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NOTE *Public Health*

Serological Differentiation of *Brucella*-Vaccinated and -Infected Domesticated Animals by the Agar Gel Immunodiffusion Test Using *Brucella* Polysaccharide in Mongolia

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ABSTRACT. To investigate *Brucella* infection in cattle, sheep, goat, reindeer and yak in Mongolia, serological reactions of *Brucella*-infected and -vaccinated domestic animals were compared by the agar gel immunodiffusion (AGID) test with a polysaccharide (poly-B) of the *B.* Abortus strain S-19. The sensitivity and specificity were compared with conventional serological tests that are commonly used in Mongolia, such as the rose Bengal test, the tube agglutination test and the compliment fixation test. A total of 73.3, 100, 100, 95.8 and 61.9% of the sera from suspected cattle, yak, goat, sheep and reindeer, respectively, that were positive in the compliment fixation test, were also positive in the AGID test. Sera from vaccinated cattle, sheep and goat were positive over 90% by conventional tests 3 months after vaccination, but were negative by the AGID. These results suggest that the AGID test may be useful to differentiate infected and vaccinated animals in the field.

KEY WORDS: AGID test, Brucellosis, poly-B.

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Brucella Abortus and *Brucella* Melitensis are bacteria that can cause abortions in domestic animals and undulant fever that may persist intermittently for years in humans. In Mongolia, young and adult domestic animals such as calves, sheep and goats have been vaccinated with attenuated live cells of *B.* Abortus strain S-19 and *B.* Melitensis strain Rev-1 to protect against brucellosis, but the protection is not an absolute [1] because brucellosis occurs every year in Mongolia. However, diagnosing brucellosis is difficult because vaccine strains have antigenicity similar to virulent strains of *Brucella* [2, 6], and consequently differentiating infected and vaccinated animals is very hard. Reports exist that vaccinated and infected animals that have checked by the agar gel immunodiffusion (AGID) test using *Brucella* polysaccharide can be differentiated [2–4, 6]. In this study, we show that the AGID test using polysaccharide (poly-B) antigen isolated from the *B.* Abortus vaccine strain S-19 can be useful in epidemiological studies to differentiate vaccinated and infected animals in Mongolia.

Poly-B antigen was prepared by the method of Diaz-Aparicio *et al.* [3]. Briefly, washed cells of *B.* Abortus strain S-19 were extracted with 2.0% acetic acid-10% NaCl at 120°C for 30 min. The cell debris was removed by centrifugation, and the supernatant was precipitated with methanol-1.0% sodium acetate. The precipitate was then chromatographed on Sphadex G-50 (Pharmacia, Uppsala, Sweden). We could not use wild-type strain for antigen preparation because of bio-safety problems in Mongolia, therefore *B.* Abortus strain S-19 was used for antigen preparation. The specificity of the AGID test using poly-B antigen was checked by using serum samples collected from 114 cattle and 42 sheep, which were selected from unvaccinated and brucellosis-free farms in Mongolia. All serum samples were negative by the rose Bengal test (RBT), the tube agglutination test (TAT) and the compliment fixation test (CFT), which were routinely used to inspect brucellosis [7] in Mongolia, and were also negative in the AGID test (data not shown), suggesting that the AGID test with poly-B antigen does not show a non-specific reaction. A whole-cell antigen of *B.* Abortus strain 99, which was the strain of antigen preparation in Mongolia, was used for three conventional serological tests (RBT, TAT, and CFT) by standard protocol [7].

To further find out the specificity of the polysaccharide antigen, serum samples collected from cattle 1, 2, 3, 6 and 10 months after inoculation with *B.* Abortus strain S-19, which is commonly used as a vaccine strain in Mongolia and the *B.* Abortus strain R-159N (a newly developed vaccine under investigation) were examined by the AGID test. Strain R-159N was attenuated from parental strain 159 by UV-irradiation. Parental strain was isolated from infected cattle in Mongolia. Serum samples at one and two months after vaccination were positive by the conventional serological tests and also by the AGID test using poly-B antigen (Table 1). However, serum samples at three months after vaccination were positive in conventional serological tests, but not in the AGID test (Fig. 1, Table 1). Since transient

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Table 1. Serological tests of cattle vaccinated with *B.* Abortus strains S-19 and R-159N

Vaccine strains[a] (number of animals)	Months after vaccination	Percentage of positive animals in the test[b]			
		RBT	TAT	CFT	AGID
B. Abortus strain S-19 n = 11	0	0	0	0	0
	1	100	100	100	81.8
	2	100	100	100	18.2
	3	90.9	100	100	0
	6	72.7	90.9	63.6	0
	10	63.6	63.6	0	0
B. Abortus Strain R159-N N=10	0	0	0	0	0
	1	100	100	30	90
	2	100	100	30	50
	3	90	50	30	0
	6	90	0	0	0
	10	70	0	0	0

a) *B.* Abortus strains S-19 and R-N159 were subcutaneously inoculated at single doses of 3×10^9 and 1.0×10^{10} cells, respectively, for the vaccination.
b) RBT: Rose Bengal test, TAT: tube agglutination test, CFT: compliment fixation test, AGID: agar gel immunodiffusion.

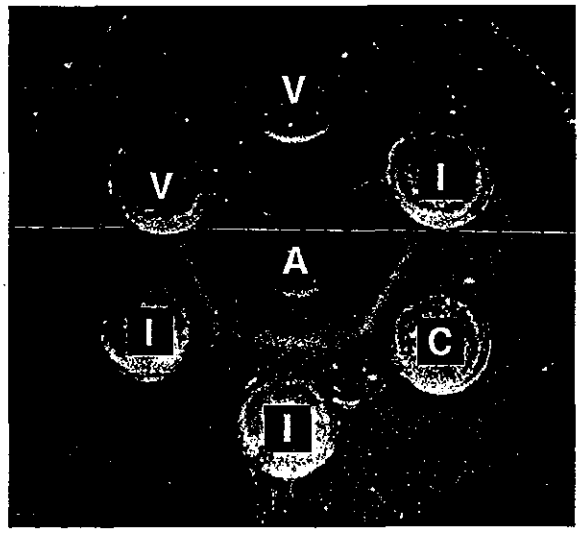

Fig. 1. AGID test using polysaccharide (poly-B) antigen of *B.* Abortus strain S-19. A, polysaccharide antigen; V, vaccinated bovine sera; I, infected bovine sera; C, positive control (rabbit immune serum). These vaccinated and infected bovine serum were collected separately.

increase of IgM antibody is observed in cattle at one or two month after vaccination [5], poly-B antigen may react with IgM antibody in serum from one and two month after vaccination. These results indicate that the AGID test using poly-B antigen would be useful to differentiate infected and vaccinated animals at least three months after the immunization. Similar results were obtained for serum samples of sheep and goats vaccinated with *B.* Melitensis Rev-1 (Table 2). Previous report showed that the AGID test using poly-B antigen was able to differentiate infected and vaccinated cattle at one and two month after vaccination [6]. Since different strains were used between that report and our study, different results were observed in serum samples at one and two month after vaccination.

As the AGID test might be useful to differentiate *Brucella*-infected and -vaccinated animals in Mongolia, we made an epidemiological survey of serum samples collected from suspected cattle, yaks, goats, sheep and reindeer by the RBT in Mongolia in 2001 (Table 3). The results showed that the AGID test eliminated 4.2–38.1% of cattle, sheep and reindeer that were positive by the CFT, but no suspected yaks and goats (0%) (Table 3).

In Mongolia and in many other countries where brucellosis is an endemic, attenuated live vaccine strains *B.* Abortus S-19 and *B.* Melitensis Rev-1 have been inoculated into domestic animals to protect against brucellosis. Both strains are good for vaccines but the antibody in the serum after vaccination is detectable for a long time when conventional serological tests are used [3]. This causes problems in differentiating between vaccinated and infected animals, especially in Mongolia where these two vaccine strains were used for immunizing young and adult animals because infected animals must be put down by law. In this study, we showed that the AGID test using poly-B antigen should be useful to differentiate between vaccinated and infected domestic animals. Therefore, we hope that the AGID test using poly-B antigen can be used in combination with other serological tests in the field by the veterinary service of Mongolia.

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Table 2. Serological tests of adult sheep and goats vaccinated with *B.* Melitensis strain Rev-1

Animals[a] (number of tested animals)	Months after vaccination	Percentage of positive animals in the test[b]			
		RBT	TAT	CFT	AGID test
Sheep (*n*=8)	0	0	0	0	0
	1	100	100	100	25
	2	100	100	100	25
	3	100	100	100	0
	4	87.5	100	100	0
Goat (n=8)	0	0	0	0	0
	1	100	100	100	12.5
	2	100	100	100	0
	3	100	100	100	0
	4	100	100	100	0

a) A single dose of 1.0×10^{10} cells of *B.* Melitensis strain Rev-1 was inoculated subcutaneously.
b) Same as in the Table 1.

Table 3. Serological tests of the domestic animals in Mongolia

Animals[a] (number of animals tested)	Percentage of positive animals in the test[b]				
	RBT	TAT	CFT	AGID test	AGID/CFT[c]
Cattle (n=16)	100	93.8	93.8	68.8	26.7
Yak (n=10)	100	40	90	90	0
Goat (n=9)	100	77.8	66.6	66.6	0
Sheep (n=72)	100	95.8	66.7	63.9	4.2
Reindeer (n =24)	100	66.6	87.5	54.2	38.1

a) All serum samples screened by RBT were collected from suspected animals with brucellosis.
b) Same as in the Table 1.
c) The percentage of elimination was determined as the percentage of AGID test divided by the percentage of CFT, and then subtracted from the percentage of RBT.

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Macrophage Plasma Membrane Cholesterol Contributes to *Brucella abortus* Infection of Mice

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***Brucella abortus* is a facultative intracellular bacterium capable of surviving inside macrophages. Intracellular replication of *B. abortus* requires the VirB complex, which is highly similar to conjugative DNA transfer systems. In this study, we show that plasma membrane cholesterol of macrophages is required for the VirB-dependent internalization of *B. abortus* and also contributes to the establishment of bacterial infection in mice. The internalization of *B. abortus* was accelerated by treating macrophages with acetylated low-density lipoprotein (acLDL). Treatment of acyl coenzyme A:cholesterol acyltransferase inhibitor, HL-004, to macrophages preloaded with acLDL accelerated the internalization of *B. abortus*. Ketoconazole, which inhibits cholesterol transport from lysosomes to the cell surface, inhibited the internalization and intracellular replication of *B. abortus* in macrophages. The Niemann-Pick C1 gene (NPC1), the gene for Niemann-Pick type C disease, characterized by an accumulation of cholesterol in most tissues, promoted *B. abortus* infection. NPC1-deficient mice were resistant to the bacterial infection. Molecules associated with cholesterol-rich microdomains, "lipid rafts," accumulate in intracellular vesicles of macrophages isolated from NPC1-deficient mice, and the macrophages yielded no intracellular replication of *B. abortus*. Thus, trafficking of cholesterol-associated microdomains controlled by NPC1 is critical for the establishment of *B. abortus* infection.**

Brucella species are facultative intracellular pathogens that survive in a variety of cells, including macrophages, and their virulence and chronic infections are thought to be due to their ability to avoid the killing mechanisms within macrophages (1). The molecular mechanisms of their virulence and chronic infections are incompletely understood. Recent studies with HeLa cells have confirmed these observations, showing that *Brucella* inhibits phagosome-lysosome fusion and transits through an intracellular compartment that resembles autophagosomes. Bacteria replicate in a different compartment, containing protein markers normally associated with the endoplasmic reticulum, as shown by confocal microscopy and immunogold electron microscopy (5, 24). *Brucella* internalizes into macrophages by swimming on the cell surface, with generalized membrane ruffling for several minutes, after which the bacteria are enclosed by macropinosomes (33). "Lipid raft"-associated molecules, such as glycosylphosphatidylinositol (GPI)-anchored proteins, GM1 gangliosides, and cholesterol, have been selectively incorporated into macropinosomes containing *Brucella abortus*. The disruption of lipid rafts on macrophages markedly inhibits the VirB-dependent macropinocytosis and intracellular replication (33). These results indicated that replicative phagosome formation of *B. abortus* is modulated by lipid raft microdomains.

The operon coding for export mechanisms specializing in transferring a variety of multimolecular complexes across the bacterial membrane to the extracellular space or into other cells has been described previously (27). These complexes, named type IV secretion systems, are in *B. abortus* (*virB* genes) (27). This operon comprises 13 open reading frames that share a homology with other bacterial type IV secretion systems involved in the intracellular trafficking of pathogens. Type IV secretion systems export four types of substrates: (i) DNA conjugation intermediates; (ii) the multisubunit pertussis toxin; (iii) monomeric proteins, including primase, RecA, and the *Agrobacterium tumefaciens* VirE2 and VirF proteins; (iv) and the *Helicobacter pylori* CagA protein (4). The RalF protein has been identified as a substrate of the type IV secretion system of *Legionella pneumophila* (20). However, substrates of the VirB secretion system of *B. abortus* and the target of the secretion system in host cells is still unclear.

In this study, we investigated the roles of plasma membrane cholesterol in internalization by the VirB system and the establishment of *B. abortus* infection in mice. Plasma membrane cholesterol associates with lipid raft microdomains. Lipid raft microdomains were originally reported by Simons and van Meer to explain sphingolipid-based sorting properties in cellular membranes (28) and were later proposed to explain cholesterol-based microheterogeneities in the membrane. Plasma membrane cholesterol and intracellular cholesterol trafficking was therefore expected to contribute to internalization and intracellular replication of *B. abortus* in macrophages, because recent evidence indicates that cholesterol sequestration from macrophages inhibits the internalization and intracellular replication of *B. abortus* (21, 33). Our results show that the plasma membrane cholesterol not only influences the bacterial inter-

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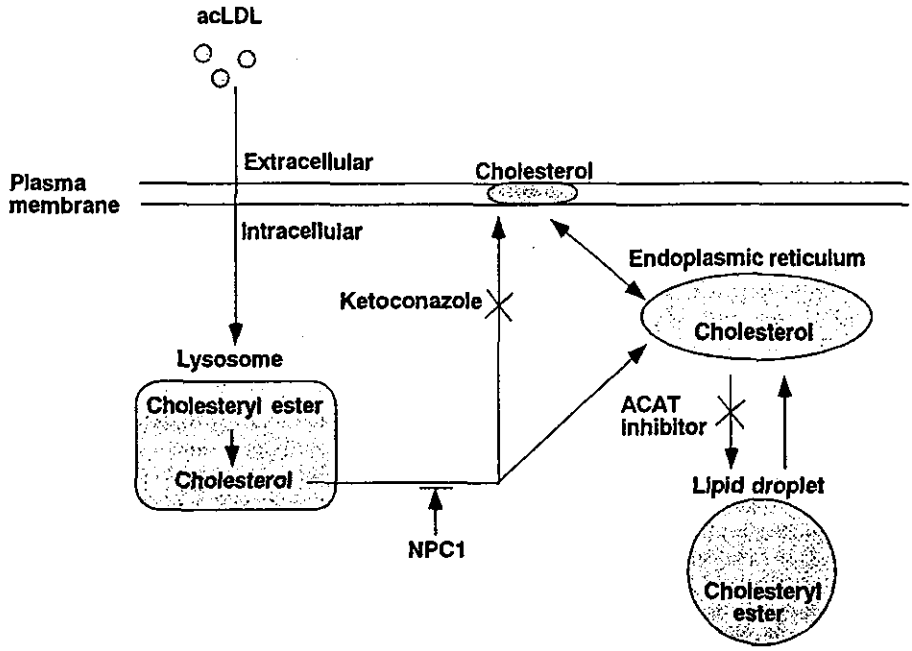

FIG. 1. Schema of cholesterol trafficking in macrophages. The effect of each pharmacological and genetic treatment examined in this study is shown.

nalization and intracellular replication, but also contributes to the establishment of *B. abortus* infection.

MATERIALS AND METHODS

Bacterial strains and mice. All *B. abortus* derivatives were from 544 (ATCC 23448) smooth virulent *B. abortus* biovar 1 strains. Ba598 (544 Δ*virB4*) has been described previously (32). Plasmid pMAW114, which encodes green fluorescence protein (GFP), was constructed by cloning the *Bam*HI-*Bgl*II fragment from the pQBI63 (GFP expression vector; TAKARA, Tokyo, Japan) into *Bam*HI- and *Bgl*II-cleaved pBBR1MCS-2. pMAW114 (GFP$^+$) was introduced into 544 (wild type) and Ba598 (Δ*virB4*), and the derivatives were designated Ba600 (wild-type GFP$^+$) and Ba604 (Δ*virB4* GFP$^+$), respectively (33).

BALB/c mice carrying the genetic mutation for NPC1 were obtained from The Jackson Laboratory (Bar Harbor, Maine) (25).

Cell culture. Bone marrow-derived macrophages from female BALB/c mice were prepared as described previously (32). The macrophages were seeded (2×10^5 to 3×10^5 in each well) in 24-well tissue culture plates for all assays. Macrophages were preloaded with or without acetylated low-density lipoprotein (acLDL) (50 μg/ml) and were treated with or without ketoconazole (10 mg/ml) or acyl coenzyme A:cholesterol acyltransferase (ACAT) inhibitor HL-004 (4 μg/ml; Taisho Pharmaceutical Co.) for 24 h (19).

Detection of intracellular bacteria by fluorescence microscopy. *B. abortus* strains were grown to an A_{600} of 3.2 in brucella broth and were used to infect mouse bone marrow-derived macrophages for various periods at an indicated multiplicity of infection (MOI). Bacteria were deposited onto the macrophages by centrifugation at 150 × *g* for 5 min at room temperature. After 0-, 5-, 15-, 25-, and 35-min incubations at 37°C, infected macrophages were washed once with medium and were fixed in periodate-lysine-paraformaldehyde (16) containing 5% sucrose for 1 h at 37°C. The samples were washed three times in phosphate-buffered saline (PBS) and wells were successively incubated three times for 5 min in blocking buffer (2% goat serum in PBS) at room temperature. The samples were stained with anti-*B. abortus* polyclonal rabbit serum diluted 1:1,000 in blocking buffer to identify extracellular bacteria. After incubating for 1 h at 37°C, the samples were washed three times for 5 min with blocking buffer, were stained with Cascade blue-conjugated goat anti-rabbit immunoglobulin G diluted 1:500 in blocking buffer, and were incubated for 1 h at 37°C. The samples were washed three times and were mounted in mounting medium. One hundred macrophages were examined per coverslip to determine the total number of intracellular bacteria.

Determination of efficiency of intracellular growth of bacteria. Bacteria were deposited onto macrophages at an MOI of 5 by centrifugation at 150 × *g* for 5 min at room temperature and then were incubated at 37°C in 5% CO_2 for 1 h. Then the macrophages were washed once with RPMI medium and were incubated with 30 μg/ml gentamicin. At different time points, the cells were washed and lysed with distilled water, and the number of bacteria on plates of a suitable dilution was determined.

Virulence in mice. The virulence was determined by quantitating the survival of the strains in the spleen after 10 days. Mice were injected intraperitoneally with approximately 10^4 CFU of brucellae in 0.1 ml of saline. Groups of five mice were injected with each strain. At 10 days after infection, their spleen was 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.

LAMP-1 staining. Infected macrophages were fixed in periodate-lysine-paraformaldehyde-sucrose for 1 h at 37°C and stained for extracellular bacteria as described above. All antibody-probing steps were carried out for 1 h at 37°C. Samples were washed three times in PBS for 5 min and then permeabilized at −20°C in methanol for 10 s. After incubating three times for 5 min with blocking buffer, samples were stained with anti-LAMP-1 rat monoclonal antibody 1D4B diluted 1:100 in blocking buffer (30). After washing three times for 5 min in blocking buffer, samples were stained simultaneously with Texas red-conjugated goat anti-rat immunoglobulin G. Samples were placed in mounting medium and visualized by fluorescence microscopy. Intracellular bacteria were detected by GFP fluorescence and absence of staining with Cascade blue.

Fluorescence labeling of lipid raft-associated molecules. Detection of the localization of GM1 gangliosides with cholera toxin B subunit (CTB) (10 μg/ml), GPI-anchored protein with aerolysin (2.5 μg/ml), and cholesterol with filipin (50 mg/ml) was described previously (33).

RESULTS

Acceleration of bacterial internalization by preloading cholesterol into macrophages. A prominent biological property of acLDL is its ability to induce lipid loading of macrophages in culture, which has been a useful model of the formation of lipid-laden macrophages (Fig. 1) (18, 19, 25). To investigate if intracellular cholesterol affects *B. abortus* internalization into

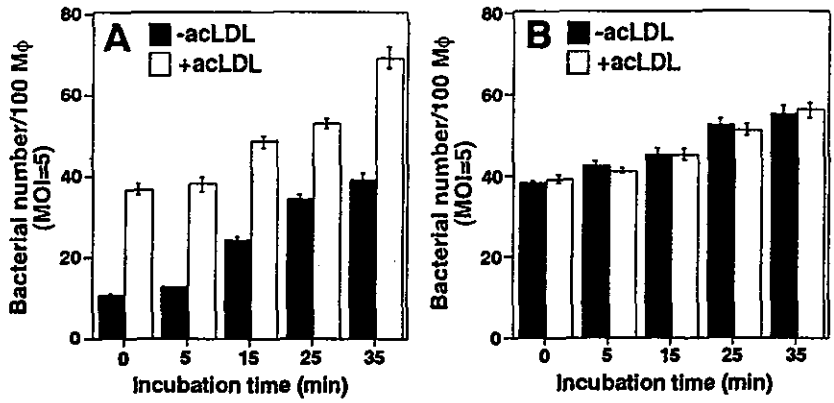

FIG. 2. Internalization of *B. abortus* accelerated by acLDL. Ba600 (wild type) (A) or Ba604 (Δ*virB4*) (B) was deposited onto bone marrow-derived macrophages with (white bars) or without (black bars) acLDL treatment and incubated at 37°C for the periods of time indicated. One hundred macrophages were examined per coverslip. Data are the average of triplicate samples from three identical experiments, and the error bars represent the standard deviation.

macrophages, bacteria were deposited onto macrophages that were preloaded with acLDL, and intracellular bacteria were quantitated microscopically at various times of incubation. The bacteria were deposited onto macrophages by centrifugation, and then Ba604 (Δ*virB4*) was rapidly internalized, with most of the associated bacteria internalized before further incubation at 37°C. In contrast, internalization of Ba600 (wild-type) was delayed and attained the same levels of internalization as Ba604 (Δ*virB4*) only after 25 min of incubation (33). Internalization of Ba600 (wild-type) accelerated by preloading with acLDL into macrophages, but internalization of Ba604 (Δ*virB4*) was not affected (Fig. 2). The percentage of bacteria in macropinosomes did not differ between macrophages treated with and without acLDL (data not shown).

Effects of agents that modulate cholesterol trafficking on bacterial internalization and intracellular replication. Macrophages take up modified lipoproteins by receptor-mediated endocytosis, and then the lipoproteins are delivered to lysosomes for degradation. This cholesterol is believed to mix with the bulk of cholesterol in the plasma membrane. Excess plasma membrane cholesterol then enters the cytoplasm, where the cholesterol is reesterified by ACAT and is stored in cellular lipid droplets (Fig. 1) (19). To investigate if intracellular cholesterol trafficking affects *B. abortus* internalization into macrophages, bacteria were deposited onto macrophages that were pretreated with ACAT inhibitor HL-004, and intracellular bacteria were quantitated microscopically at various times of incubation. HL-004 treatment accelerated the internalization of Ba600 (wild-type) into macrophages preloaded with acLDL (Fig. 3B). Under the same conditions, HL-004 did not accelerate the internalization of Ba604 (Δ*virB4*) (Fig. 3C and D). Macropinosome formation of Ba600 (wild-type) was also enhanced by HL-004 treatment, but the percentage of bacteria in macropinosomes did not differ (data not shown). These results suggest that intracellular cholesterol transport would contribute to VirB-dependent internalization and macropinocytosis of *B. abortus*.

To confirm that plasma membrane cholesterol contributes to *B. abortus* internalization, we tested the effect of ketoconazole, which inhibits cholesterol transport from lysosomes to the cell surface (Fig. 1). Ketoconazole greatly diminished the internalization of Ba600 (wild-type) into macrophages preloaded with or without acLDL (Fig. 3A and B), but under the same conditions, it did not block the internalization of Ba604 (Δ*virB4*) (Fig. 3C and D).

To determine whether intracellular cholesterol trafficking has a role in bacterial replication in macrophages, macrophages were treated with acLDL, ketoconazole, or HL-004 and then were infected with Ba600 (wild type). As reported previously (32, 33), Ba600 (wild-type) replicated in macrophages without ketoconazole treatment, but Ba600 (wild-type) failed to replicate in macrophages treated with ketoconazole (Fig. 3E). Although 12% ± 2.0% of internalized Ba600 (wild type) was observed under ketoconazole treatment (mean ± standard deviation) (Fig. 3A and B), the internalized bacteria did not replicate in the macrophages. Intracellular replication was not affected by acLDL and HL-004 (Fig. 3E). We consistently found that approximately 15% of internalized Ba600 (wild-type) into untreated macrophages target improperly into a LAMP-1 positive compartment (32, 33). These results suggest that other uptake pathways of *B. abortus* by macrophages exist, but replicative phagosome formation requires the uptake pathway associated with plasma membrane cholesterol.

Role of cholesterol trafficking in establishment of *B. abortus* infection. The most prominent cellular feature of Niemann-Pick type C (NPC) disease is lysosomal accumulation of free cholesterol, caused by impaired relocation of cholesterol derived from LDL from the lysosome to other cellular sites, such as the plasma membrane and endoplasmic reticulum (Fig. 1) (23). To investigate if NPC1 contributes to the recruitment of lipid raft-associated molecules, fluorescence-labeled lipid raft-associated molecules, such as cholesterol, GM1 gangliosides, and GPI-anchored proteins, were observed by microscopy. These molecules were in the plasma membrane and intracellular vesicles of macrophages from wild-type mice (Fig. 4). In contrast, these molecules accumulated only in intracellular vesicles in macrophages from NPC1-deficient mice (Fig. 4). Localization of the transmembrane protein CD44, which is not associated with lipid rafts, was not affected by NPC1 (Fig. 4).

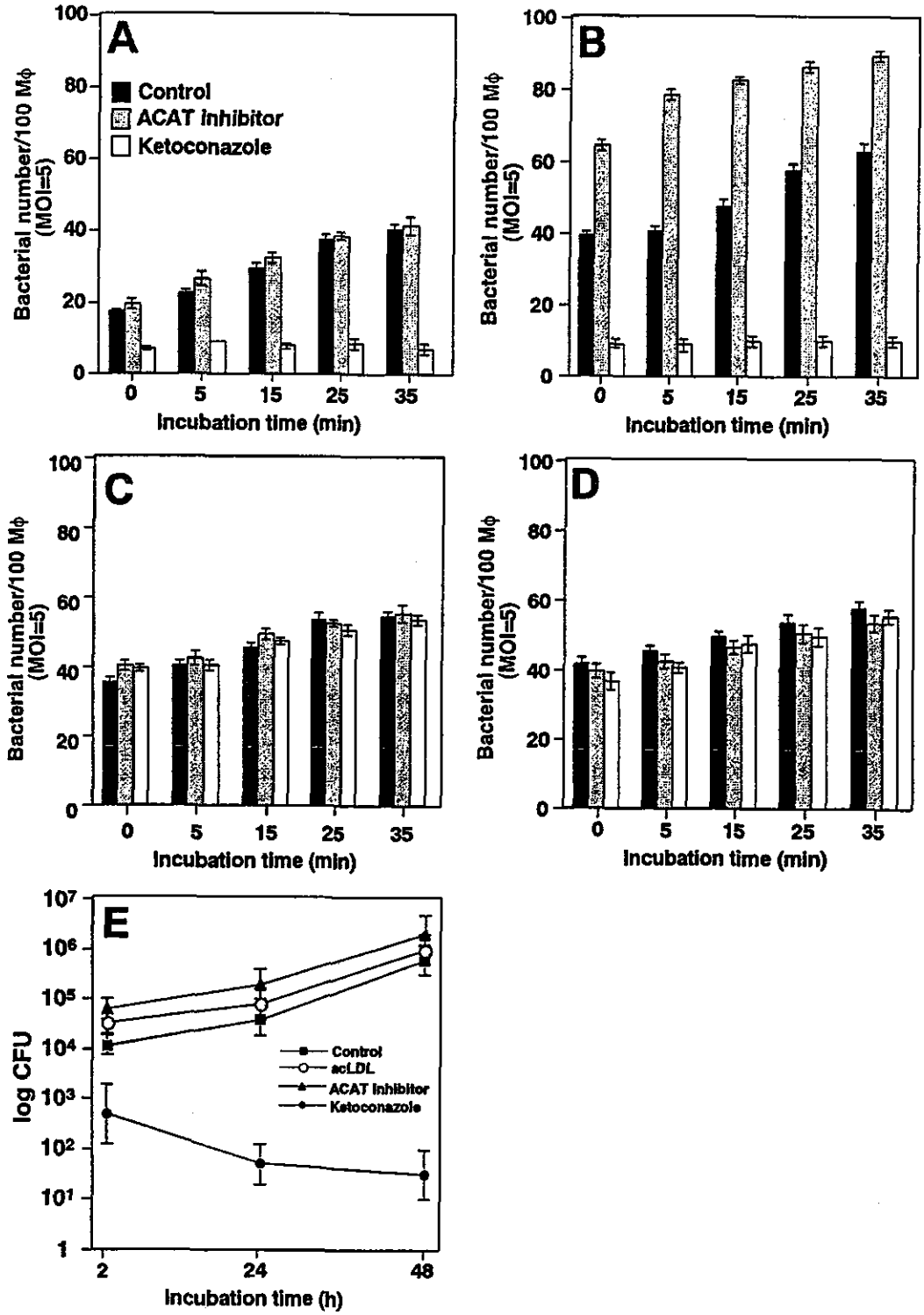

FIG. 3. Internalization of *B. abortus* into macrophages was influenced by plasma membrane cholesterol. Ba600 (wild-type) (A and B) or Ba604 (Δ*virB4*) (C and D) was deposited onto bone marrow-derived macrophages with ketoconazole (white bars) or with ACAT inhibitor HL-004 (gray bars), or without drug treatment (black bars) in the presence (B and D) or absence (A and C) of acLDL and incubated at 37°C for the periods of time indicated. One hundred macrophages were examined per coverslip. Data are the average of triplicate samples from three identical experiments, and the error bars represent the standard deviation. (E) Ketoconazole inhibited intracellular replication of *B. abortus* in macrophages. Macrophages in the presence or absence of ketoconazole, ACAT inhibitor HL-004, or acLDL were infected with Ba600 (wild type) as described in Materials and Methods. 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, respectively.

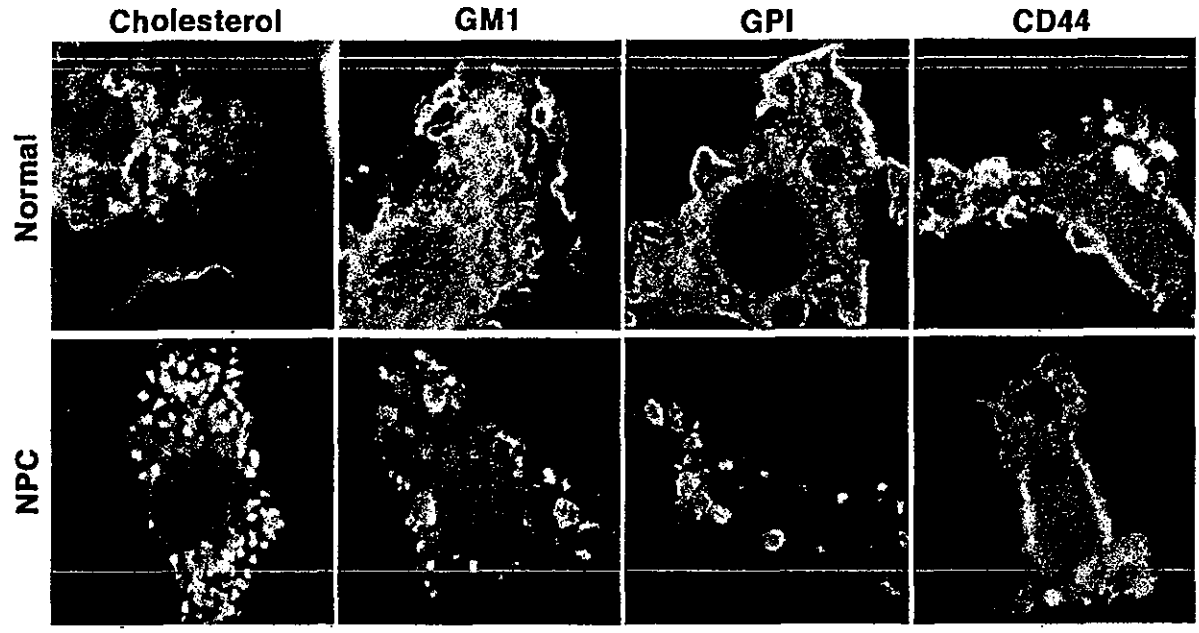

FIG. 4. Intracellular distribution of lipid raft-associated molecules in NPC1-deficient macrophages. Macrophages of wild-type (upper panels) or NPC1-deficient (lower panels) mice were labeled with indicated molecules.

These results suggest that NPC1 influences lipid raft formation on the macrophage surface.

We next investigated if NPC1 contributes to the internalization and intracellular replication of *B. abortus*. *B. abortus* was infected to bone marrow derived-macrophages from wild-type or NPC1-deficient BALB/c mice, and the intracellular bacteria were quantitated microscopically at various periods of incubation. Macrophages from wild-type mice supported internalization and intracellular replication of Ba600 (wild-type), but not macrophages from NPC1-deficient mice (Fig. 5A). Fewer bacteria were internalized in macrophages from NPC1-deficient mice, but they did not replicate in the macrophages (Fig. 5C). Macrophages from wild-type and NPC1-deficient mice showed no significant difference in the internalization of Ba604 (Δ*virB4*) (Fig. 5B). In NPC1-deficient mice, Ba600 (wild-type) failed to block phagosome maturation, as shown by colocalization of phagosomes containing the bacteria and the late endocytic marker, LAMP-1, at 1 h after infection (82.8% ± 3.4% positive) (Fig. 6B and D). In contrast, Ba600 (wild-type) prevented phagosome-lysosome fusion, and therefore phagosomes containing Ba600 (wild-type) do not have endocytic and lysosomal marker proteins in macrophages from wild-type mice (Fig. 6A and C). These results suggest that replicative phagosome formation required uptake pathway associated with NPC1.

To find if this defect in internalization and intracellular replication of *B. abortus* correlates with an inability to establish infection in the host, we experimentally infected wild-type or NPC1-deficient mice with *B. abortus*. Many bacteria were recovered from the spleen of wild-type mice infected with Ba600 (wild type) at 10 days after infection, but fewer bacteria were recovered from NPC1-deficient mice, based on the number of CFU in each spleen (Fig. 5D). These results indicated that *B. abortus* proliferation was promoted by NPC1.

DISCUSSION

In this study we showed that internalization and intracellular replication of *B. abortus* in mouse macrophages are influenced by plasma membrane cholesterol and that intracellular cholesterol trafficking is essential to establish *B. abortus* infection in the mouse model. These events were dependent on the presence of the VirB system. Studies of the cellular trafficking of cholesterol derived from the metabolism of LDL showed that after hydrolysis of LDL cholesteryl ester in lysosomes, most LDL-derived cholesterol traffics to the plasma membrane (14). The internalization of *B. abortus* into macrophages was accelerated by preloading with acLDL, indicating that the cholesterol on the plasma membrane of macrophages contributes to the internalization of *B. abortus*. To confirm this possibility, we used two inhibitors of intracellular cholesterol trafficking, HL-004 and ketoconazole. As HL-004 is a specific ACAT inhibitor (18), alterations in cholesterol metabolism by HL-004 can be attributed to the intracellular ACAT inhibition in macrophages (19). Macrophages incorporate modified LDL via scavenger receptors, which is not down-regulated by cellular sterol levels (8). The incorporated cholesteryl ester is delivered to lysosomes, and is hydrolyzed to free cholesterol, which forms the intracellular free cholesterol pool. Excess free cholesterol is esterified by ACAT and the cholesteryl ester is stored in cytoplasmic inclusions. This cholesteryl ester is the substrate for neutral cholesteryl ester hydrolase. Thus, a cholesteryl ester cycle of de-esterification exists because of the hydrolase and reesterification by ACAT. Free cholesterol can move between intracellular pools and the plasma membrane (3). Internalization and intracellular replication of *B. abortus* in macrophages were modulated by cholesterol-rich microdomains, so-called lipid rafts, on plasma membrane surfaces (33). As the cholesterol microdomains are induced in macrophages when esteri-

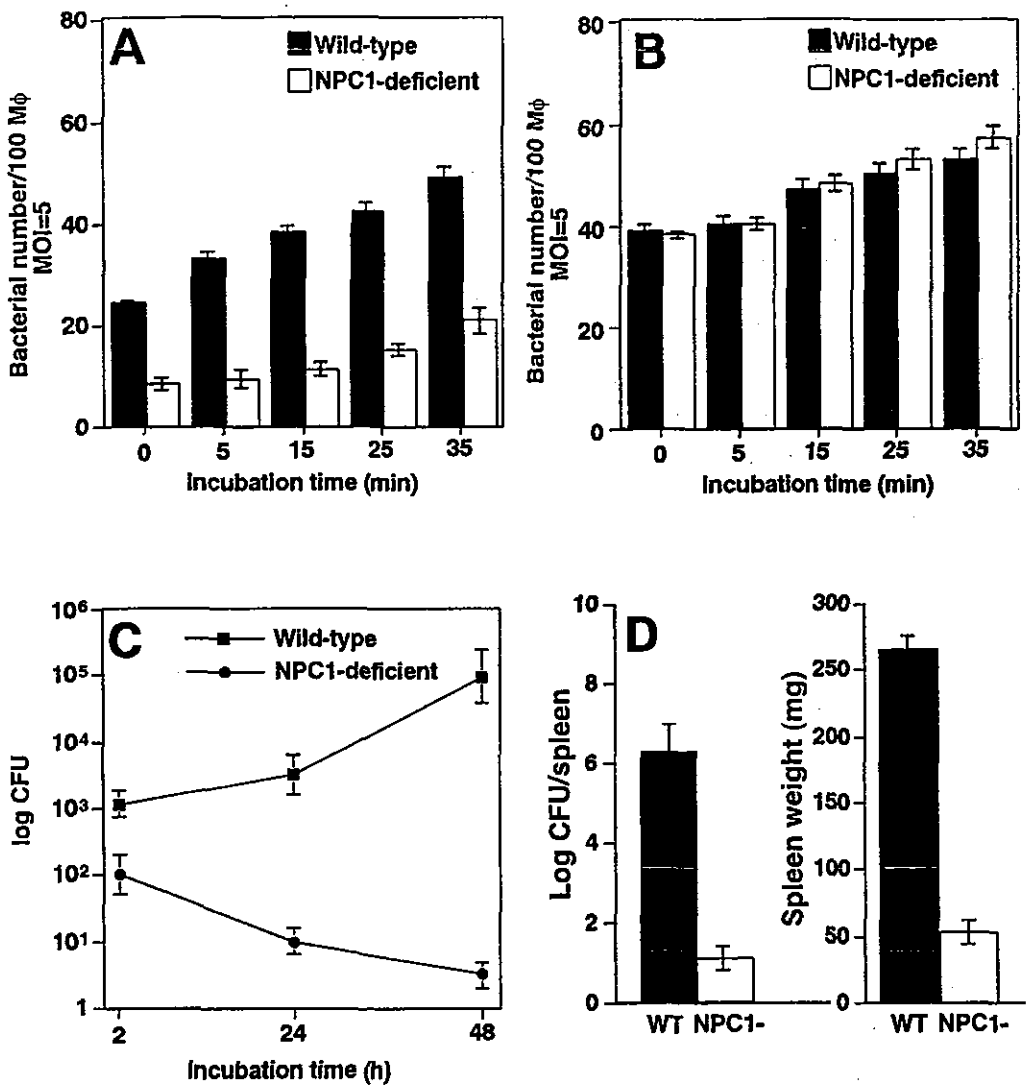

FIG. 5. NPC1-influenced *B. abortus* infection. (A and B) Internalization of *B. abortus*. Macrophages from wild-type (black bars) or NPC1-deficient (white bars) mice were infected with virulent Ba600 (wild type) (A) or Ba604 (Δ*virB4*) (B) for the periods of time indicated. Data are the average of triplicate samples from three identical experiments, and the error bars represent the standard deviation. (C) Intracellular replication of *B. abortus*. Macrophages from wild-type or NPC1-deficient mice were infected with Ba600 (wild type). 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, respectively. (D) Proliferation in mice. Wild-type (black bar) or NPC1-deficient (white bars) mice were infected with virulent *B. abortus*. Recovery of viable bacteria from the spleen and the weights of spleens of infected mice at 10 days postinfection are shown. Error bars indicate standard deviations.

fication of excess LDL-derived cholesterol is blocked with ACAT inhibitor (10), the internalization of *B. abortus* by uptake pathway associated with lipid rafts into macrophages increased by ACAT inhibitor treatment. In contrast, ketoconazole treatment greatly diminished the internalization of *B. abortus* into macrophages. Ketoconazole interferes with trafficking of cholesterol from lysosomes to the plasma membrane (12). As the appearance of cholesterol microdomains can be inhibited by ketoconazole (10), the internalization of *B. abortus* by uptake pathway associated with lipid rafts into macrophages decreases upon ketoconazole treatment. These results suggest that the plasma membrane cholesterol should influence the internalization of *B. abortus*. Fewer *B. abortus* cells were internalized into macrophages treated with ketoconazole, but the internalized bacteria did not replicate, suggesting that replicative phagosome formation would require correct intracellular cholesterol trafficking and plasma membrane cholesterol.

To confirm this hypothesis, macrophages from NPC1-deficient mice were infected with *B. abortus*. The gene that causes NPC disease, referred to as *NPC1*, has been mapped to a region of chromosome 18 in both humans and mice and has been cloned (15). Although the function of NPC1 remains undefined, studies have shown a crucial role for this protein in cholesterol metabolism (13). NPC1-deficient mice share many of the pathophysiological abnormalities observed in patients with NPC, including accumulation of cholesterol in tissues (15). Our results showed that lipid raft-associated molecules accumulate in intracellular vesicles in macrophages from

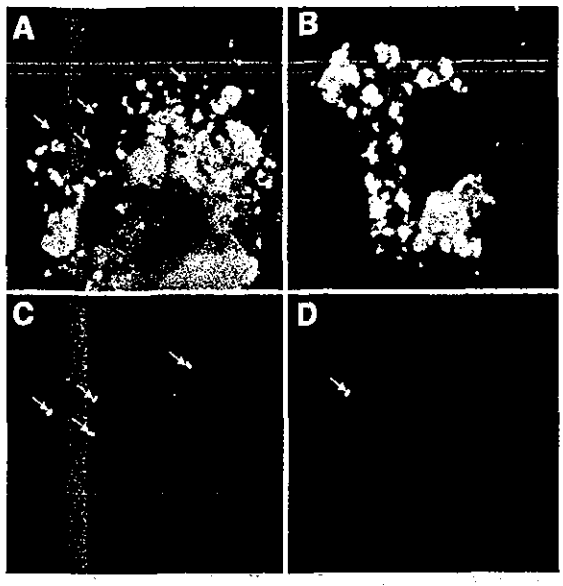

FIG. 6. Colocalization of *B. abortus* with late endosomal and lysosomal marker LAMP-1 in macrophages from NPC1-deficient mice by immunofluorescence microscopy. Macrophages from wild-type mice (A and C) or NPC1-deficient mice (B and D) were infected with Ba600 (wild type) for 1 h, fixed, and stained for LAMP-1 colocalization (A and B) and intracellular bacteria (C and D). Arrows point to bacteria.

NPC1-deficient mice. NPC1 is recruited to the site of free cholesterol accumulation by enrichment of cellular cholesterol or by pharmacological intervention of cholesterol egress from the lysosomes (34). Intracellular trafficking of GM1 ganglioside in NPC1-deficient Chinese hamster ovary cells has been shown by using CTB as a probe (29). CTB-labeled vesicles contain the early endosome marker Rab5 but not LAMP-2, indicating that they represent early endosomes. Similarly, CTB accumulate in intracellular vesicles of human NPC fibroblasts that contain both Rab5 and early endosomal antigen 1 (29). Presumably, these results, together with our results, indicate that cholesterol or GM1 ganglioside accumulate in lysosomes or early endosomes in macrophages from NPC1-deficient mice. Therefore, the internalization of *B. abortus* by uptake pathway associated with lipid rafts was inhibited in macrophages from NPC1-deficient mice.

The role of mouse macrophages in mediating resistance or susceptibility among mouse strains to some intracellular pathogens has been shown by studies of the *Ity/Lsh/Bcg* resistance model; resistance to *Salmonella enterica* serovar Typhimurium, *Leishmania donovani*, and mycobacterial species is regulated by the polymorphism of the *Nramp1* gene that controls macrophage function (6). Bovine Nramp1 is a major candidate for controlling the in vivo resistant phenotype against *B. abortus* infection (2). Our results indicate that NPC1 promotes the internalization and intracellular replication of *B. abortus* and also contributes to bacterial proliferation in mice. However, control of *B. abortus* infections is a multigenic trait (9), and further investigation is needed to clarify the genetic control of *B. abortus* infection.

Cholesterol or GPI-anchored proteins is included in apicomplexan *Toxoplasma gondii* and *Plasmodium falciparum* vacuoles (11, 17). Cholesterol is essential for the uptake of *Mycobacterium bovis* by macrophages (7). Cholesterol accumulates at the site of *M. bovis* entry, depleting plasma membrane cholesterol specifically inhibits *M. bovis* uptake, and *M. bovis* has a high binding capacity for cholesterol (7). Macropinosomes harboring *L. pneumophila* also include lipid raft-associated macromolecules (31). A similar process of selective lipid recruitment has been described during human immunodeficiency virus or influenza virus budding from mammalian cells (22, 26). Since lipid rafts are thought to be involved in signaling pathways in immune cells, uptake processes associated with lipid rafts might lead microorganisms into compartments that avoid fusion with the lysosomal network, and that is essential for the establishment of infection. The results of this study will provide a new target of prevention against infection by intracellular pathogens.

ACKNOWLEDGMENTS

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PATHOGENESIS

Membrane sorting during swimming internalization of *Brucella* is required for phagosome trafficking decisions

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Brucella infects macrophages by swimming internalization, after which it is enclosed in macropinosomes. We investigated the role of the uptake pathway in phagosome trafficking, which remains unclear. This study found membrane sorting during swimming internalization and is essential in intracellular replication of *Brucella*. The *B. abortus virB* mutant replicated intracellularly when it was in the macropinosome established by wild-type *B. abortus* that retained its ability to alter phagosome trafficking. Lipid rafts-associated molecules, such as GM1 ganglioside, were selectively included into macropinosomes, but Rab5 effector early endosome autoantigen (EEA1) and lysosomal glycoprotein LAMP-1 were excluded from macropinosomes containing *B. abortus* induced by swimming internalization. In contrast, when the swimming internalization was bypassed by phorbol myristate acetate (PMA)-induced macropinocytosis, lipid raft-associated molecules were excluded, and EEA1 and LAMP-1 were included into macropinosomes containing bacteria. The phosphatidylinositol 3-kinase inhibitor wortmannin that inhibits PMA-induced macropinocytosis blocked internalization of *virB* mutant, but not of wild-type of *B. abortus* and wortmannin treatment did not affect intracellular replication. Our results suggest that membrane sorting requires swimming internalization of *B. abortus* and decides the intracellular fate of the bacterium, and that *Brucella*-induced macropinosome formation is a different mechanism from PMA-induced macropinocytosis.

Key words: Membrane sorting, macrophage, brucellosis, intracellular replication.

Introduction

Phagocytosis by 'professional' phagocytes, which are macrophages and neutrophils, is central to the pathogenesis of intracellular pathogens, such as *Brucella*, and is the initial step in the degradation of dying cells, inert particles and live infectious agents. It has a critical part in essential biological functions, such as inflammation, immunity and development [1]. After uptake by professional phagocytes, inert particles are found in a membrane-derived phagosome, which undergoes maturation to a

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hydrolase-rich phagolysosome. Intracellular pathogens control the membrane-derived phagosomes, circumventing host defences and further degrading and transforming their compartments into replicative phagosomes [2, 3].

Brucella species are facultative intracellular pathogens that survive in a variety of cells, including macrophages, and their virulence and chronic infections are thought to be due to their ability to avoid the killing mechanisms within macrophages [4]. The molecular mechanisms of their virulence and chronic infections are incompletely understood. Recent studies with HeLa cells have confirmed that *Brucella* inhibits phagosome-lysosome fusion and transits through an intracellular compartment that resembles autophagosomes. Bacteria replicate in a different compartment, containing protein markers normally associated with the endoplasmic reticulum, shown by confocal microscopy and immunogold electron microscopy [5–7]. *Brucella* internalizes into macrophages by swimming on the cell surface with generalized membrane ruffling for several minutes, after which the bacteria are enclosed by macropinosomes [8]. Lipid raft-associated molecules, such as glycosylphosphatidylinositol (GPI)-anchored proteins, GM1 ganglioside and cholesterol, are selectively incorporated into macropinosomes containing *B. abortus*. The disruption of lipid rafts on macrophages markedly inhibits VirB-dependent macropinocytosis and intracellular replication [8]. Thus replicative phagosome formation of *B. abortus* is modulated by lipid raft microdomains.

We here describe the swimming internalization of *B. abortus* into macrophages is essential for targeting properly and formation of replicative phagosome. Macropinosomes induced by PMA, which cannot alter phagosome trafficking, are distinct from macropinosomes formed after the swimming internalization. This indicates that the internalization strategy of *B. abortus* into macrophages contributes to membrane sorting and is an important role in phagosome trafficking.

Results

Macropinosomes containing *B. abortus* are replicative phagosomes

Our previous results showed that *B. abortus* altered phagosomes into a specialized organelle permissive for bacterial replication through specific actions of the VirB system [8]. To further investigate if replicative phagosomes formed by wild-type *B. abortus* can support the intracellular replication of *virB* mutant, bone marrow-derived macrophages were co-infected simultaneously with 544 (wild-type, GFP-negative) and Ba604 (Δ*virB4* GFP-positive), and then macropinosomes containing both bacteria were identified. After 15 min infection, macropinosomes containing bacteria were analyzed by fluorescence microscopy. Seventy-nine percent of macropinosomes contained 544 (wild-type), and 18% macropinosome contained both 544 (wild-type) and Ba604 (Δ*virB4*) [Figs 1(A)–(D), (I)]. To examine in detail the distribution of bacteria inside macropinosomes containing both strains, the number of bacteria inside macropinosomes was scored. Fifty-two percent of the macropinosomes contained single bacteria of 544 (wild-type) and Ba604 (Δ*virB4*), and 21–25% of the macropinosomes contained single or double bacteria of these strains; macropinosomes containing more than double bacteria of both strains were hardly detectable in this procedure [Fig. 1(J)].

To determine whether synchronous uptake of wild-type and mutant strains can rescue the replication of the mutant strain, macrophages were co-infected simultaneously with 544 (wild-type) and Ba604 (Δ*virB4*) and then replicative phagosomes containing both strains were identified. After 24 h infection, macrophages harboring bacteria were analyzed by fluorescence microscopy. Thirty-five percent of macrophages contained no bacteria, and 25–27% of the macrophages contained 544 (wild-type) or/and Ba604 (Δ*virB4*) [Fig. 1(K)]. Macrophages containing more than six bacteria of both strains were observed (18%), indicating that the replicating mutant strain was rescued [Figs 1(E)–(H), (K)]. Replication of *virB* mutant was only supported in the macropinosome containing wild-type strain, because it was not observed replication of *virB* mutant only.

Internalization of *B. abortus* by PMA-induced macropinocytosis

To investigate if the swimming internalization of *B. abortus* requires replicative phagosome formation, PMA-treated macrophages were infected with bacteria. As PMA stimulates both membrane ruffling and macropinocytosis in macrophages [9, 10], bacteria may internalize accidentally into macrophages by

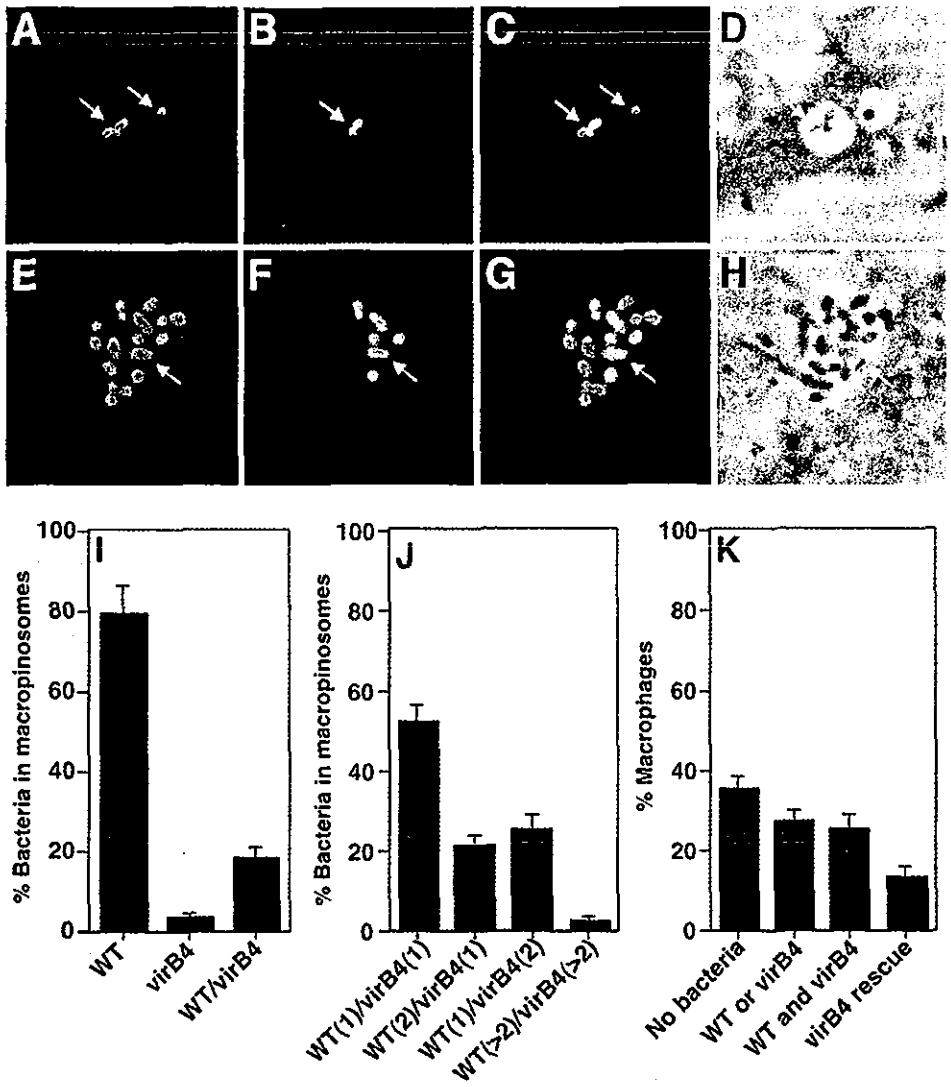

Figure 1. *Brucella abortus* creates an organelle permissive for intracellular replication by VirB-dependent macropinocytosis. (A–H) Mouse bone marrow-derived macrophages were infected simultaneously with 544 (wild-type, GFP-negative) and Ba604 (Δ*virB4*, GFP-positive), and fixed after 15 min (A–D, I and J) or 24 h (E–H and K) incubation. Red, anti-*B. abortus* staining; green, GFP-Δ*virB4* mutant. The Δ*virB4* mutant (green, panel B) internalized in a macropinosome that corresponds to phase contrast image (D) were localized by GFP fluorescence and anti-*B. abortus* staining (yellow) with wild-type strain (red) in the merged image (C). The Δ*virB4* mutant (green, panel F) replicating intracellularly that corresponds to the phase contrast image (H) were localized by GFP fluorescence and anti-*B. abortus* staining (yellow) with wild-type strain (red) in the merged image (G). White arrows point to both wild-type and Δ*virB4* mutant (A–H), and the blue arrow points wild-type strain in macropinosome (A–D). (I–K) Number of *B. abortus* wild-type and Δ*virB4* mutant in co-infected macrophages were scored by fluorescence microscopy (see Experimental procedures). (I) Numbers of macropinosomes containing wild-type *B. abortus* (WT), Δ*virB4* mutant (virB4) and both strains (WT/virB4) were scored. Data for macropinosomes are from triplicate coverslips representing 50 macropinosomes per coverslip, and the error bars represent the standard deviation. (J) Distribution of bacterial numbers in macropinosomes containing both strains. Numbers of bacteria in macropinosomes containing both strains are shown in parentheses. Data for macropinosomes are from triplicate coverslips representing 30 macropinosomes containing both strains per coverslip, and the error bars represent the standard deviation. (K) After 24 h infection, bacterial phagosomes were scored. Macrophages that were uninfected (no bacteria), harboring phagosomes containing only a single strain *B. abortus* (WT or virB4), harboring both strains (WT and virB4) or containing replicating Δ*virB4* mutant (virB4 rescue) were scored. One hundred macrophages were examined per coverslip. Data are the average of triplicate samples from three identical experiments, and the error bars represent the standard deviation.

macropinocytosis without the swimming internalization. Time-lapse videomicroscopy was used to follow the internalization of Ba600 (wild-type) into macrophages. Bacteria moved from the site of initial bacterial contact with PMA-untreated macrophages for several minutes [Fig. 2(A)]. Internalization of Ba600 (wild-type) into PMA-treated macrophages, in contrast, was much quicker and bacteria were enclosed by macropinosomes within a minute without swimming [Fig. 2(B)]. Some bacteria, however, showed the same swimming internalization with PMA-treated macrophages as with PMA-untreated macrophages. From analysis of time-lapse videomicroscopy, some Ba600 (wild-type) internalized into PMA-treated macrophages by macropinocytosis without swimming. To analyze this further, samples were fixed after brief incubation of macrophages with *B. abortus*. The differences in the rate of phagocytosis and the formation of macropinosomes for PMA-treated and -untreated macrophages were quantitated-microscopically at various times of incubation, using strategies that allowed as synchronous an infection as possible (see Experimental procedures). In PMA-untreated macrophages, Ba604 (Δ*virB4*) was rapidly internalized, with most of the associated bacteria internalized before further incubation, but the internalization of Ba600 (wild-type) was delayed [Fig. 2(C) and (E)]. By 15 min after deposition of Ba600 (wild-type), 55.6% of the bacteria were in macropinosomes. Phagosomes containing Ba604 (Δ*virB4*) were relatively devoid of the fluid phase marker [Fig. 2(D)]. In PMA-treated macrophages, in contrast, Ba600 (wild-type) rapidly internalized and showed similar intracellular bacterial numbers as Ba604 (Δ*virB4*) [Fig. 2(F) and (H)]. In macropinosome formation, more than 20% of both wild-type and mutant strains were in macropinosomes at 0 and 5 min [Fig. 2(G)]. These results at 0 and 5 min after infection indicated that PMA-induced macropinocytosis can take up extracellular bacteria more efficiently and that about 20–40% of bacteria internalized into macrophages by PMA-induced macropinocytosis without swimming internalization.

Localization of membrane markers on *B. abortus* or PMA-induced macropinocytosis

To investigate differences in phagosome maturation between normal (PMA−) or abnormal (PMA+) uptake pathways, the infected PMA-treated and -untreated macrophages were stained with several membrane markers. We previously showed that lipid raft-associated molecules, such as GPI-anchored proteins, GM1 ganglioside and cholesterol, are selectively incorporated into macropinosomes containing Ba600 (wild-type) [8]. To determine if GM1 ganglioside is incorporated into PMA-induced macropinosomes containing *B. abortus*, the macropinosomes were probed with biotin-labeled CTB. In PMA-untreated macrophages, the kinetics and degree of association of CTB-labeled GM1 ganglioside with internalized Ba600 (wild-type) showed maximal association after 15 min incubation at 37°C [41%, Figs 3(A), 4(A)]. The association of CTB-labeled GM1 ganglioside with internalized Ba600 (wild-type) showed a marked high efficiency in macropinosomes [>78%, Figs 3(A), 4(C)]. Colocalization of CTB-labeled GM1 ganglioside with Ba604 (Δ*virB4*) was much less pronounced [Figs 3(A), 4(A) and (C)]. In PMA-treated macrophages, the degree of association of CTB-labeled GM1 ganglioside with internalized Ba600 (wild-type) was less than 23.3% after 15 min incubation at 37°C [Figs 3(A), 4(B)]. The association of CTB-labeled GM1 ganglioside with internalized Ba600 (wild-type) showed a lower efficiency in macropinosomes [<42%, Figs 3(A), 4(D)]. Similar results were obtained from aerolysin-labeled GPI-anchored proteins and filipin-labeled cholesterol (data not shown). These results indicated that about 36% macropinosomes containing Ba600 (wild-type) targeted improperly in PMA-treated macrophages, and thus membrane sorting might correlate with the uptake pathway.

To confirm these indications, acquisition of Rab5 effector early endosome autoantigen (EEA1) in macropinosomes was tested. In PMA-untreated macrophages, more than 88.2% of the phagosomes and 96.4% of the macropinosomes containing Ba600 (wild-type) failed to colocalize with EEA1, whereas Ba604 (Δ*virB4*) was found predominantly colocalized with EEA1 [Figs 3(B), 4(E) and (G)]. In PMA-treated macrophages, in contrast, acquisition of EEA1 of the phagosomes or macropinosomes containing Ba600 (wild-type) increased (10.3–22.4% or 32.1–46.3%, respectively) [Figs 3(B), 4(F) and (H)]. Similar results were obtained from LAMP-1 acquisition in PMA-treated and -untreated macrophages [Figs 3(C) and 4(I–L)]. In PMA-untreated macrophages, Ba600 (wild-type) prevented phagosome-lysosome fusion, and therefore

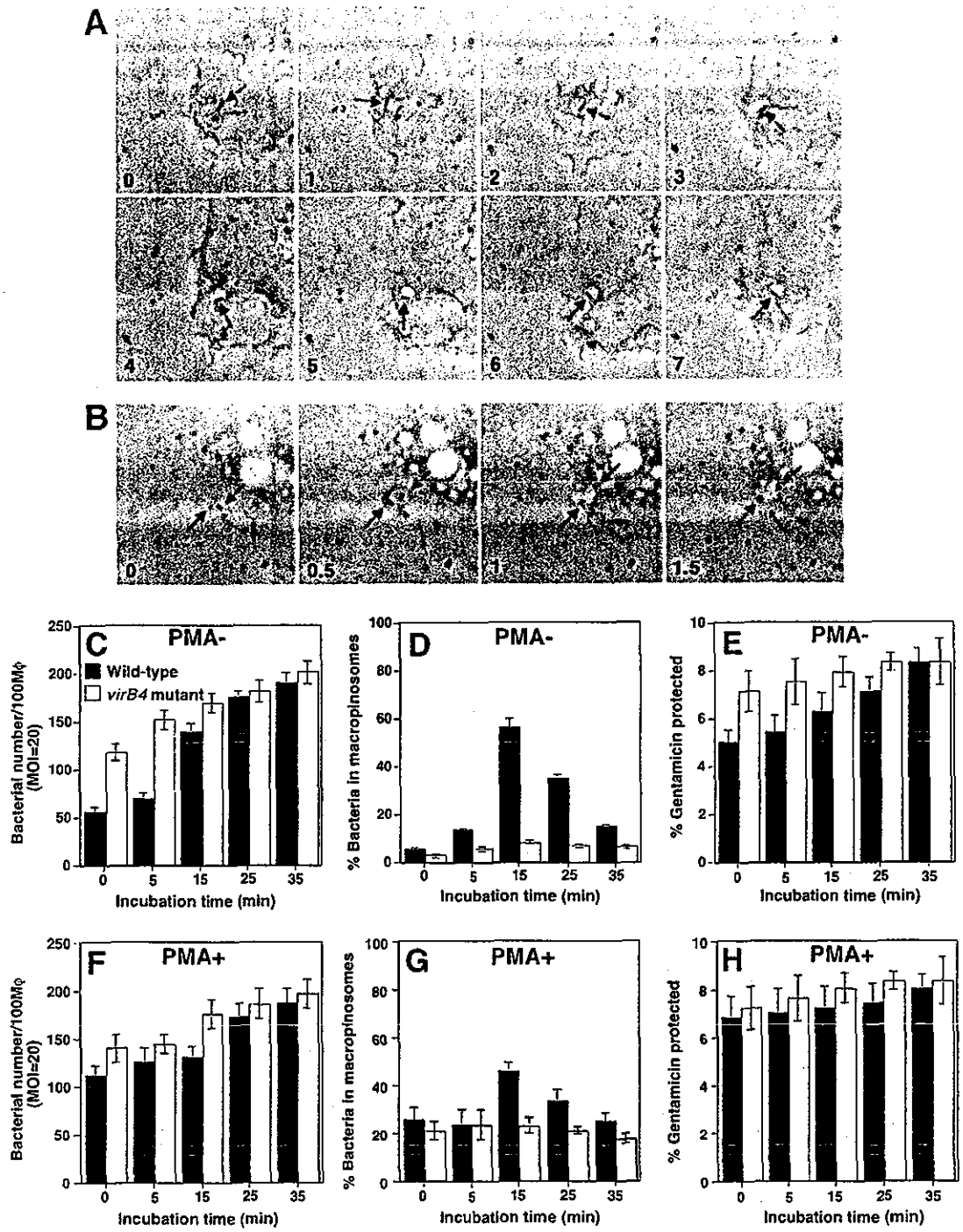

Figure 2. Effect of PMA treatment on delayed phagocytosis and macropinosome formation of *B. abortus*. (A and B) Selected time-lapse videomicroscopic images of wild-type *B. abortus* entry into mouse bone marrow-derived macrophages with (B) without (A) PMA treatment. Elapsed time in minutes is indicated at the bottom of each frame. Arrows point to bacteria. (C–H) Uptake and macropinosome formation increased by PMA treatment. Bacteria were deposited onto bone marrow-derived macrophages with (F–H) or without (C–E) PMA treatment and incubated at 37°C for the periods indicated. Uptake (C, E, F and H) or macropnosome formation (D and G) were quantitated as described (see Experimental procedures). C, D, F and G, 100 macrophages were examined per coverslip; E and H, uptake efficiency by macrophages was determined by protection of internalized bacteria from gentamicin killing. Black bars, Ba600 (wild-type); open bars, Ba604 (Δ*virB4*). Data are the average of triplicate samples from three identical experiments, and the error bars represent the standard deviation.

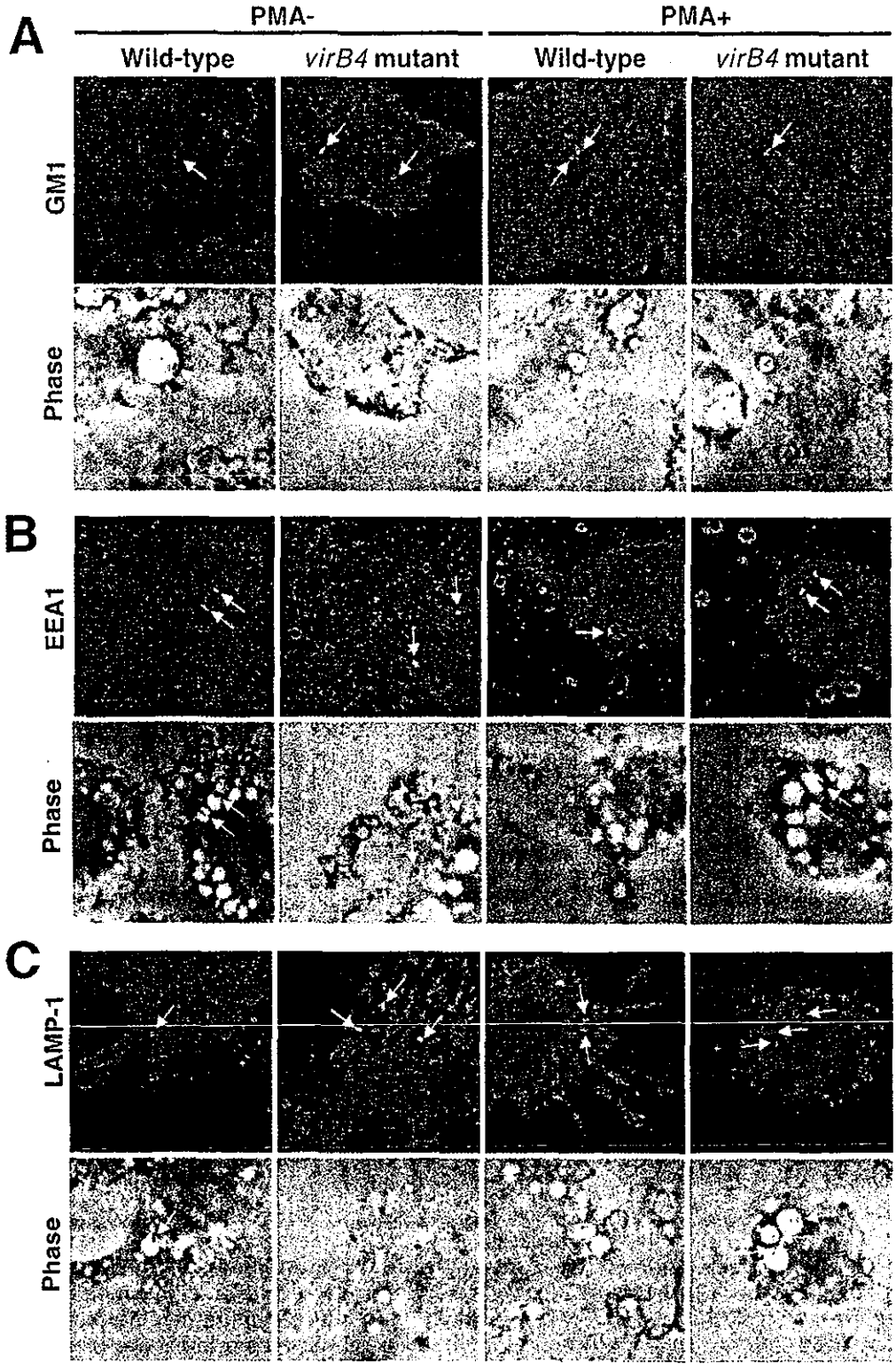

Figure 3. Association of membrane markers with *B. abortus*, macropinosomes. Bone marrow-derived macrophages with (PMA+) or without (PMA−) PMA treatment were incubated with wild-type or *virB4* mutant of *B. abortus*, and GM1 ganglioside (A), EEA1 (B) or LAMP-1 (C) was localized by immunofluorescence, as described (see Experimental procedures). Shown are merged images of the GFP (bacteria) and TRITC (GM1 ganglioside, EEA1 or LAMP-1) channels up-and-down with phase contrast images of the identical cells. Arrows point to bacteria.

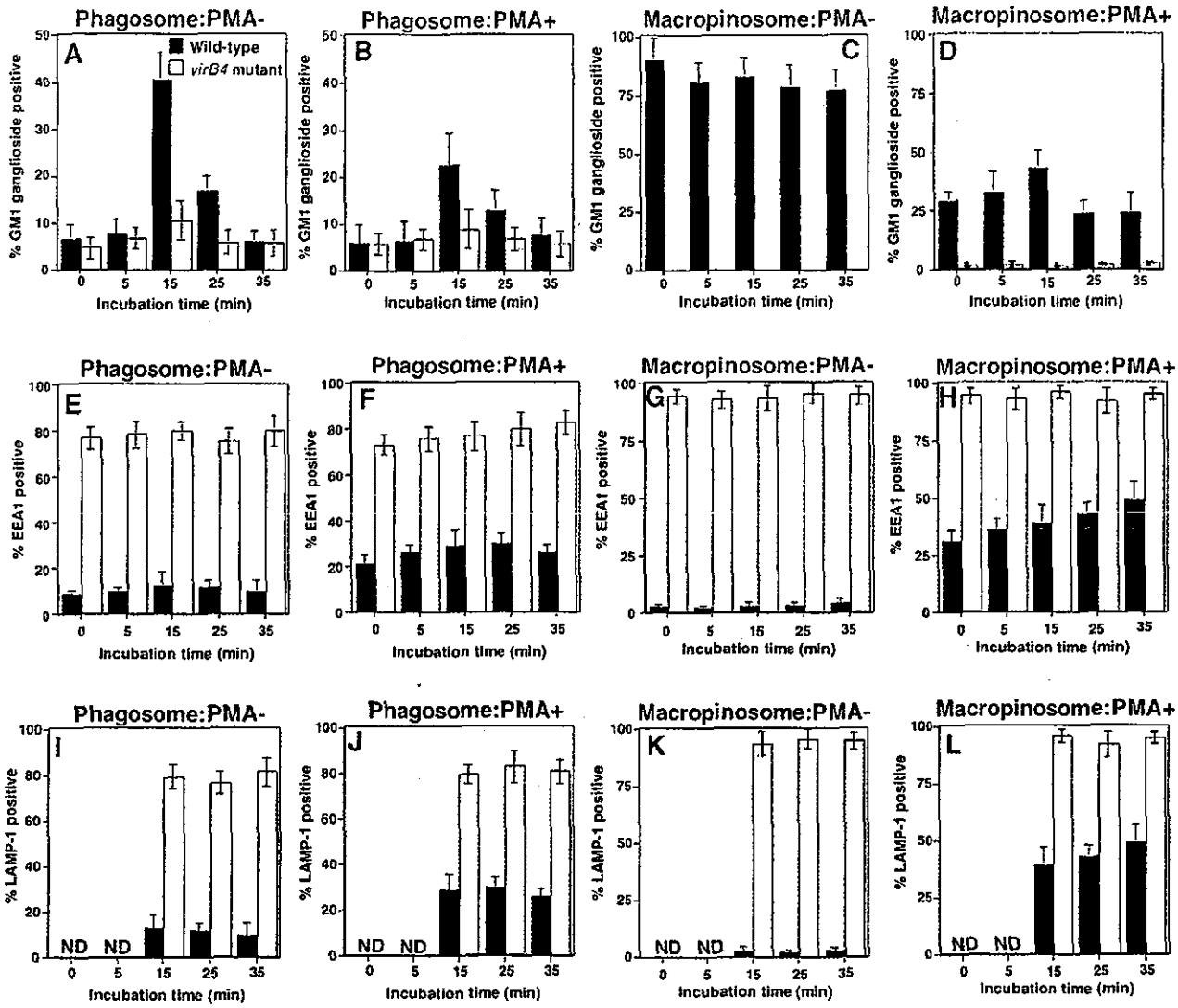

Figure 4. Kinetics of colocalization membrane markers with phagosomes containing *B. abortus*. Ba600 (wild-type) (black bars) or Ba604 (Δ*virB4*) (open bars) was deposited onto macrophages with (Phagosome: PMA+) or without (Phagosome: PMA−) PMA treatment, then incubated for the periods indicated at 37°C before fixation and probing with biotin-labeled CTB, anti-EEA1 or anti-LAMP-1 antibody (see Experimental procedures). '% GM1ganglioside, EEA1 or LAMP-1 positive' refers to percentage of internalized bacteria that showed co-staining with the GM1 ganglioside (A and B), EEA1 (E and F) or LAMP-1 (I and J) based on observation of 100 bacteria per coverslip. Data are the average of triplicate samples from three identical experiments, and the error bars represent the standard deviation. Macrophages with (Macropinosome: PMA+) or without (Macropinosome PMA−) PMA treatment incubated with Ba600 (wild-type) (black bars) or Ba604 (Δ*virB4*) (open bars) for the indicated time at 37°C were probed for GM1 ganglioside, and macropinosomes harboring bacteria were identified by fluorescence and phase microscopy. The macropinosomes were then observed for the presence of GM1 ganglioside (C and D), EEA1 (G and H) or LAMP-1 (K and L). '% GM1 ganglioside, EEA1 or LAMP-1 positive' refers *only* to those phagosomes with a macropinocytotic morphology, and represents the percentage of macropinosomes that show costaining with the GM1 ganglioside, EEA1 or LAMP-1. Data for macropinosomes are from triplicate coverslips representing 50 macropinosomes per coverslip, and the error bars represent the standard deviation. ND, not detectable.

phagosomes containing Ba600 (wild-type) do not have endocytic and lysosomal marker protein LAMP-1 in macrophages [Figs 3(C), 4(I) and (K)]. In PMA-treated macrophages, in contrast, Ba600 (wild-type) failed to block phagosome maturation comparatively, as shown by colocalization of phagosomes or macropinosomes containing the bacteria and the LAMP-1 at 35 min after

infection (35.5 or 49.2% positive, respectively) [Figs 3(C), 4(J) and (L)]. Bacteria in LAMP-1 positive phagosomes cannot replicate [11]. Thus, these results suggest that replicative phagosome formation requires an uptake pathway associated with swimming internalization.

B. abortus-induced macropinocytosis is a PI3-kinase-independent mechanism

To further investigate if *B. abortus*-induced macropinocytosis requires replicative phagosome formation, the role of phosphoinositide 3-kinase (PI3-kinase) in the internalization of *B. abortus* was assessed. PI3-kinase is not necessary for receptor-mediated stimulation of pseudopod extension, but rather functions in the closure of macropinosomes and phagosomes into intracellular organelles [12]. Ba600 (wild-type) or Ba604 (Δ*virB4*) was infected with macrophages treated with or without wortmannin, an inhibitor of PI3-kinase [13]. The internalization and macropinosome formation of Ba600 (wild-type) were not affected markedly by wortmannin [Fig. 5(A)–(C)]. However, wortmannin treatment appeared to decrease the internalization ability of Ba604 (Δ*virB4*) [Fig. 5(A)–(C)]. Consistent with these results, LAMP-1 acquisition of both Ba600 (wild-type) and Ba604 (Δ*virB4*) was not affected markedly by wortmannin [Fig. 5(D)]. To determine if PI3-kinase has a role in bacterial replication in macrophages, macrophages were treated with wortmannin and then were infected with Ba600 (wild-type) or Ba604 (Δ*virB4*). Ba600 (wild-type) replicated in macrophages without drug treatment. The intracellular replication of Ba600 (wild-type) was not affected markedly by wortmannin [Fig. 5(E)]. In contrast, Ba604 (Δ*virB4*) failed to replicate in macrophages with or without wortmannin treatment [Fig. 5(E)].

Discussion

This study showed membrane sorting is during the swimming internalization of *Brucella abortus* into mouse bone marrow-derived macrophages and is essential for intracellular replication. These events were dependent on the presence of the VirB system. As our previous study showed that *B. abortus* induced macropinosome formation [8], we speculated that co-infected *virB* mutant with wild-type *B. abortus* was easy to detect in macropinosomes containing wild-type *B. abortus* by microscopy. As expected, the *virB* mutant of *B. abortus* grew intracellularly when the bacteria were internalized by VirB-dependent macropinocytosis, suggesting that *B. abortus*-induced macropinosomes support intracellular replication of bacteria. However, this result suggested the important question of the role of macropinosomes in intracellular replication of *B. abortus*. Macropinosomes are generally large organelles, and their surface-to-volume ratio is lower than for smaller vesicles. Macropinosomes internalize true fluid-phase solutes more efficiently for each unit area of membrane than smaller vesicles [14]. Therefore, macropinosomes provide a comparatively efficient, though non-selective, mechanism to internalize extracellular macromolecules [15].

PMA and macrophage colony-stimulating factor stimulate macropinocytosis in macrophages [9, 10]. To clarify our question of the role of macropinosomes in intracellular replication of *B. abortus*, we investigated if *B. abortus* enclosed in PMA-induced macropinosomes can alter phagosome trafficking. Our results showed that PMA-induced macropinosomes containing wild-type *B. abortus* could not sort a phagosomal membrane, suggesting that the uptake pathway is more important than macropinosome formation for the alteration of phagosome trafficking. *Brucella abortus* internalizes into macrophages by swimming internalization [8]. Presumably, the membrane sorting is during swimming internalization, and then replicative phagosomes are established. Indeed, in this study, membrane sorting did not occur when bacteria internalized into macrophages by bypassing the swimming internalization. *Brucella abortus*-induced macropinosomes are thought to result from swimming internalization, because bacteria moving round from the site of initial bacterial contact with the macrophages were observed. Macropinosome formation induced by *B. abortus* is clearly distinct from that induced by PMA. The swimming of the bacteria on the macrophage surface often lasted for several minutes with generalized plasma membrane ruffling before eventual enclosure in macropinosomes. In contrast, bacterial internalization into PMA-treated macrophages is much quicker. After stimulation by macrophage colony stimulating factor or phorbol esters, circular ruffles appear in macrophages. Membrane ruffling becomes longer and broader, and frequently close into large macropinosomes [16]. The circular ruffles are clearly different from the