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Novel Pathogenetic Mechanism in a Clinical Isolate of *Yersinia enterocolitica* KU14

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Yersinia enterocolitica **induces a broad range of gastrointestinal syndromes, including acute enteritis. We previously reported that the clinical isolate, *Y. enterocolitica* KU14, which lacks pYV, was still capable of causing clinical infection. The present study demonstrated that KU14 did not trigger the death of macrophages *in vitro*, unlike WA-314 (ATCC51871, which harbors the pYV virulence plasmid). However, the intracellular growth of KU14 in the macrophages was greater than that of WA-C (ATCC51872, a non-plasmid harboring the derivative pYV plasmid). Treatment with a cholesterol-binding drug (β-cyclodextrin) that affected lipid rafts resulted in a dramatic reduction in the intracellular growth of KU14. These data clearly indicate that the enhanced intracellular growth of KU14 is related to lipid raft-mediated infection.**

Keywords: *Yersinia enterocolitica*, intracellular growth of bacteria and lipid-rafts

The genus *Yersinia* includes three species that are pathogenic to humans (Straley *et al.*, 1993). Among these, *Y. pestis* is the etiological agent for plague, whereas *Y. pseudotuberculosis* and *Y. enterocolitica* induce a broad range of gastrointestinal syndromes, including acute enteritis, which can sometimes be complicated by septicemia (Cornelis *et al.*, 1997). Despite their different routes of infection, these bacteria share in common the ability to escape the host's immune response, allowing for their extracellular survival and intracellular growth (Simonet *et al.*, 1990; Cornelis *et al.*, 1997).

The intracellular survival of these bacteria relies on the inhibition of phagocytosis and the suppression of the oxidative burst in the case of professional phagocytes, including macrophages (Schwan *et al.*, 1994; Visser *et al.*, 1995; Chakravortty *et al.*, 2002). In *Y. enterocolitica*, this ability depends on the presence of a 70-kb plasmid (pYV) encoding for virulence effector proteins referred to as the *Yersinia* outer proteins (Yops), as well as a type III secretion system, which mediates the injection of Yops into the cytosol of eukaryotic cells (Hueck, 1998; Cornelis *et al.*, 1998; Lee *et al.*, 2001; Navarro *et al.*, 2005). Among these proteins, YopP is the primary virulence factor inducing the apoptosis of macrophages and altering the expression of cytokines (Mills *et al.*, 1997; Zhang *et al.*, 2005). The other primary virulence factor is tissue invasion, which is a pivotal first step in the pathogenesis of infections caused by *Y. enterocolitica*. The genes involved in the initial step of invasion are referred to as invasion (*inv*) and *yadA* (Revell and Miller, 2000; Roggenkamp *et al.*, 2003). The *inv* gene is located on the bacterial chromosome, and activates the primary invasion factor (Revell and Miller, 2000; Grosdent *et al.*, 2002). The *yadA* gene is dependent on pYV, but also plays a critical role in the initial step of invasion (Roggenkamp *et al.*, 2003; Grosdent *et al.*, 2002). Mechanisms such as the type III secretion system and the virulence genes have also been detected in gram-negative bacteria, including *Shigellae* spp. and *Salmonella typhimurium* (Sansonetti, 2001; Guiney, 2005). These mechanisms appear to promote the survival of bacteria within a host. Bacteria also require a mechanism allowing them to escape or evade phagocytosis. For example, *Listeria* grows as an intracellular parasite, which is able to replicate inside both epithelial cells and macrophages (Dussurget *et al.*, 2004).

Recently, the inhibition of phagosome-lysosome fusion was suggested as a mechanism enabling the intracellular survival of *Brucella abortus* in both professional and nonprofessional phagocytes (Watarai *et al.*, 2002). *B. abortus* invades host cells via the accumulation of lipid rafts in macropinosomes. In fact, lipid rafts are becoming the focus of increasing attention, due to their possible role as a significant portal of entry into host cells for a broad variety of pathogenic microorganisms (Shin and Abraham, 2001; Shin and Abraham, 2001). The molecules comprising lipid

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rafts include GPI- anchored proteins, which harbor sphingolipid-rich and cholesterol-rich microdomains (Shin and Abraham, 2001; Shin and Abraham, 2001). Pretreatment with the cholera toxin B subunit, which binds to GM1 molecules expressed on the cell plasma membrane of the host with a high degree of affinity and specificity, modulates the functions of the lipid rafts, and greatly diminishes the degree to which wild-type *B. abortus* is internalized by bone marrow-derived macrophages or J774.1 cells (Naroeni and Porte, 2002). Moreover, pretreatment with phosphatidylinositol phosopholipase C (PI-PLC) to remove GPI-anchored molecules from the cell surface, as well as pretreatment with cholesterol-scavenging or cholesterol-binding drugs (β-cyclodextrin, nystatin and filipin), has also been shown to markedly attenuate the internalization of *B. abortus* by macrophages (Watarai *et al.*, 2002; Naroeni and Porte, 2002; Kim *et al.*, 2002). These results suggest that bacterial invasion via lipid rafts may constiute a prerequisite step to the subsequent intracellular growth of bacteria within host cells.

In a previous study, we reported a case of *Y. enterocolitica* infection characterized by symptoms that were quite different from those associated with the typical infection caused by this bacterium (Hosaka *et al.*, 1997). Interestingly, the strain isolated from blood cultures (KU14) exhibited virulence, in spite of its lack of the pYV virulence plasmid. In the present study, we determined that the KU14 strain of *Y. enterocolitica* evidenced enhanced intracellular growth in macrophages after lipid raft-mediated invasion.

Materials and Methods

Bacterial strains

The *Y. enterocolitica* strains tested in this study were as follows: WA-314 (ATCC51871), harboring the pYV virulence plasmid (serogroup O:8); WA-C (ATCC51872), a non-plasmid-bearing derivative of WA-314; and the clinical isolate *Y. enterocolitica* KU14, which also lacks the pYV virulence plasmid (Hosaka *et al.*, 1997). After an overnight culture at 26°C and dilution to 1:100 in fresh Luria-Bertani (LB) broth, incubation continued for an additional 4 hours at 37°C (Ruckdeschel *et al.*, 1998). The bacteria were then resuspended in LB broth at a concentration of 10^8/ml, with the concentration being adjusted via optical density measurements at 530 nm, and confirmed by plating serial dilutions of the cultures on LB agar and counting the colony forming units (CFUs) after 24 hours of incubation at 26°C.

Cell culture

A murine macrophage-like cell line (J774.1) and a human monocytic cell line (THP-1) were grown on RPMI 1640 medium (Sigma-Aldrich Chemical Co., USA) supplemented with 10% heat- inactivated fetal calf serum (FCS) (Hyclone, USA), and penicillin/streptomycin (100 U/ml) (Gibco BRL, USA) in a humidified atmosphere with 5% CO_2 at 37°C. For the experiments, 5×10^4 J774.1 cells were seeded onto 24-well plates and cultured for 2 days, while the THP-1 cells were suspended in microtubes, at a concentration of 1×10^5 cells/ml.

Infection of J774.1 or THP-1 cells with Y. enterocolitica and intracellular growth assay

Prior to the infection assay, the J774.1 and THP-1 cells were washed with PBS. The cells were then infected at a multiplicity of infection (moi) of 10 bacteria/cell. The J774.1 cells were thoroughly washed 3 times with PBS after 1 hour of infection. Then, in order to remove extracellular bacteria, the J774.1 cells were incubated on RPMI 1640 medium supplemented with 10% FCS and 100 μg/ml gentamicin, for the indicated periods. The THP-1 cells were washed with PBS and centrifuged (1,000 × g for 10 min) after 1 hour of infection, and were then transferred to 24-well culture plates and incubated for the indicated periods. After incubation, both the J774.1 cells and THP-1 cells were thoroughly washed 5 times with PBS. The intracellular bacteria were then harvested after the lysis of the J774.1 cells or THP-1 cells via the addition of water (1 ml) to each of the wells. The desired bacterial concentrations were confirmed by plating serial dilutions of the samples on LB agar, and counting CFUs after 24 hours of incubation at 26°C. After the J774.1 cells had been infected for 3 hours with *Y. enterocolitica*, the cells were Giemsa stained (Merck, Germany).

Drug treatment

Treatment with phorbol myristate acetate (PMA, Sigma-Aldrich Chemical Inc, USA) and β-cyclodextrin (Sigma-Aldrich Chemical Inc, USA) was conducted in accodance with the method described by Kim *et al.*, 2002. The J774.1 cells and THP-1 cells were incubated with RPMI 1640 medium containing 100 ng/ml PMA for 16 hours, or with 5 mM β-cyclodextrin for 30 minutes, at 37°C. After washing with medium containing PMA or β-cyclodextrin, the macrophages were infected with *Y. enterocolitica*, as described above.

FACS analysis of apoptosis

In order to quantify the apoptosis of J774.1 cells in response to bacterial infection, apoptotic cells were labeled with fluorescein isothiocyanate-conjugated annexinV (FITC-AnnexinV) and stained using the DNA stain propidium iodide (PI), to differentiate the apoptotic cells from the necrotic cells, using an

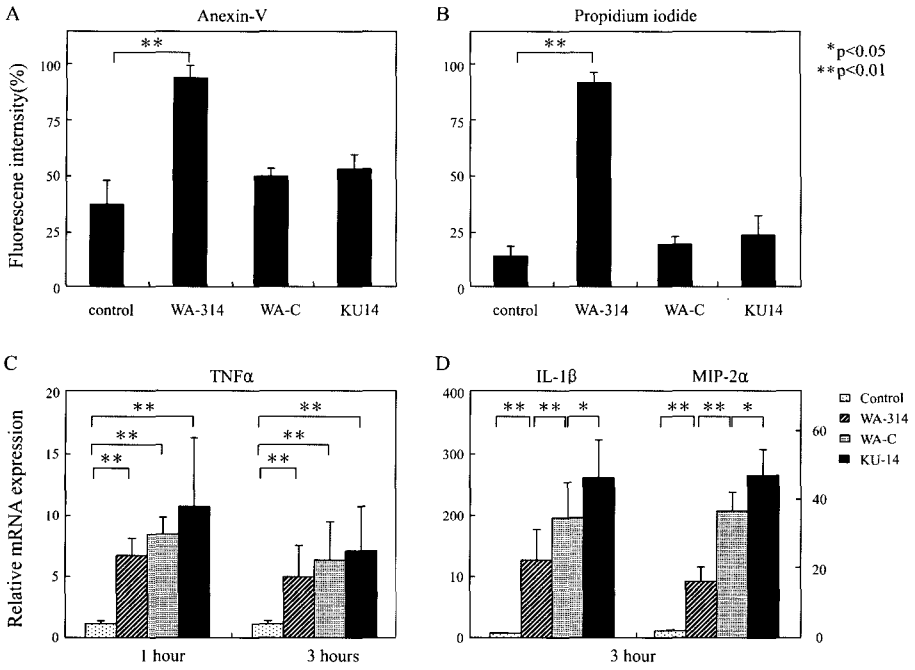

Fig. 1. Induction of apoptosis and influence on cytokine expression by Y. enterocolitica-infected macrophages. Y. enterocolitica WA-314, WA-C, and KU14 were administered at an MOI of 10 bacteria per cell, as shown in the Methods section. A, B: J774.1 cells were washed in PBS after 3 h of infection, and were then stained with FITC-AnnexinV and PI, prior to a FACScan analysis. C, D: TNFα, IL-1β, and MIP-2α mRNA expression was assessed by real-time PCR after total RNA had been extracted from the cells. Results are expressed as means ± SD for one representative experiment out of five.

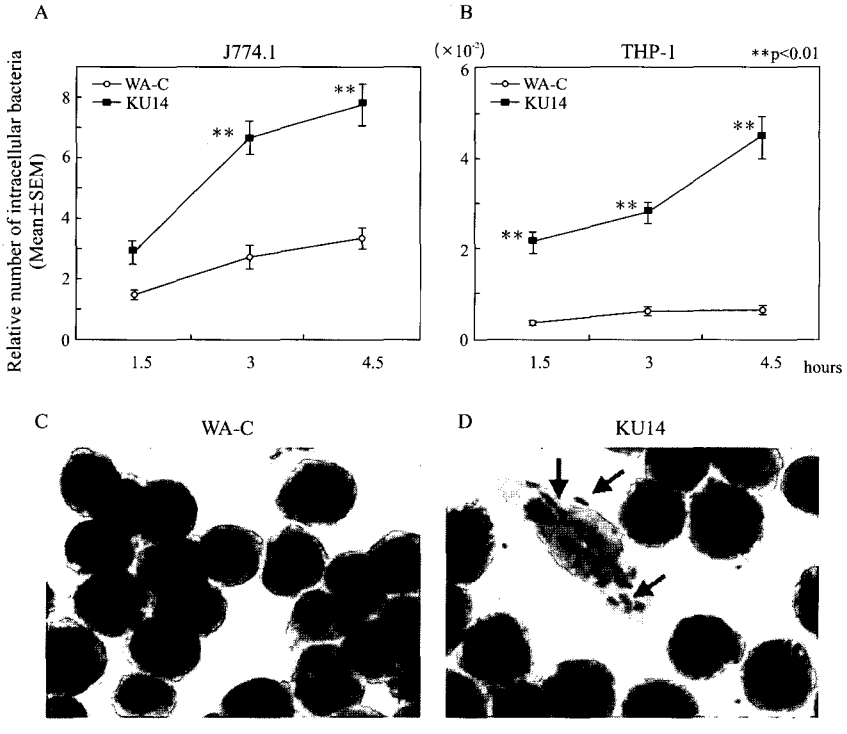

Fig. 2. Intracellular replication of Y. enterocolitica in macrophages. A, B: The infection assay was performed at an MOI of 10 bacteria per cell for the indicated periods (1.5, 3, and 4.5 hr). Bacteria were harvested after the lysis of J774.1 cells with 1 ml of water per well. The intracellular growth of bacteria was then assessed via the counting of CFUs after 24 hours of incubation at 26°C on LB agar plates using serially diluted samples. Results are expressed as the means ± SD for one representative experiment out of six. C, D: Giemsa staining was conducted 3 h after infection. as shown in the Methods section.

FITC-AnnexinV fluorescein staining kit (Wako, Japan). Subsequently, the J774.1 cells were washed with PBS, and then harvested via centrifugation (1,000 × g for 5 min). The J774.1 cells were added to the staining buffer (100 μl/samples), and incubated for an additional 15 minutes. The apoptotic cells were detected by flow cytometry with a FACScan (Becton Dickinson, USA). The J774.1 cells were identified with FITC-AnnexinV and PI, and also by their scatter properties, via flow cytometry. The gated populations were then assessed with regard to the surface expression of annexinV or PI DNA staining. The apoptotic cells were quantified as the mean percentage of FITC-annexinV positive J774.1 cells from the total number of cells. The necrotic cells were quantified as the mean percentage of PI-positive J774.1 cells out of the total number of cells. The results were expressed as the mean percentage of fluorescent apoptotic cells detected in three independent experiments.

Gene expression analysis

The levels of expression of genes for a variety of proinflammatory mediators were determined via real-time PCR. After infection, total RNA was extracted from the J774.1 cells with an RNeasy Mini kit (Qiagen GmbH, Germany). 1 μg of the RNA was then amplified via real-time PCR (Quantitect SYBR Green PCR, Qiagen), using the following oligonucleotide primers: 5'-TTGGATCCGTCAGTGCCGGCCTCGTCTCATAG -3' and 5'-TTCT CGAGGACCCTTTTGGCACCACCC TTCAG-3' for murine GAPDH, 5'-ATGATCCGCGAC GTGGAA-3' and 5'-CTGCCACAAGCAGGAATGAG -3' for murine TNFα, 5'-ACCTGGGCTGTCCTGATG AGAG-3' and 5'-CCACGGGAAAGACACAGGTAG C-3' for murine IL-1β, and 5'-CAAGAACATCCAG AGCTTGAGTGT-3' and 5'-CTTGAGAGTGGCTATG ACTTCTGTCT-3' for murine MIP- 2α. Murine GAPDH was used as an internal control for the quantification of TNFα, IL-1β and MIP-2α in the real-time PCR. The real-time PCR was conducted as follows: 40 cycles of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. The PCR products were subsequently analyzed using the ABI Sequence Detection System (ABI PRISM 7700).

Statistics

Results are expressed as the means ± SD or SEM. The Student's *t*-test was used for multiple comparisons, and differences were considered to be statistically significant at $p<0.05$ or $p<0.01$.

Results

Induction of apoptosis and expression of TNFα, IL-1β, and MIP-2α mRNA in J774.1 cells with Y. enterocolitica infection

In order to determine the virulence of KU14, we evaluated the induction of apoptosis in the infected J774.1 cells. When the cells were infected with WA -314, WA-C, or KU14 for 3 hours, the induction of apoptosis witnessed in the WA-C and KU14-infected cells was significantly less prominent than in the cells infected by WA-314 (Fig. 1A, 1B). This indicated that KU14 does not possess a virulence factor that promotes apoptosis. TNFα, IL-1β, and MIP-2α mRNA expression in the infected J774.1 cells was assessed via the real-time PCR amplification of total RNA. In the KU14-infected cells, TNFα mRNA expression was upregulated compared to what was observed in the WA-C-infected cells at 1 and 3 hours (Fig. 1C), whereas the expression levels of IL-1β and MIP-2α mRNA in the KU14-infected cells were significantly higher than was seen in the WA-C-infected cells at 3 hours (Fig. 1D). These results suggested that KU14 influenced the production of cytokines by host cells.

Intracellular growth of bacteria after infection of macrophages with Y. enterocolitica

As is shown in Fig. 1, KU14 appears to influence the production of cytokines by the host cells. After the J774.1 cells and THP-1 cells had been infected with WA-C or KU14, the intracellular growth of the bacteria was evaluated over the indicated periods. The intracellular growth of KU14 in the J774.1 cells was determined to be significantly greater than that of WA-C, after 3 and 4.5 hours (Fig. 2A). Moreover, the intracellular growth of KU14 in the THP-1 cells was also significantly greater than that of WA-C from the 1.5-hour mark and afterward (Fig. 2B). When the J774.1 cells were infected with KU14, bacteria were observed in the cytosol by Giemsa staining after 3 hours, but were not observed at this time in the WA-C-infected cells (Fig. 2C, 2D). These results suggested that KU14 could avoid phagocytosis, and could grow intracellularly within the host.

Influence of PMA-induced macropinocytosis on intracellular growth of Y. enterocolitica

PMA is known to induce macropinosomes and promote phagosome-lysosome fusion, without causing the accumulation of lipid rafts (Araki *et al.*, 1996). Therefore, to assess the possible influence of the lipid rafts on intracellular growth, PMA-treated macrophages (J774.1 cells and THP-1 cells) were infected with KU14 or WA-C for the indicated periods. Although the level of intracellular growth of KU14 in the PMA-treated J774.1 cells was

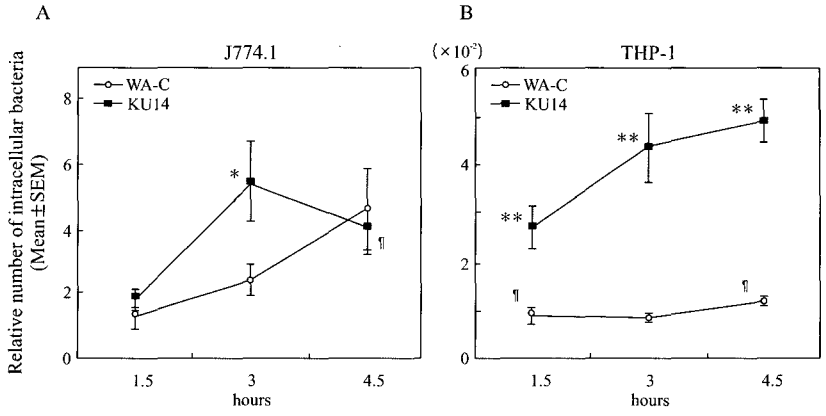

Fig. 3. Effect of PMA on infection of macrophages by Y. enterocolitica. The infection assay was performed at an MOI of 10 bacteria per cell for the indicated periods (1.5, 3, and 4.5 hr) after the J774.1 cells and THP-1 cells were incubated for 16 hours with 100 ng/ml of PMA. Intracellular bacterial growth was assessed by counting CFUs after 24 hours of incubation at 26°C on LB agar plates using serially diluted samples. Results are expressed as the mean ± SEM for one representative experiment out of six. *, $p<0.05$ **, $p<0.01$ (WAC-infected PMA-treated cells (WAC) vs. KU14-infected PMA-treated cells (KU14)). ¶$p<0.05$, ¶¶$p<0.01$ (WAC- or KU14-infected untreated cells vs. WAC- or KU14-infected PMA-treated cells).

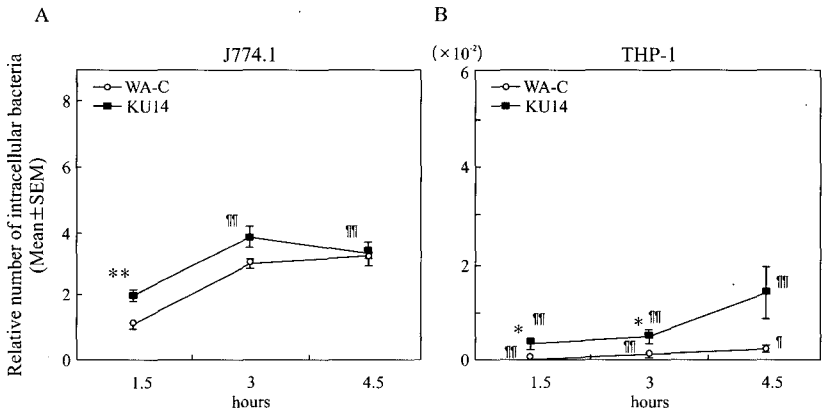

Fig. 4. Effects of β-cyclodextrin on the intracellular growth of Y. enterocolitica. The infection assay was performed at an MOI of 10 bacteria per cell for the indicated periods (1.5, 3, and 4.5 hr) after the J774.1 and THP-1 macrophages had been incubated for 30 minutes with 5 mM β-cyclodextrin (β-CD). Intracellular bacteria were assessed by counting CFUs after 24 hours of incubation at 26°C on LB agar plates using serially diluted samples. Results are expressed as the mean ± SEM for one representative experiment out of six. *, $p<0.05$ **, $p<0.01$ (WAC-infected β-CD-treated cells (WAC) vs. KU14-infected β-CD-treated cells (KU14)). ¶$p<0.05$, ¶¶$p<0.01$ (WAC- or KU14-infected untreated cells vs. WAC- or KU14-infected β-CD-treated cells).

found to be significantly greater than that of WA-C after 3 h, this level of growth had not changed significantly at the 4.5-hour mark (Fig. 3A). The intracellular growth of KU14 in the PMA-treated THP-1 cells was significantly greater than that of WA-C for the periods shown in Fig. 3B. Additionally, the intracellular growth of KU14 in the PMA-treated THP-1 cells evidenced little inhibition compared with what was observed in the untreated THP-1 cells, while such intracellular growth was not detected after WA-C infection. These results indicated that KU14 was capable of survival in the J774.1 cells, due to its ability to avoid phagosome-lysosome fusion after macropinosome formation. However, KU14 could not survive in the phagolysosomes. The PMA-treated THP-1 cells experiment, however, showed that WA-C could not survive in the phagolysosomes and KU14 was a little growth in it.

Effect of β-cyclodextrin on intracellular growth

Recently, it has been reported that β-cyclodextrin pretreatment strongly inhibits the intracellular growth of *B. suis* in J774.1 cells (Naroeni and Porte, 2002). This finding suggests that the bacteria invade these cells via the lipid rafts. Therefore, in order to characterize the possible role of the lipid rafts in *Yersinia* infection, β-cyclodextrin-treated J774.1 cells and THP-1 cells were infected with WA-C and KU14 for the indicated periods. The intracellular growth of KU14 in the β-cyclodextrin-treated J774.1 cells did not differ significantly from that of WA-C after 3 and 4.5 h (Fig. 4A), but was significantly lower than the growth observed in the untreated cells. The intracellular growth of KU14 in β-cyclodextrin-treated THP-1 cells also did not differ significantly from that of WA-C after 4.5 h, although the results at 1.5h and 3h post-infection were statistically significantly different (Fig. 4B). Additionally, the intracellular growth of KU14 in the β-cyclodextrin-treated THP-1 cells was significantly less prominent than in the untreated cells. These findings suggested that KU14 was capable of survival in the macrophages, via the evasion of phagosome-lysosome fusion after macropinosome formation, a process which putatively operates through the modulation of cholesterol on the cell surface.

Discussion

Y. enterocolitica induces a broad range of gastrointestinal symptoms, particularly acute enteritis (Straley *et al.*, 1993). The virulence of this organism is dependent on the pYV virulence plasmid, which encodes for a group of virulence factors (Yops). We found, in a previous study, that *Y. enterocolitica* KU14, which lacks this pYV plasmid, was still able to cause clinical disease (Hosaka *et al.*, 1997). In the present study, an *in vitro* infection assay demonstrated that KU14, unlike WA-314, did not trigger macrophage apoptosis. In addition, the degree of intracellular growth of KU14 in the macrophages was higher than that observed for WA -C. Treatment with cholesterol-binding drugs that affect the lipid rafts resulted in a dramatic reduction in the intracellular growth of KU14. These data clearly indicate that the enhanced intracellular growth of KU14 is dependent on lipid-raft mediated infection, which points to the existence of a heretofore-unknown *Y. enterocolitica* virulence factor.

Our previous research has shown that KU14 does not possess the pYV virulence plasmid. Yops (YopP, E, H, and O), encoded for by this plasmid, were also undetectable in KU14 by PCR (data not shown). In this study, when J774.1 cells were infected for 3 hr with WA-314, WA-C, or KU14, no apoptotic induction was detected after WA-C and KU14 infection, although this was clearly observed in the case of infection with WA-314 (Fig. 1A, 1B). This suggests strongly that KU14 does not possess pYV-dependent virulence factors. However, KU14 was still capable of causing clinical infection and symptoms (Hosaka *et al.*, 1997). Therefore, we attempted to evaluate the production of proinflammatory mediators occurring after the infection of the J774.1 cells. A variety of proinflammatory mediators have been determined to contribute to the development of sepsis (Dinarello, 2000; Ashare *et al.*, 2005). As is shown in Figs. 1C and 1D, KU14 elicited higher expression levels of TNFα, IL-1β, and MIP-2α mRNA compared with WA-C, after 3 hours. However, when the J774.1 cells were stimulated with heat-killed WA-C or KU14, the degree to which proinflammatory mediators were induced did not differ significantly between the two bacterial strains (data not shown). These findings indicated that the heat-labile components of KU14 affected the production of cytokines by the host cells.

Some gram-negative bacteria induce the expression proinflammatory cytokines, as the result of cellular responses to intracellular pathogens (Cossart and Sansonetti, 2004). Therefore, the intracellular growth of KU14 was assessed in J774.1 cells and THP-1 cells. Our results indicated that the KU14 bacterial line exhibited a marked increase in intracellular growth, by approximately 4-fold, as compared with the WA-C bacteria after 4.5 hours (Fig. 2A, B). In addition, KU14 organisms were observed in the cytosol at 3 hours post-infection (Fig. 2D). These results suggested that the intracellular growth of KU14 resulted in the induction of proinflammatory mediator generation by the macrophages. However, a question remains as to the mechanism by which KU14 evaded phagocytosis. Two genes that are known to be crucial in invasion are the *inv* gene and the *ymoA* gene, which modulates *inv* (Revell and Miller, 2000; Ellison *et al.*, 2003). When we analyzed the *inv* and *ymoA* genes, KU14 did not demonstrate mutations in either gene (data not shown). This suggests that KU14 possesses biological virulence factors that disrupt the functions of the host cell.

Recent research has demonstrated that bacterial invasion via the lipid rafts results in replication within the host cells (Shin and Abraham, 2001; Shin and Abraham, 2001; Naroeni and Porte, 2002; Watarai *et al.*, 2002). Lipid rafts are known to be involved in the inhibition of phagosome-lysosome fusion, whereas PMA induces macropinosomes and phagosome-lysosome fusion, but does not induce an accumulation of lipid rafts (Araki *et al.*, 1996). Therefore, the influence of PMA-induced macropinocytosis on the intracellular growth of *Y. enterocolitica* was evaluated. Our results indicated that the intracellular growth of KU14 in the

PMA-treated J774.1 cells did not differ significantly from that of WA-C after 4.5 h, and that the growth of KU14 in the PMA-treated cells was significantly lower than that observed in the untreated cells (Fig. 3A). However, the intracellular growth of KU14 in the PMA-treated THP-1 cells exhibited little inhibition as compared with that seen in the untreated cells after 4.5 hours (Fig. 3B). Although the growth of KU14 did not decrease substantially in the PMA-treated THP-1 cells, this is a human monocytic cell line, and thus may possess some weak phagosome-lysosome activity (Auwerx, 1991; Nishizuka, 1995). Therefore, it is suggested that the inhibition of bacterial growth, similar to that detected in the J774.1 cells, might also occur in the THP-1 cells, if they possess similar phagolysosome activity. These results also show that KU14 is incapable of survival in the phagolysosomes of macrophages. Furthermore, treatment with the cholesterol-binding drug, β-cyclodextrin, was shown to affect the intracellular growth of KU14. In fact, the intracellular growth of KU14 in the β-cyclodextrin-treated J774.1 cells and THP-1 cells did not differ significantly from that of WA-C (Fig. 4A, 4B), although the intracellular growth of KU14 in the β-cyclodextrin-treated J774.1 cells and THP-1 cells was significantly lower than that observed in the untreated cells. These results indicate that KU14 was able to survive in the macrophages by evading phagosome-lysosome fusion after macropinosome formation, but was not able to survive in the phagolysosomes. The results also suggest strongly that KU14 invaded the cells via the lipid rafts.

Although *Yersinia* has been reported to possess a virulence plasmid, few reports have been conducted regarding the virulence factors contributing to the intracellular growth of *Y. enterocolitica* in the macrophages. The results of this study suggest that a clinical isolate of *Y. enterocolitica* (KU14) exploited a mechanism involving the inhibition of phagosome-lysosome fusion after infection via the lipid rafts. However, the actual factor that prevents phagosome-lysosome fusion remains to be identified. *Brucella* has a type IV secretion system, which is necessary for intracellular survival after infection via the lipid rafts (Ceil *et al.*, 2003), but *Y. enterocolitica* has no such system. In the future, we will conduct further investigations into this unknown bacterial factor, in order to elucidate the mechanism involved in the inhibition of phagosome-lysosome fusion evidenced by *Y. enterocolitica*.

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