC and promote hBD-2 expression via mitogen-activated protein kinase.

Antimicrobial peptides play an important role in host defense against bacteria, fungi, and viruses (1, 2). Defensins are antimicrobial cationic peptides that are stabilized by three intramolecular disulfide bonds. In humans, the defensin family consists of α- and β-defensins, with positions of the conserved cysteines defining type. Six α-defensins (HD[1]-1 to HD-6) have been reported. HD-1, HD-2, HD-3, and HD-4 were found in granulocytes (3), whereas HD-5 and HD-6 were identified in the Paneth cells of small intestinal crypts (4, 5) and in female reproductive tissue (6). Six β-defensins (hBD-1 to hBD-6) have also been described. HE2β1, identified as a splicing variant of the human EP2 gene, also contains the β-defensin-specific cysteine motif (7). hBD-1 was purified from plasma (8) and detected in several epithelial tissues (9). hBD-2 was purified from skin and shown to be expressed in the lung, trachea, and uterus (10). hBD-3 was purified from psoriatic scales and detected in several organs (*e.g.* skin and tonsil) (11). hBD-4 was strongly expressed in the testis and gastric antrum (12). hBD-5, hBD-6, and HE2β1 were specifically expressed in the epididymis (13). Although hBD-1 was expressed constitutively, hBD-2, hBD-3, and hBD-4 were induced following bacterial infections (10–12, 14, 15).

In addition to their antimicrobial activity, hBD-2 and murine β-defensins can potentially function as chemokines for immature dendritic cells and memory T cells through interaction with chemokine receptor CCR6 (16, 17). Recently, it was reported that murine β-defensin 2 is a ligand for Toll-like receptor (TLR) 4 on immature dendritic cells (18). TLRs function as pattern recognition molecules for various microbial components and endogenous ligands and mediate activation of the innate immune response (19, 20). Thus, induction of hBD-2 may also affect inflammatory reactions.

Salmonella is a Gram-negative bacterium that causes gastroenteritis and enteric fever in humans. *Salmonella* infection of cultured intestinal epithelial cells resulted in hBD-2 induction (15). However, the signaling pathways involved in hBD-2 induction in Caco-2 cells, a human colon carcinoma line, by *Salmonella* are still unclear. From our previous work to evaluate the role of hBD-2 in *Salmonella enteritidis* infection, we concluded that *S. enteritidis* flagellin (FliC) increased hBD-2 promoter activity and mRNA levels in Caco-2 cells via NF-κB

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[1] The abbreviations used are: HD, human defensin; AP-1, activator protein 1; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; Me_2SO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; FCS, fetal calf serum; FGF-2, fibroblast growth factor-2; FITC, fluorescein isothiocyanate; hBD, human β-defensin; HE, human epididymis secretory protein; IL, interleukin; MAP, mitogen-activated protein; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PPMP, DL-*threo*-2-hexadecanoylamino-3-morpholino-1-phenylpropanol; TBS, Tris-buffered saline; TLR, Toll-like receptor; CHO, Chinese hamster ovary.

activation (21). Other studies demonstrated that the flagellin of *Salmonella dublin* (22) or *Salmonella typhimurium* (23) induced nitric-oxide synthase and IL-8 secretion, respectively.

It has been demonstrated that TLR5 is a receptor for flagellin of Gram-negative, as well as Gram-positive, bacteria (24). After binding of FliC to TLR5 (24, 25), myeloid differentiation factor 88 was involved in NF-κB activation, resulting in IL-6 expression (24). We reported recently that *S. enteritidis* FliC increased intracellular Ca^{2+} concentration via activation of phospholipase C, leading to translocation of NF-κB to the nucleus and increased hBD-2 promoter activity (26). However, the signal transduction pathways involved in hBD-2 induction by FliC are still unclear.

Gangliosides, sialic acid-containing glycosphingolipids, are ubiquitous components of eukaryotic cell membranes that have been identified as receptors for bacterial toxins and viruses (27–30). Although TLR5 was determined to be a FliC receptor, gangliosides such as GM1, GD1a, and asialo-GM1 also bound *Pseudomonas aeruginosa* flagellin (31). In particular, asialo-GM1, serving as a *P. aeruginosa* flagellin receptor, was critical for flagellin-dependent signaling in epithelial cells (32). Recently, Yu *et al.* (33) reported that induction of p38 MAP kinase phosphorylation by *S. typhimurium* flagellin was mediated by activation of TLR5 rather than through asialo-GM1.

In this study, to evaluate signal transduction pathways used by FliC, we examined the effect of gangliosides and TLR5 on FliC induction of hBD-2 expression in Caco-2 cells, focusing on the potential role of complex gangliosides as TLR co-receptors.

MATERIALS AND METHODS

Antibodies and Reagents—Ganglioside mixture was obtained from IsoSep AB; GM1, asialo-GM1, GM2, GM3, GD1a, GD1b, GD3, and GT1b were from Wako; anti-GD1a, GD1b, and GT1b antibodies were from Seikagaku Corp.; anti-asialo-GM1 antibody was from Wako. DL-*threo*-PPMP hydrochloride was from Wako. FITC-labeled goat anti-rabbit IgG was from BIOSOURCE International, Inc.; anti-phospho-p38 and phospho-ERK1/2 antibodies were from Cell Signaling Technology, Inc.; horseradish peroxidase-conjugated anti-rabbit IgG or mouse IgG antibodies were from DAKO; horseradish peroxidase-conjugated anti-V5 antibody was from Invitrogen; and transfection reagent (DAC-30) was from Eurogentec. Plasmid encoding V5-tagged TLR5 (pEF6V5/HIS TOPO::TLR5) (24) was a gift from Dr. Kelly D. Smith and Dr. Alan Aderem. *Helicobacter pylori* vacuolating cytotoxin (VacA) was purified as described previously (34).

Cell Culture—Caco-2 (human colon carcinoma cells) and Chinese hamster ovary (CHO) cells were grown in DMEM (Sigma) supplemented with 10% FCS. CHO-K1, which do not synthesize complex gangliosides (35), were grown in Ham's F-12 medium (IWAKI, Asahi Techno Glass) supplemented with 10% FCS.

Transfection and Luciferase Assay—Transfection and luciferase assays were performed as described previously (21) with the following modifications. To assess hBD-2 promoter activity, Caco-2 cells were seeded in 24-well culture plates (0.5 or 1.0×10^5 cells in 1 ml of DMEM per well) and incubated at 37 °C for 24 h. 2.5 μg of the hBD-2 promoter linked to a luciferase reporter gene (pGL3-2110 or pGL3-938) or a mutated hBD-2 promoter construct (pGL3-938/NF-κBmt or pGL3-938/AP1mt) were incubated with 0.5 μg of an internal control *Renilla* luciferase expression vector (pRL-TK) and 10 μl of 3.2 mM dendritic poly-(L-lysine) (KG6) (36) in 250 μl of FCS-free DMEM at room temperature for 15 min before addition to Caco-2 cells. After incubation at 37 °C for 3 h, 1 ml of DMEM was added, followed by incubation at 37 °C for 24 h.

To initiate experiments, the medium was replaced with 200 μl of fresh FCS-free DMEM, and 50 μl of the sample to be assayed were added. After incubation at 37 °C for 3 or 6 h, cells were washed with 1 ml of PBS and lysed by adding 300 μl of lysis buffer (Toyo Ink Co.). After 15 min at room temperature, the lysate was centrifuged (18,000 × *g*, 5 min, 4 °C). Luciferase activity of the supernatant, measured using a luminometer (Berthold), was expressed relative to the activity of an internal control *Renilla* luciferase.

To assess the effects of TLR5 expression and GD1a loading on FliC induction of hBD-2 promoter activity, CHO-K1 cells were seeded in 24-well culture plates (1.0×10^5 cells per well) and incubated at 37 °C for 24 h. 1.5 μg of pGL3-2110 were incubated with 0.5 μg of pRL-TK, 1 μg of TLR5 expression vector (pEF6V5/HIS TOPO::TLR5), and 5 μl of DAC-30 (2.5 μg) in 250 μl of FCS-free medium at room temperature for 20 min before addition to cells. After incubation at 37 °C for 4 h, CHO-K1 cells were incubated without or with GD1a (50 μg/ml) in FCS-free medium at 37 °C for 24 h. At the end of incubation, cells were washed with medium and then incubated with the indicated concentrations of FliC at 37 °C for 6 h. hBD-2 promoter activity was determined by the luciferase reporter gene assay.

Isolation of Flagellin from Bacterial Cells—S. enteritidis FliC and *P. aeruginosa* flagellin were prepared as previously described (21) with the following modifications. *S. enteritidis*, and *P. aeruginosa* grown in 2 liters of tryptic soy broth at 37 °C for 16 h were pelleted by centrifugation (5,000 × *g*, 4 °C, 30 min) and dispersed in 40 ml of PBS, which was adjusted to pH 2 with 1 M HCl and maintained at that pH with constant stirring at room temperature for 30 min. After centrifugation (100,000 × *g*, 4 °C, 1 h), the pH of the supernatant containing soluble monomeric flagellin was adjusted to 7.2 with 1 M NaOH, and solid $(NH_4)_2SO_4$ was added to 65% saturation. After incubation overnight at 4 °C, the mixture was centrifuged (15,000 × *g*, 4 °C, 15 min). The precipitate was dissolved in distilled water, dialyzed against distilled water, then heated at 65 °C for 15 min, placed on ice, and centrifuged (100,000 × *g*, 4 °C, 1 h). To the supernatant, which contained depolymerized FliC, solid $(NH_4)_2SO_4$ was added to 65% saturation. After incubation overnight at 4 °C, the mixture was centrifuged at 15,000 × *g* at 4 °C for 15 min. The precipitate was dissolved in distilled water and dialyzed against PBS.

Effect of Exogenous Ganglioside on FliC Induction of hBD-2 Promoter Activity—Caco-2 cells, transfected with luciferase reporter gene, were incubated with FliC (100 ng/ml) and the indicated concentration of ganglioside at 37 °C for 3 or 6 h. hBD-2 promoter activity was determined using the luciferase reporter gene assay.

Effect of Anti-ganglioside Antibodies on FliC Induction of hBD-2 Promoter Activity—Caco-2 cells were seeded in 24-well culture plates (5×10^4 cells per well) and incubated at 37 °C for 24 h before transfection with luciferase reporter gene as described above and incubation with the indicated concentration of anti-GD1a, -GD1b, or -GT1b antibodies at 37 °C for 1 h followed by incubation with FliC (100 ng/ml) at 37 °C for 6 h. hBD-2 promoter activity was determined using the luciferase reporter gene assay.

Flow Cytometric Analysis—Caco-2 cells (1×10^7 cells) were harvested in TNE (40 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM EDTA), washed twice with PBS, and suspended in 1 ml of PBS. Samples (90 μl) were incubated at 4 °C for 1 h with FliC, 0, 10, or 100 μg/ml FliC, or with FliC (100 μg/ml) plus 100 μM GD1a. After two washes with PBS containing 2% BSA, cells were incubated (4 °C, 1 h) with anti-FliC antibody diluted 1:100 with PBS (21), washed twice with PBS containing 2% BSA, and incubated at 4 °C for 30 min with FITC-labeled goat anti-rabbit IgG (1:100). After three washes with PBS containing 2% BSA, samples (10,000 cells) were analyzed by flow cytometry (BD Pharmingen Immunocytometry system) with excitation at 488 nm and emission at 530 nm.

Proteolytic Digestion of FliC—Protection against tryptic digestion of FGF-2 by gangliosides has been reported (37). *S. enteritidis* FliC (3 μg), VacA (3 μg), or *P. aeruginosa* flagellin (3 μg) was incubated in 50 mM Tris-HCl, pH 7.5, with or without the indicated amount of ganglioside at 37 °C for 5 min, followed by addition of trypsin (Sigma), 60 ng in a final volume of 100 μl, and incubation at 37 °C for 3 h, before addition of an equal volume of SDS sample buffer (0.1 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.1% (w/v) bromphenol blue, 50 mM dithiothreitol), and heating at 95 °C for 5 min. Samples were subjected to SDS-PAGE in 10% gels, which were stained with Coomassie Brilliant Blue.

Inhibition of Ganglioside Synthesis and Incorporation of Exogenous GD1a into Caco-2 Cell Membranes—Caco-2 cells were seeded in 24-well culture plates (5×10^4 cells per well), incubated for 24 h at 37 °C, and then further incubated (37 °C, 48 h) without or with 5 μM PPMP (38, 39). pGL3-2110-transfected Caco-2 cells were incubated with GD1a (50 μg/ml) in FCS-free medium without or with 5 μM PPMP for 24 h at 37 °C (39, 40), before washing with DMEM and incubation (37 °C, 3 h) with the indicated concentration of FliC without or with 5 μM PPMP. hBD-2 promoter activity was determined by the luciferase reporter gene assay.

Western Blotting Analysis—Caco-2 cells were seeded in 6-well culture plates (3×10^5 cells per well). After 24 h, the medium was replaced with FCS-free DMEM, and the cells were incubated at 37 °C for 24 h. FliC (10 μg/ml) was added, and after 30 min at 37 °C, cells were washed with PBS and lysed in 300 μl of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 0.1% (w/v) bromphenol blue, 50 mM

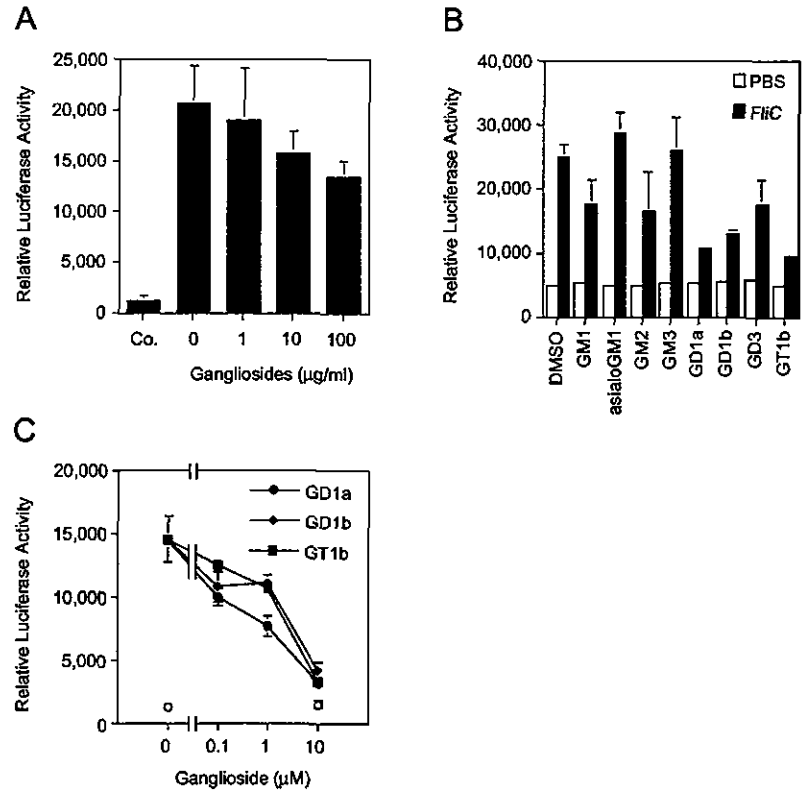

FIG. 1. **Effect of added ganglioside on FliC induction of hBD-2 promoter activity in Caco-2 cells.** *A*, pGL3-2110-transfected Caco-2 cells were incubated (37 °C, 6 h) with FliC (100 ng/ml) plus the indicated concentration of ganglioside mixture dissolved in Me_2SO (final concentration of Me_2SO is 1%) before assay of *hBD-2 promoter activity* (luciferase reporter gene). There is no difference between the effect of 1% Me_2SO and no Me_2SO. *B*, pGL3-2110-transfected Caco-2 cells were incubated (37 °C, 6 h) without (PBS) or with FliC (100 ng/ml) and 10 μM GM1, asialo-GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b, or vehicle (Me_2SO) before luciferase activity assay. *C*, pGL3-2110-transfected Caco-2 cells were incubated (37 °C, 3 h) without (*open symbols*) or with FliC (100 ng/ml) plus the indicated concentration of GD1a (●), GD1b (◆), or GT1b (■) before assay of luciferase activity. Data in this figure are means ± S.D. of values from three separate experiments with assays in duplicate. *DMSO*, Me_2SO_4.

dithiothreitol, 1 mM Na_3VO_4). Proteins were separated by SDS-PAGE in 10% gels and transferred to polyvinylidine difluoride membranes, which were washed with TBS (20 mM Tris-HCl, 133 mM NaCl, 1 mM Na_3VO_4) at room temperature for 5 min, and then incubated with 5% (w/v) milk powder in TBS-T (20 mM Tris-HCl, 133 mM NaCl, 0.1% Tween 20, 1 mM Na_3VO_4) at room temperature for 1 h. Membranes were washed with TBS-T, followed by incubation in a 1:1,000 dilution of anti-phospho-p38 or -phospho-ERK1/2 antibodies overnight at 4 °C. After washing in TBS-T, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1:4,000 dilution) at room temperature for 1 h and then washed in TBS-T, followed by incubation for 5 min in TBS before detection by ECL system (Amersham Biosciences).

To assess *TLR5 expression*, *CHO or CHO-K1* cells transfected with TLR5 expression vector were washed with PBS and lysed in 200 μl of SDS sample buffer. Proteins were resolved by SDS-PAGE in 10% gels and transferred to polyvinylidine difluoride membranes, which were washed with TBS at room temperature for 5 min, and then incubated with 5% (w/v) milk powder in TBS-T at room temperature for 1 h. Membranes were washed with TBS-T, followed by incubation in a 1:2,000 dilution of horseradish peroxidase-conjugated anti-V5 antibody at room temperature for 1 h, then washed in TBS-T, followed by incubation for 5 min in TBS before detection by ECL system.

RESULTS

Effect of Exogenous Gangliosides on FliC Induction of hBD-2 Promoter Activity in Caco-2 Cells—The hBD-2 promoter was coupled to luciferase, and activity was quantified in pGL3-2110-transfected Caco-2 cells incubated with FliC (100 ng/ml) and various amounts of ganglioside mixtures. Gangliosides inhibited hBD-2 promoter activity in a concentration-dependent manner (Fig. 1*A*). To determine the specificity of ganglioside inhibition, several gangliosides were tested in the luciferase reporter gene assay. Asialo-GM1 and GM3 had no effect, whereas GM1, GM2, and GD3 were weakly inhibitory, and GD1a, GD1b, and GT1b strongly inhibited FliC action (Fig. 1*B*). To examine further the inhibitory effects of GD1a, GD1b, and GT1b, we used the luciferase reporter gene assay. Gangliosides GD1a, GD1b, and GT1b each inhibited FliC induction of hBD-2 promoter activity in a concentration-dependent manner (Fig. 1*C*), with more than 90% inhibition at 10 μM. These results showed that exogenous gangliosides suppress FliC action when added to Caco-2 cell culture medium.

Effects of Anti-ganglioside Antibodies on FliC Induction of hBD-2 Promoter Activity in Caco-2 Cells—To confirm the role of FliC interaction with gangliosides on signal transduction, gangliosides on the cell surface were masked by reaction with anti-ganglioside antibody, and then FliC induction of hBD-2 promoter activity was evaluated with the luciferase assay. After masking of native GD1a, GD1b, and GT1b on Caco-2 cells by antibodies, FliC induction of hBD-2 promoter activity was inhibited in a concentration-dependent manner (Fig. 2). Anti-asialo-GM1 and isotype control antibodies had no effects (data not shown). These results suggested that native gangliosides on Caco-2 cells are functionally important for FliC induction of signaling, leading to hBD-2 expression.

Exogenous GD1a Binds to FliC and Prevents Binding of FliC to Caco-2 Cells—Rusnati *et al.* (37) reported that gangliosides directly bind to fibroblast growth factor 2 (FGF-2) and protect it from tryptic digestion. Furthermore, they recently reported (41) that GM1 acts as *functional co-receptor for FGF-2*. To determine whether gangliosides directly bind to FliC, we determined whether ganglioside interaction with FliC can prevent its digestion by trypsin. GD1a protected FliC from tryptic digestion in a concentration-dependent manner, but asialo-GM1 had no effect (Fig. 3*A*), and GD1a did not protect an unrelated bacterial toxin VacA from tryptic digestion (Fig. 3*B*). Both GD1a and asialo-GM1 had no effect on tryptic digestion of *P. aeruginosa* flagellin (Fig. 3*C*).

We investigated the effect of exogenous GD1a on binding of FliC to Caco-2 cells using fluorescence-activated cell sorting. FliC bound to Caco-2 cells in a concentration-dependent manner, and exogenous GD1a suppressed FliC binding to Caco-2 cells (Fig. 4), consistent with the notion that exogenous GD1a directly interacts with FliC and prevents binding of FliC to Caco-2 cells.

Effects of Ganglioside Depletion and GD1a Loading on FliC Induction of hBD-2 Promoter Activity in Caco-2 Cells—To con-

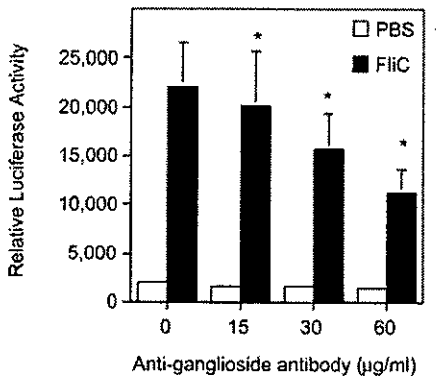

FIG. 2. **Effect of anti-ganglioside antibodies on FliC induction of hBD-2 promoter activity in Caco-2 cells.** pGL3-2110-transfected Caco-2 cells were incubated (37 °C, 1 h) first with the indicated concentration of a mixture of equal amounts (each 5, 10, and 20 μg/ml) of anti-GD1a, -GD1b, and -GT1b antibodies and then without (PBS) or with FliC (100 ng/ml) before assay of luciferase activity. Data are means ± S.D. of values from three separate experiments with assays in duplicate. *, $p < 0.05$ *versus* values without anti-ganglioside antibody.

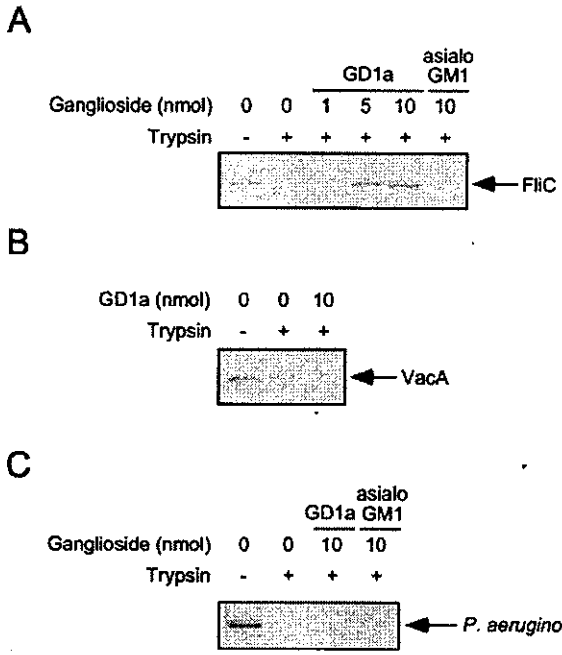

FIG. 3. **Protection of FliC from tryptic digestion by ganglioside GD1a.** 3 μg of *S. enteritidis* FliC (*A*), *H. pylori* VacA (*B*), or *P. aeruginosa* flagellin (*C*) was incubated (37 °C, 3 h) with 60 ng of trypsin without or with 1, 5, or 10 nmol of GD1a or 10 nmol of asialo-GM1 before separation of proteins by SDS-PAGE (10% gel) and staining with Coomassie Brilliant Blue. Data are representative of three experiments.

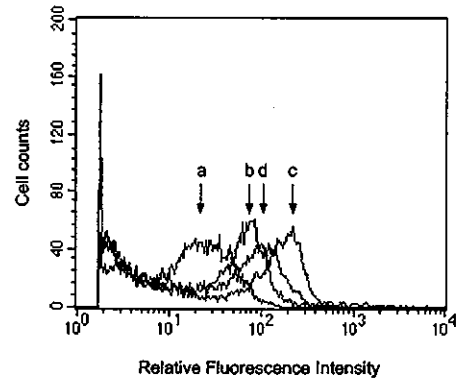

FIG. 4. **Effect of exogenous GD1a on binding of FliC to Caco-2 cells.** Caco-2 cells were incubated (4 °C, 1 h) with 0 (*a*), 10 (*b*), 100 μg/ml FliC (*c*), or 100 μg/ml FliC plus 100 μM GD1a (*d*) and then with anti-FliC antibody for 1 h, followed by 30 min with FITC-labeled goat anti-rabbit IgG and FACScan analysis. Data are representative of three experiments.

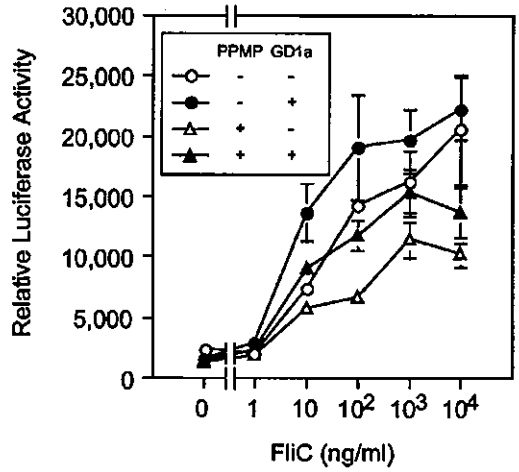

FIG. 5. **Effects of PPMP and GD1a on FliC induction of hBD-2 promoter activity in Caco-2 cells.** Caco-2 cells were incubated (37 °C, 48 h) without or with 5 μM PPMP before transfection with pGL3-2110 luciferase reporter gene and incubation (37 °C, 24 h) without or with GD1a (50 μg/ml) and/or 5 μM PPMP as indicated. Cells were then washed with DMEM and incubated (37 °C, 3 h) with the indicated concentration of FliC without or with 5 μM PPMP before assay of luciferase activity. Data are means ± S.D. of values from three separate experiments with assays in duplicate.

firm that native gangliosides in Caco-2 cells serve as a FliC co-receptor, Caco-2 cells were incubated with 5 μM PPMP, an inhibitor of glucosylceramide synthase before stimulation with FliC. FliC induction of hBD-2 promoter activity was reduced in PPMP-treated Caco-2 cells. GD1a-loaded Caco-2 cells exhibited greater hBD-2 expression by following FliC stimulation than did untreated cells. Furthermore, incubation of ganglioside-depleted cells with GD1a restored FliC responsiveness (Fig. 5). These results also supported the hypothesis that ganglioside is functionally important as a FliC co-receptor.

Effects of TLR5 Expression and GD1a Loading on FliC induction of hBD-2 Promoter Activity in CHO-K1 Cells—To confirm further that the gangliosides serve as a FliC co-receptor, we determined the effects of TLR5 expression and GD1a loading on FliC induction of hBD-2 promoter activity in CHO-K1 cells. First TLR5 expression in cells was confirmed using Western blotting analysis (Fig. 6*A*). Both lines of TLR5-expressing cells respond to FliC in a concentration-dependent manner (Fig. 6, *B* and *C*). Induction of hBD-2 was increased by prior loading of TLR5-expressed CHO-K1 cells with GD1a, but CHO-K1 cells not expressing TLR5 were unresponsive to FliC (Fig. 6*D*). These results supported the hypothesis that gangliosides serve as a co-receptor for FliC.

Effects of Exogenous Gangliosides on FliC Induction of MAP Kinase Phosphorylation in Caco-2 Cells—To examine the effect of ganglioside on MAP kinase phosphorylation, Caco-2 cells were incubated with FliC plus gangliosides. p38 and ERK1/2 phosphorylation by FliC were significantly inhibited by the presence of GD1a, GD1b, or GT1b (Fig. 7).

Effect of Mutation in the AP-1 Site of the hBD-2 Promoter on Response of Caco-2 Cells to FliC—To determine the importance of AP-1 for induction of hBD-2, we constructed pGL3-938/AP-1mt, a plasmid having a mutated AP-1 site. FliC increased activity in cells transfected with pGL3-938 but not pGL3-basic (empty vector), pGL3-938/NF-κBmt, and pGL3-938/AP-1mt (Fig. 8). This result suggested that AP-1 is also an important transcriptional factor for hBD-2 induction in Caco-2 cells by FliC.

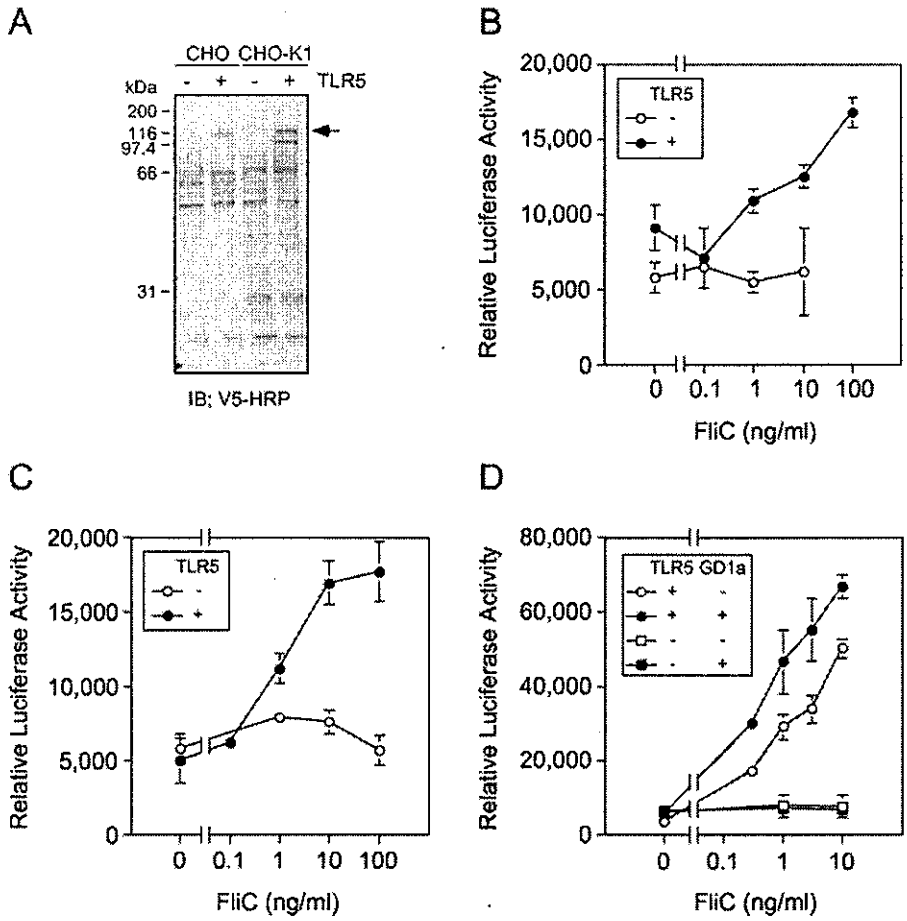

FIG. 6. **Effects of TLR5 expression and GD1a-loading on FliC induction of hBD-2 promoter activity in CHO or CHO-K1 cells.** *A*, CHO or CHO-K1 cells were transfected with pEF6V5/HIS TOPO::TLR5, lysed in SDS sample buffer, and then analyzed by Western blotting using anti-V5 antibody. *Arrow* indicates V5-tagged TLR5. Data are representative of three experiments. CHO (*B*) or CHO-K1 (*C*) cells were transfected with pGL3-2110 luciferase reporter gene without or with pEF6V5/HIS TOPO::TLR5 and then incubated with the indicated concentrations of FliC at 37 °C for 6 h. Luciferase activity was then determined. *D*, CHO-K1 cells were transfected with pGL3-2110 luciferase reporter gene without or with pEF6V5/HIS TOPO::TLR5, and then incubated without or with GD1a (50 μg/ml) at 37 °C for 24 h. At the end of incubation, cells were washed with serum-free medium, followed by incubation with the indicated concentrations of FliC at 37 °C for 6 h. Luciferase activity was then assayed. Data are means ± S.D. of values from three separate experiments with assays in duplicate.

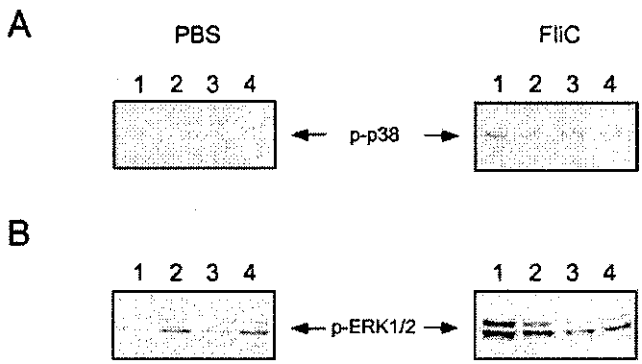

FIG. 7. **Effect of added ganglioside on FliC induction of p38 and ERK1/2 phosphorylation in Caco-2 cells.** Caco-2 cells were incubated (37 °C, 30 min) with PBS (*left panel*) or FliC (10 μg/ml, *right panel*) with Me_2SO (*lane 1*), 10 μM of GD1a (*lane 2*), 10 μM of GD1b (*lane 3*), or 10 μM of GT1b (*lane 4*) before lysis and separation of proteins by SDS-PAGE and immunoblotting with anti-phospho-p38 (*A*) and phospho-ERK1/2 (*B*) antibodies. Data are representative of three experiments.

DISCUSSION

Binding of *P. aeruginosa* flagellin to gangliosides such as GM1, GD1a, and asialo-GM1 had been reported in 1998 (31). In addition, asialo-GM1 on epithelial cells interacted with *P. aeruginosa* flagellin, leading to activation of phospholipase C, Ca^{2+} mobilization, phosphorylation of ERK1/2, and activation of mucin transcription (32). We demonstrate here that higher order gangliosides serve as a *S. enteritidis* FliC co-receptor and influence FliC induction of hBD-2 promoter activity in Caco-2 cells.

FliC induction of hBD-2 promoter activity in Caco-2 cells was inhibited by exogenous gangliosides, GD1a, GD1b, and GT1b (Fig. 1). These three gangliosides have a common backbone (GM1) plus additional one or two sialic acids (Fig. 9). Because exogenous GD1a protected FliC from tryptic digestion (Fig. 3*A*) and suppressed binding of FliC to Caco-2 cells (Fig. 4), it appears that exogenous GD1a directly interacts with FliC. Moreover, these data are consistent with the inhibitory effects of GD1a on FliC induction of hBD-2 promoter activity. In contrast to the *S. enteritidis* FliC, *P. aeruginosa* flagellin digestion by trypsin was not inhibited by GD1a and asialo-GM1 (Fig. 3*C*), suggesting that the sialic acids components of gangliosides have an important role in FliC binding and induction of promoter activity. In agreement, anti-GD1a, GD1b, and GT1b antibodies partially blocked FliC induction of hBD-2 promoter activity (Fig. 2). Increasing the GD1a content of Caco-2 cells enhanced FliC responsiveness; similarly, inhibition of ganglioside synthesis by treatment with PPMP decreased sensitivity to FliC, which was restored by GD1a loading (Fig. 5). In addition, as shown in Fig. 6, GD1a loading of TLR5-expressing CHO-K1 cells increased hBD-2 induction by FliC, whereas GD1a-loaded CHO-K1 cells were not responsible to FliC.

Glycosphingolipid, particularly gangliosides, sphingomyelin, and glycosylphosphatidylinositol-anchored proteins exist in glycosphingolipid-enriched microdomain (lipid rafts) in the plasma membrane. Li *et al.* (42) provided a perspective on the biological effect of gangliosides on cell proliferation. Depletion of the gangliosides conceivably abolishes the formation of glycosphingolipid clusters in the cell plasma membrane, and consequently, this could be one mechanism leading to blocked growth factor-induced proliferation. Conversely, preincubation of the cells with gangliosides, which causes enrichment of gangliosides (GD1a) in the plasma membrane and thereby potentially enhances the formation of glycosphingolipid cluster, promoted growth factor-induced proliferation (42) through

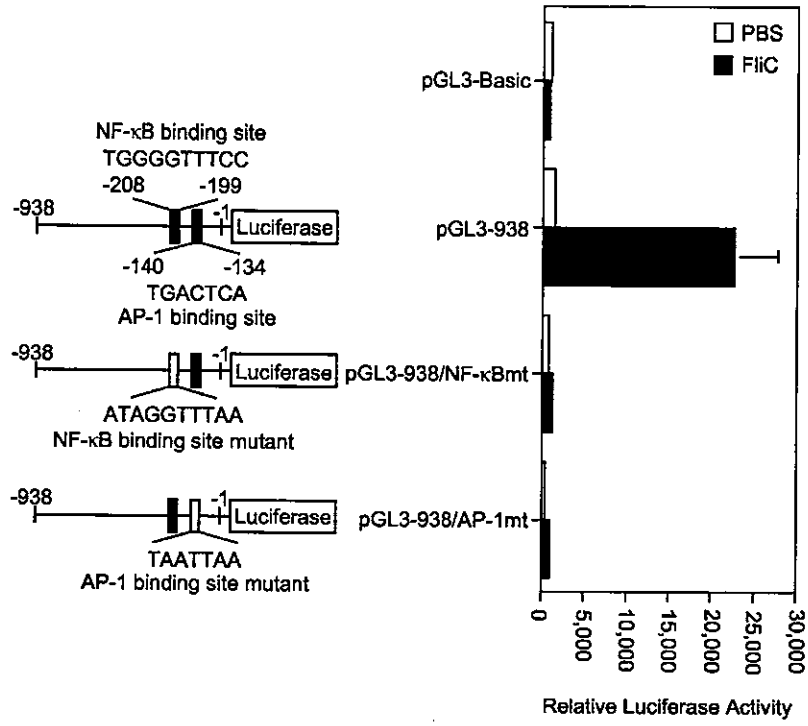

FIG. 8. **Effect of mutation in the AP-1 site of the hBD-2 promoter on response of Caco-2 cells to FliC.** Luciferase reporter gene constructs of the hBD-2 promoter region with putative binding elements for NF-κB and AP-1 are depicted diagrammatically on the *left* with their activities ($\times 10^{-3}$) in the luciferase assay on the *right*. *Open bars* represent activities without and *solid bars* with FliC (100 ng/ml) incubation. Data are the means ± S.D. of values from three separate experiments with assays in duplicate.

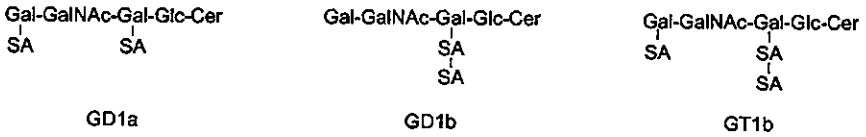

FIG. 9. **Structure of GD1a, GD1b, and GT1b, which inhibited FliC induction of hBD-2 promoter activity in Caco-2 cells.** *Cer*, ceramide; SA, sialic acid.

enhancement of growth factor signaling and activation of Src kinase by gangliosides (43). In addition, by coupling to glycosphingolipid, *Escherichia coli* P fimbriae utilize the TLR4-dependent pathway to trigger cytokine responses, consistent with the conclusion that the glycosphingolipid receptors for P fimbriae recruit TLR4 as a co-receptor (44, 45). Thus, these results supported our hypothesis that cell membrane gangliosides such as GD1a, GD1b, and GT1b function as co-receptors for FliC with TLR5.

TLRs can activate not only NF-κB signaling pathways but also MAP kinase-dependent signaling. MAP kinase pathways are thought to play a major role in the activation of gene transcription by proinflammatory cytokines and antimicrobial peptides. Hobbie *et al.* (46) showed that the inflammatory response induced by *S. typhimurium* may be because of the specific stimulation of MAP kinase signaling pathways, leading to nuclear responses. In agreement, FliC increased phosphorylation of MAP kinases, p38 and ERK1/2, whereas exogenous GD1a, GD1b, and GT1b blocked FliC induction of p38 and ERK1/2 phosphorylation (Fig. 7). The promoter region of the hBD-2 gene contains an AP-1-binding site as well as NF-κB-binding sites. AP-1 is a homo- or heterodimer of proteins of the Jun and Fos families, transcription factors activated by MAP kinase pathways. Recently, Krisanaprakornkit *et al.* (47) reported that MAP kinase pathways involving the AP-1 transcription family are important for *Fusobacterium nucleatum*-dependent hBD-2 induction in gingival epithelial cells. We had reported that FliC enhanced hBD-2 promoter activity in Caco-2 cells transfected with pGL3-938, containing the 5′-flanking region of the hBD-2 gene (−938 to −1) linked to a luciferase reporter but did not enhance activity with pGL3-938/NF-κBmt containing a mutated NF-κB-binding site (21). We constructed a reporter gene pGL3-938/AP-1mt with a mutated AP-1 site. FliC increased luciferase activity in Caco-2 cells transfected with pGL3-938 but not with pGL3-basic (empty vector), PGL3–938/NF-κBmt, and pGL3-938/AP-1mt (Fig. 8). These data suggest that both NF-κB and AP-1 are important for hBD-2 induction by *S. enteritidis* FliC in Caco-2 cells.

P. aeruginosa can activate MAP kinase signaling pathways through effects on intracellular Ca^{2+} concentration (48). Gouy *et al.* (49) showed that an increase in intracellular Ca^{2+} concentration can be elicited by human Jurkat T cell line by using cholera toxin B subunits to ligate ganglioside GM1. It would appear that GM1 may act as a cell activation molecule associated with unidentified transmembrane protein(s) (49). We reported previously (26) that FliC increased intracellular Ca^{2+} concentrations, followed by induction of hBD-2 expression via an NF-κB-dependent pathway. In this study, we reported that exogenous gangliosides inhibited not only FliC induction of hBD-2 promoter activity but also MAP kinase phosphorylation. Endogenous gangliosides by serving as co-receptors with TLR5 may increase intracellular Ca^{2+} concentration and MAP kinase phosphorylation by FliC. Moon *et al.* (50) showed that an Src-dependent Raf-MEK1/2-ERK signaling pathway is required for IL-1α-induced β-defensin 2 in human middle ear epithelial cells. Hazeki *et al.* (51) also showed that TLR regulates the function of paxillin through an Src family-dependent mechanism. FliC-initiated signals may activate related pathways because TLRs and the IL-1 receptor have homologous cytoplasmic domains and can induce expression of some of the same target genes.

In summary, we have shown that gangliosides such as GD1a, GD1b, and GT1b act as co-receptors for FliC and promote hBD-2 induction in Caco-2 cells. FliC can activate not only the NF-κB pathway but also MAP kinase pathways followed by enhancement of hBD-2 induction. Understanding the signaling pathways used by FliC could lead to novel therapeutic strategies for preventing the toxic effects of bacterial products.

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A Multicenter, Open-Label Clinical Study of Micafungin (FK463) in the Treatment of Deep-seated Mycosis in Japan

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The efficacy and safety of micafungin (FK463), which is a new lipopeptide antifungal agent of the echinocandin class and is active against both Aspergillus and Candida species, were investigated in patients with deep-seated mycosis in this study. 70 patients were treated with micafungin 12.5–150 mg/d intravenously for up to 56 d. The overall clinical response rates were 60% (6/10) in invasive pulmonary aspergillosis, 67% (6/9) in chronic necrotizing pulmonary aspergillosis, 55% (12/22) in pulmonary aspergilloma, 100% (6/6) in candidemia, and 71% (5/7) in esophageal candidiasis. The response rates for patients with prior antifungal treatment which was considered ineffective or toxic, were similar to rates for patients without prior treatment. Mycological eradication was observed in patients infected with Aspergillus fumigatus, Aspergillus flavus, Aspergillus terreus, Aspergillus niger, Candida albicans, Candida glabrata, or Candida krusei. Adverse events related to micafungin were reported in 21 patients (30%), and there was no dose-related occurrence of any adverse event. It is concluded that treatment with micafungin as monotherapy seems to be effective and safe in patients with deep-seated mycosis.

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INTRODUCTION

Recently, the incidence of systemic fungal infections has continued to increase, as more patients have undergone intensive chemotherapy or transplantation and have received long-term treatment with immunosuppressive agents (1–4). These therapies have successfully improved the survival of patients, but have produced a greater number of immunocompromized hosts at risk of fungal infections over longer periods (1). Systemic fungal infections are the most frequent causes of serious morbidity and mortality among these patients (2, 3, 5), indicating the difficulty in treating fungal infections. This may be explained by various factors, including difficulties in making an early diagnosis, and limited choices of antifungal therapy (2–6). Amphotericin B (AMPH-B) is considered to be the standard for systemic antifungal treatment. However, it is associated with significant toxicity, including dose-limiting nephrotoxicity (7, 8). Flucytosine is a pyrimidine class antifungal drug, but its utility is limited by its narrow spectrum of activity, toxic effects (nausea, bone marrow suppression, etc.), and the rapid emergence of resistance (7). The azole antifungal agents represent a recent advance in the management of systemic fungal infections. However, miconazole, which is the first azole drug, and is classified as an imidazole agent, has no remarkable advantages of efficacy or safety in comparison with other antifungal agents (7). As for triazole agents, fluconazole (FLCZ) and itraconazole (ITCZ) are generally well tolerated, but FLCZ has low activity against Aspergillus species (spp.), and its potential has been limited by the emergence of resistance of some Candida spp. (7). ITCZ has a broad spectrum of activity against both Aspergillus and Candida spp., but its gastrointestinal absorption is often poor in severely ill patients (9). Voriconazole is a new triazole agent with a broad-spectrum of activity and with both oral and intravenous formulations, but it sometimes causes reversible visual disturbance (10). These azole agents have the potential for drug interactions, as they alter hepatic metabolism via the cytochrome p-450 system (7, 11). Some new antifungal agents with a novel mechanism of antifungal activity have been developed recently. Caspofungin is classified in the echinocandin family. It has been investigated for efficacy and safety in invasive aspergillosis and esophageal candidiasis (12, 13).

Micafungin (FK463) is a new semisynthetic lipopeptide antifungal agent in the echinocandin class, the same class as caspofungin. The echinocandin agents have a novel mechanism of action by interference with (1, 3)-β-D-glucan synthesis. Non-clinical studies of micafungin indicated its broad-spectrum fungistatic activity against Aspergillus spp. and fungicidal activity against Candida spp. (14). In vivo studies have also demonstrated its antifungal activity in various animal models of fungal infection (15–17). Micafungin was well tolerated at doses ranging from 2.5 to 150 mg/d in

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