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Regulation of the Mitogen-Activated Protein Kinases by *Brucella* spp. Expressing a Smooth and Rough Phenotype: Relationship to Pathogen Invasiveness

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By comparing smooth wild-type *Brucella* spp. to their rough mutants, we show that the LPS O chain restricted the activation of the ERK1/2 and p38 mitogen-activated protein kinase (MAPK) pathways, thus preventing the synthesis of immune mediators that regulate host defense. We conclude that the MAPKs are a target for immune intervention by virulent smooth *Brucella*.

Brucella spp. are major veterinary pathogens that can cause serious human disease. The strains which are pathogenic for humans (*Brucella abortus*, *Brucella suis*, and *Brucella melitensis*) carry a smooth lipopolysaccharide (LPS) involved in the virulence of the bacteria. The mutants which derive from these strains and express a rough phenotype are attenuated in animals or isolated macrophages compared to the parental *Brucella* (1, 9, 11, 16, 31, 38, 39, 48), suggesting that they can be used as live vaccine strains. The properties of the LPS O chain, a linear homopolymer of α1,2-linked perosamine (5), explain this attenuation. The LPS O chain protects the bacteria from cellular cationic peptides (13, 30), oxygen metabolites (45), and complement-mediated lysis (10, 27), is a key molecule for *Brucella* invasion and development (36), and impairs the apoptotic or necrotic signals directed against the infected cells (11, 35). Recently, we showed that immune molecules that elicit macrophage defense are produced at higher levels in rough *Brucella*-infected macrophages than in smooth *Brucella*-infected macrophages (26, 40). Production of immune molecules depends on intracellular signaling pathways that connect receptor-mediated events to transcriptional response within the nucleus. One important group of signaling pathways, the mitogen-activating protein kinase (MAPK) signaling cascade, is implicated in bacterial pathogenesis as demonstrated by the induction or inhibition of ERK1/2 and p38 MAPKs during infection with *Salmonella enterica* serovar Typhimurium (21), *Yersinia* spp. (34, 42), *Listeria monocytogenes* (46, 47), and *Mycobacterium* spp. (43). Therefore, the discrepancies in the virulence of smooth and rough *Brucella* could be linked to the MAPK pathways triggered by these bacteria. We thus analyzed these pathways and their possible relationship with the divergent responses of macrophages infected with smooth or rough *Brucella*. The results obtained with intact live *Brucella* greatly suggest that because of their relationship with the LPS O chain, the MAPKs are a target for immune intercession by virulent smooth *Brucella*.

Table 1 shows the *Brucella* strains used in the study. As mentioned in previous reports (1, 14, 26, 36), all of the rough *Brucella* assessed displayed a great attenuation in mouse or isolated macrophages compared to smooth *Brucella*. To analyze the activation of p38 and ERK1/2 kinases during infection, J774.A1 cells (10^6/well) were incubated with the different *Brucella* strains (multiplicity of infection [MOI] = 40) at 37°C. They were then rinsed twice with phosphate-buffered saline (PBS) and lysed in 150 μl of buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 10 mM NaF, 10 mM iodoacetamide, 1% NP-40, 1 mM Na_2VO_3, and 1 μg/ml of each protease inhibitor (leupeptin, apoprotein, and chymostatin) and centrifuged. The cytosolic fraction denatured by the addition of reducing buffer and boiling was analyzed by Western blotting with anti-phospho-p38 MAPK and anti-phospho-ERK 1/2 (Cell Signaling Technology, Beverly, MA), which recognize the phosphorylated (i.e., activated) form of the MAPKs (28, 29). Pan p38 (anti-p38MAPK) (Cell Signaling) and pan ERK2 (anti-ERK2) (Santa Cruz Biotechnology, Inc., CA), which detect both active and inactive forms of kinases, were applied on stripped blots to verify that equivalent amounts of proteins were loaded per lane (28, 29). Figure 1A shows that at 30 min postinfection (p.i.), the activation process triggered by the rough *Brucella* resulted in a potent phosphorylation of the p38 and ERK1/2 MAP kinases. Infections with smooth *Brucella* induced a markedly weaker (or no visible) stimulation of these kinases, with *B. suis* and *B. abortus* 2308 demonstrating a slightly higher capacity of activation than *B. melitensis*, a difference which could be due to the genetic background of the different bacteria. Figure 1B shows that *B. suis manB*-induced phosphorylation of ERK1/2 and p38 MAPKs occurred within 5 min of infection and increased until at least 4 h p.i. This was true for the other rough *Brucella*, demonstrating that the activation resulted from a long active process which involved the attachment, ingestion, and early death of the bacteria, as shown with macrophages infected with virulent mycobacteria (41). The weak phosphorylation triggered by *B. suis*, which was visible at 30 min p.i., increased modestly but always remained

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TABLE 1. *Brucella* strains used in the study[a]

Strain	Genotypic and/or phenotypic description	CFU/spleen at 3 wk p.i.[b]	DI for J774A.1 cells[c]
B. melitensis 16M	Spontaneous smooth nalidixic acid-resistant mutant of *B. melitensis* 16M (49)	$>10^4$	50–200
B. melitensis R5	Natural rough mutant of *B. melitensis* 16M (CITA collection, Zaragoza, Spain)	<10	<0.002
B. melitensis B3B2	Rough mini-Tn*5* insertion mutant of *B. melitensis* 16M invalidated on the perosamine synthase gene, kanamycin resistant (16)	<10	<0.01
B. suis 1330	Smooth wild-type *B. suis* (ATCC 23444)	$>5 \times 10^5$	100–500
B. suis manB	Rough mini-Tn*5* insertion mutant of *B. suis* 1330 invalidated on phosphomannose gene, kanamycin resistant (12)	<10	<0.02
B. ovis REO198	Natural rough strain, CO_2-independent *B. ovis.* Laboratory collection, CITA, Zaragoza, Spain	<10	<0.02
B. abortus 2308	Smooth wild-type *B. abortus.* Laboratory collection, CITA, Zaragoza, Spain	$>10^6$	50–200
B. abortus RB51	Vaccine strain. Rough mutant of *B. abortus* 2308, rifampin resistant. G. G. Schurig, Virginia (44)	<10	<0.1
B. abortus 45/20	Vaccine strain. Rough mutant of *B. abortus* 2308. Laboratory collection, CITA, Zaragoza, Spain	ND	<0.1

[a] The smooth and rough phenotype of the different *Brucella* strains was assessed by crystal violet staining and verified by immunoblotting techniques involving monoclonal antibodies which recognize the smooth LPS or rough LPS of *Brucella* (8). ND, not done.

[b] BALB/c mice were injected intraperitoneally with 5×10^4 CFU of one of the following bacteria: *B. suis* 1330, *B. suis manB*, *B. melitensis* 16M, *B. melitensis* B3B2, *B. melitensis* R5, *B. ovis* Reo 198, *B. abortus* 2308, and *B. abortus* RB51. Six mice per group were killed by CO_2 asphyxiation 3 weeks postinoculation. Spleens were aseptically removed and homogenized with 10 ml of PBS for determining *Brucella* counts (CFU/spleen) as indicated in references 24 and 25.

[c] J774A.1 cells were infected with the different strains of *Brucella* (MOI = 40) as indicated in the table. The number of intracellular bacteria was then measured at 90 min p.i. and 48 h p.i. The development index [DI = ($CFU_{48\ h}$/well)/($CFU_{90\ h}$/well)] was then calculated for each bacterium at 48 h p.i. (6). Each infection was performed at least three times.

at a much lower level than the phosphorylation induced by *B. suis manB*. Therefore, the slight activation observed at 30 min p.i. was not due to a delayed kinetic of activation but reflected the poor capacity of activation of the bacteria. In *Salmonella*, the LPS O chain is crucial for the signaling triggered by the direct interaction between the bacterial LPS and macrophages (32). Furthermore, the LPS lipid As of smooth and rough *B. abortus* are structurally different (4). There was no significant difference in ERK1/2 and p38 MAPK activation elicited by the LPS from virulent smooth *B. abortus* 2308 or rough vaccine strain *B. abortus* 45/20 (Fig. 1C). Moreover, when added at 100 ng in the assays, the LPS from *B. abortus* 2308 did not modify the ERK1/2 and p38 MAPK activation induced by *B. abortus* 45/20 or *B. suis manB* (MOI = 40) (data not shown). Therefore, neither a direct effect of the O chain on macrophages, nor the sole structural change of the lipid A explain the potent ability of intact rough *Brucella* to activate MAPKs compared to smooth *Brucella*. Owing to the absence of the O chain, the MAPK activation could be due to increased numbers of lipid A molecules exposed at the surface of the bacteria. However, the amount of LPS carried by 4×10^7 rough *Brucella*, which corresponds to a MOI of 40 and is theoretically lower than 50 ng LPS (33), promoted no perceptible activation of MAPKs (Fig. 1C) and therefore eliminated such a possibility. Finally, in accordance with its poor endotoxic potential (17, 23), the LPS appears to be a minor actor of MAPK activation by intact rough *Brucella*. This result appears to coincide with recent reports claiming that TLR2 but not TLR4 are involved in macrophage stimulation by heat-killed *Brucella* (15, 22). Phagocytes express a variety of receptors that participate in *Brucella* recognition and internalization (3). Because of the exposition of ligands normally hidden by the O chain in smooth *Brucella* at the surface of the bacteria, the rough mutants bind to macrophages and penetrate into these cells to a much higher extent than the parental smooth *Brucella* (9, 31, 36, 40). The rough *Brucella*-elicited activation could result from a saturation of the macrophage receptors engaged by these different ligands. However, the MAPK activation triggered by *B. ovis* REO198 eliminated this possibility. *B. ovis* REO198 binds to and penetrate macrophages or J774A.1 cells to a similar extent as smooth *Brucella* (reference 14 and data not shown), but it activated the MAPKs at a level similar to those of other rough *Brucella* spp. (Fig. 1). It is thus likely that only few favored receptors elicited MAPK activation during infection with rough *Brucella*. This possibility agrees with the data in Fig. 1D, which compare the activation of ERK1/2 triggered by *B. suis* and *B. suis manB* when the rough mutant MOI decreased from 40 to 5. Under these conditions, which tended to equalize the uptake of *B. suis* ($18{,}500 \pm 5{,}210$ CFU/10^6 cells for a MOI of 40) and *B. suis manB* ($25{,}430 \pm 7{,}840$ CFU/10^6 cells for a MOI of 5), the discrepancy in ERK1/2 activation was still observed.

J774A.1 cells infected with rough *Brucella* synthesize nitric oxide (NO) (26), a deleterious radical that controls the intracellular development of *Brucella* at the onset of infection (19, 50, 51). NO is not produced in smooth *Brucella*-invaded cells, and the purified LPS of *B. abortus* 2308 (100 ng) did not impair the production of NO elicited by rough *Brucella* (data not shown). The expression of the inducible nitric oxide synthase (iNOS) explains these data, with iNOS being induced only in rough *Brucella*-infected cells (Fig. 2A). In phagocytes, the transcription of the *nos2* gene can be triggered by p38 and/or ERK1/2 MAPK pathways (7). Therefore, iNOS could constitute a link between the MAPK responses induced by rough and smooth *Brucella* and the relative capacity of invasiveness of these bacteria. To determine whether ERK1/2 and p38 MAPKs were involved in iNOS induction, iNOS in cells infected with *B. suis manB* in the presence or absence of specific pharmacological inhibitors of these kinases was measured. SB203580 (which inhibits p38MAPK activation) and PD98059 (which represses activation of MEK-1, the kinase upstream of ERK-1/2) (28, 29), respectively, impaired the *B. suis manB*-triggered phosphorylation of p38 and ERK1/2 MAPKs in a

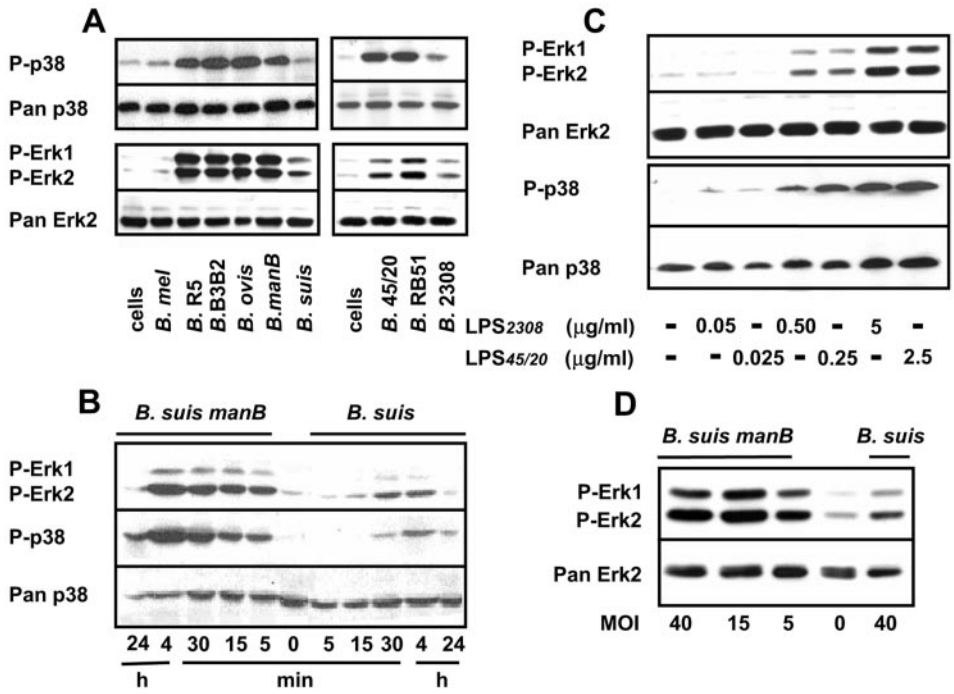

FIG. 1. Activation of ERK1/2 and p38 MAPKs in J774A.1 cells treated with different *Brucella* strains or with *Brucella* LPSs. ERK1/2 and p38 MAPKs were determined by Western immunoblotting of J774A.1 cells treated (A) for 30 min with different strains of smooth or rough *Brucella* (MOI = 40), (B) for different periods of time with *B. suis manB* or *B. suis* (MOI = 40), or (C) for 30 min with different concentrations of purified LPSs from smooth *B. abortus* 2308 (0.050 μg/ml to 5 μg/ml) or rough *B. abortus* 45/20 (0.025 μg/ml to 2.5 μg/ml). The LPSs were obtained from I. Moriyón (University of Navarra, Pamplona, Spain). They were carefully solubilized by sonication. Their purification and properties have been reported elsewhere (2, 14). The effect of the smooth LPS was assessed at concentrations double those for the rough LPS to compare the lipid A of both LPSs (I. Moriyón, personal communication) (D) or for 30 min with *B. suis manB* (MOI = 40, 15, and 5) or *B. suis* (MOI = 40); 2×10^6 J774A.1 cells were cultured at 37°C for the indicated periods of time in 150 μl of RPMI 1640 alone (cells) or supplemented with *B. suis*, *B. melitensis* 16 M (*B. mel*), *B. abortus* 2308 (*B*. 2308), *B. ovis* REO198 (*B*. *ovis*), *B. suis manB* (*B. manB*), *B. melitensis* R5 (*B*. R5), *B. melitensis* B3B2 (*B*. B3B2), *B. abortus* RB51 (*B.* RB51) or *B. abortus* 45/20 (*B.* 45/20), or with different concentrations of the purified LPS from *B. abortus* 2308 or *B. abortus* 45/20. They were then rinsed, lysed, and after sodium dodecyl sulfate-10% acrylamide gel electrophoresis and transfer to nitrocellulose membrane, analyzed with phosphospecific antibodies against ERK1/2 or p38 active kinases. The blots were stripped and reprobed with pan antibodies (A and C). In panel B, the same blot was sequentially analyzed for phospho-ERK1/2, phospho-p38, and pan p38 MAPKs. Results are representative of three separate experiments which gave identical results.

dose-dependent manner (Fig. 2B and C), with a complete inhibition at 25 μM for PD98059 and 20 μM for SB203580. In parallel, PD98059 impaired the induction of iNOS, while SB20380 consistently had no effect on the expression of this enzyme (Fig. 2E), a result confirmed by the effect of the inhibitors on NO release (data not shown). It demonstrated that only the ERK1/2 MAPK pathway was meaningful for iNOS induction and that the weak activation of the ERK1/2 MAPK induced by smooth *Brucella* was not sufficient to induce iNOS expression (Fig. 2A). This explains why in macrophages invaded by these bacteria the expression of iNOS requires an additional signal elicited, for instance, by exogenous gamma interferon (19, 50). The rough *Brucella* were capable of bypassing this signal, perhaps because they potently activated the ERK1/2 MAPK pathway, these kinases being one possible branch of gamma interferon signaling (37). However, other unidentified pathways, not related to p38 MAPK, could also participate in iNOS induction.

To verify whether the ERK1/2 MAPK activation pathway influences the intracellular outcome of *Brucella*, we tested the influence of PD98059 on macrophage infection. Assays were performed with 24-well plates (Falcon; Becton Dickinson, Meylan, France). J774A.1 cells in RPMI 1640-10% fetal calf serum (FCS) (10^6 cells ml^{-1} per well) were pretreated with PD98059 at 25 μM for 30 min prior to infection; they were then incubated for 30 min at 37°C with a suspension of *B suis manB* or *B. suis* (MOI = 40) in the presence of the drug. After washing three times with PBS to remove extracellular bacteria, the infected macrophages were reincubated with PD98059 for an additional 4 h in RPMI-10% FCS supplemented with 30 μg/ml gentamicin before the drug was washed and the cells were cultured in the same medium. In these conditions, PD98059 did not induce any toxicity as determined by microscopic observations and trypan blue exclusion (41 and data not shown). At several times p.i., the cells were washed twice and the number of viable intracellular bacteria was determined after cell lysis in 0.1% Triton X-100 (18). As with HeLa cells infected with *Brucella* (20), PD98059 had no effect on the phagocytosis of *B. suis manB* ($P > 0.3$) (or *B. suis*, $P > 0.4$). However, in macrophages, it significantly reversed the elimination of intracellular *B. suis manB* which occurred in the absence of drug (Fig. 3A) (26, 36) ($P < 0.005$ at 48 h). This indicated that the ERK1/2 MAPK pathway triggered by *B. suis manB*, which had no influence on the entrance of the bacteria,

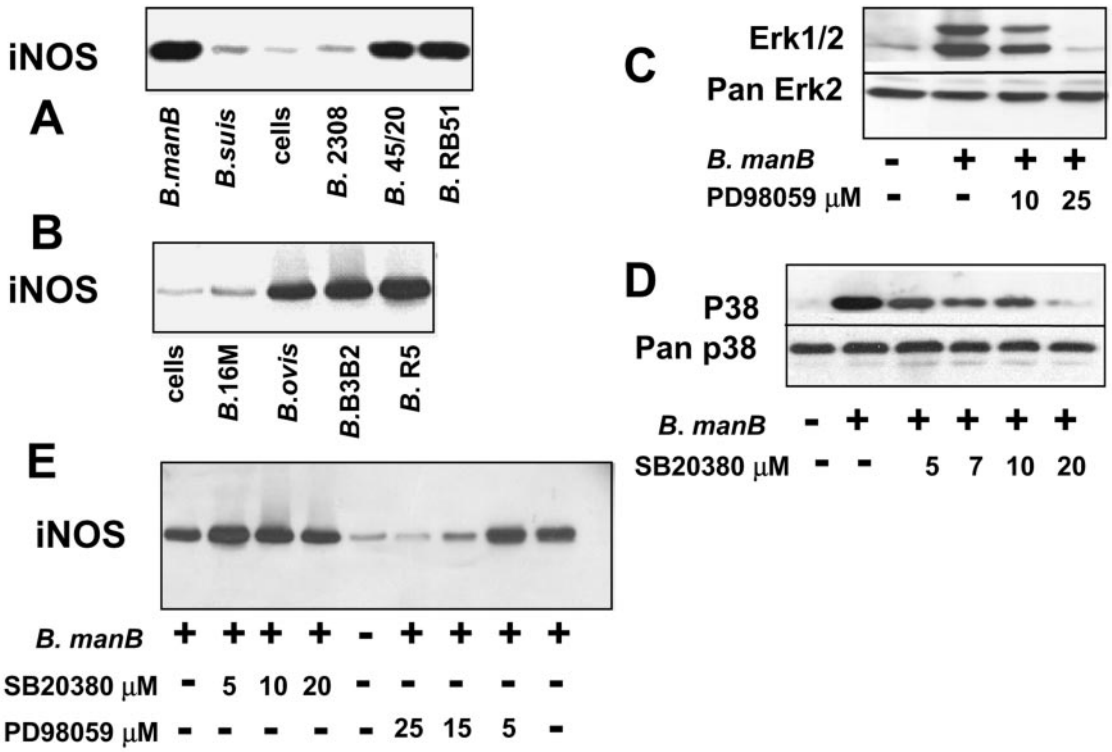

FIG. 2. (A and B) Analysis of iNOS expression in J774A.1 cells infected with different *Brucella* strains. J774A.1 cells (10^6) in 100 μl of RPMI 1640 were infected for 30 min at 37°C with the different *Brucella* strains analyzed in Fig. 1 (MOI = 40) or not infected (cells). They were then extensively washed and cultured in gentamicin-supplemented RPMI 1640-10% FCS. At 24 h p.i., they were lysed and analyzed by Western immunoblotting, with a mouse anti-iNOS serum (Alexis Corp., San Diego, Calif.) as previously described (19). (C and D) Inhibition of ERK1/2 and p38 MAPK activation elicited by *B. suis manB*. J774A.1 cells were pretreated for 30 min with the indicated concentrations of PD98059 or SB20380 in RPMI 1640 at 37°C. They were then infected (or not) for 30 min with *B. suis manB* (MOI = 40) in the presence of the MAPK inhibitor applied during the pretreatment. They were lysed and analyzed by Western immunoblotting as mentioned in Fig. 1, with phosphospecific antibodies against ERK1/2 or p38 active kinase. The stripped blots were then reprobed with pan antibodies. (E) Effect of PD98059 and SB20380 on iNOS expression in J774A.1 cells infected with *B. suis manB*. J774A.1 cells were pretreated (or not) for 30 min with the indicated concentrations of PD98059 or SB20380 in RPMI 1640 at 37°C. They were then infected for 30 min with *B. suis manB* in the presence of the MAPK inhibitor applied during the pretreatment, cultured for a further 4 h in RPMI 1640-10% FCS supplemented with 30 μg/ml gentamicin in the presence of PD98059 or SB20380. The MAPK inhibitors were then removed by washing, and the infected cells were cultured again in the gentamicin-supplemented medium. At 24 h p.i., the expression of iNOS was analyzed by Western immunoblotting as in panels A and B. Each experiment was performed at least three times. The MAPK inhibitors were dissolved in dimethyl sulfoxide (DMSO) so that the DMSO dilution was always lower than 0.1% in assays. At this dilution, the DMSO affected neither MAPKs nor iNOS induction. *B. manB*, *B. suis manB*; *B.* 2308, *B. abortus* 2308; *B.* 45/20, *B. abortus* 45/20; *B.* RB51, *B. abortus* RB51; *B.* 16M, *B. melitensis* 16 M; *B. ovis*, *B. ovis* REO198; *B.* B3B2, *B. melitensis* B3B2; *B.* R5, *B. melitensis* R5.

was determinant for its future. Blocking the ERK1/2 signaling pathway was beneficial to the bacteria, which meant that its activation favored the elimination of the *B. suis manB* once they were phagocytosed. These results paralleled those observed with infection performed in the presence or absence of L-NAME (*N*-ω-nitro-L-arginine methyl ester), a specific inhibitor of iNOS (Fig. 3B). Altogether, the data showed that the *B. suis manB*-induced ERK1/2 MAPK pathway regulated the killing of bacteria and that it could be through the induction of iNOS. This possibility was in accordance with the results observed during the infection of J774A1 cells with *B. suis*: when iNOS was not induced, PD98059 (or L-NAME) did not significantly modify the bacterial invasiveness (Fig. 3) (19) ($P > 0.5$ or $P > 0.2$ at 48 h compared to *B. suis* alone). Therefore, the *B. suis manB*-triggered ERK1/2 MAPK activation armed the host cells with a NO-generating system. The capacity of defense of the cells against the bacteria was thus reinforced by a microbicidal weapon which was missing during infections with smooth wild-type *B. suis*. Regarding the data of Fig. 1 and 2, the ERK1/2 MAPK activation and its consequence on iNOS induction appeared to be a general property of the rough *Brucella* spp. which participate in the clearance of the bacteria during an active infection. In addition, when it is produced in large amounts, NO is deleterious to cells. Therefore, in certain conditions of infections, the rough *Brucella*-elicited activation could favor the death of the host cells and thus negatively affect the apparent development of the bacteria. This could be the case in infections performed at elevated MOIs (35) or in the absence of serum (11). In any case, by indirectly preventing the ERK1/2 MAPK activation, the LPS O chain impaired NO formation and thus favored the intramacrophagic development of smooth *Brucella*.

We evaluated the effect of MAPK activation on the intracellular development of *Brucella* by analyzing the infected mac-

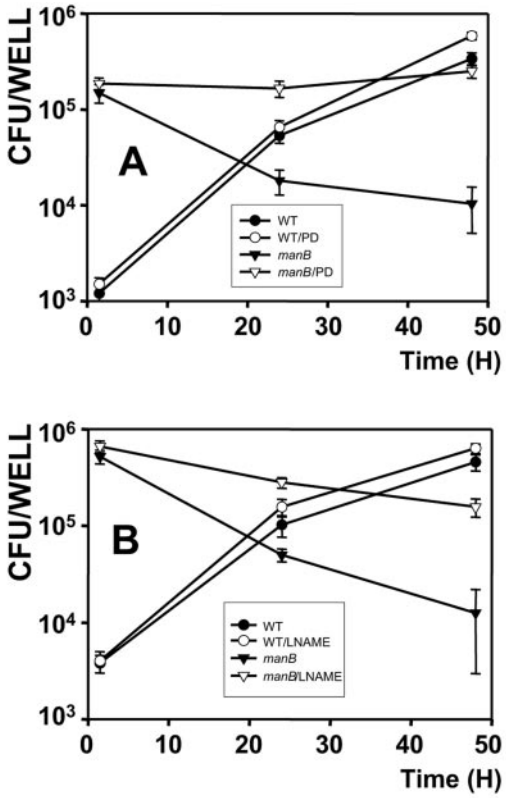

FIG. 3. Intracellular behavior of *B. suis manB* or *B. suis* within J774A.1 cells treated with PD98059 (A) or L-NAME (B). (A) J774A.1 cells pretreated for 30 min with 25 μM PD98059 (▽, ○) or not treated (▼, ●) were infected with *B. suis manB* (▼, ▽) or *B. suis* (●, ○) as described in the text. They were then washed and cultured for a further 4 h in gentamicin-supplemented RPMI 1640-10% FCS in the presence 25 μM PD98059 (▽, ○) or not (▼, ●). The MAPK inhibitor was then removed by washing, the infected cells being cultured in the gentamicin-supplemented medium. At different times p.i., the intracellular number of bacteria was measured as described in reference 26. (B) J774A.1 cells were infected with *B. suis manB* (▼, ▽) or *B. suis* (●, ○) (MOI = 40). Thirty minutes after the onset of the infection, they were cultured in gentamicin-supplemented RPMI 1640-10% FCS in the presence (▽, ○) or absence of 3 mM L-NAME (▼, ●). At different times, the intracellular number of bacteria was measured as described in reference 26. Infections were performed in triplicate. Results were expressed as CFU/well ± standard deviation. DMSO used to dissolve PD98059 did not affect the infection of J774A.1 cells with *Brucella*, a dilution of 0.1% having been applied in the assays. Both experiments were repeated at least four times, giving similar results. Where indicated in the text, CFU values relative to different assays at one time p.i. were compared by using the Student *t* test.

rophages for the induction of iNOS. This particular protein was chosen because it is known that NO was involved in the elimination of *Brucella* in the first 48 h p.i. (19, 50). However, it is evident that other pathways are involved in the elimination of intracellular rough *Brucella* (see introduction). Inflammatory cytokines and chemokines (interleukin-1, tumor necrosis factor alpha, MIP-1α and others), for instance, participate in the clearance of *Brucella* (26, 40). In numerous situations, the production of these mediators depends on the activation of the p38 and ERK1/2 MAPKs alone or in coordination (7). Therefore, our results, which indicated that the p38 and ERK1/2 MAP kinases were strongly activated upon infection of mouse macrophages with rough *Brucella*, but not upon infection with smooth *Brucella*, defined the MAPKs as critical signaling molecules in the reactions of macrophages to *Brucella*. Although the LPS O chain has no direct action on the MAPK pathway, it interferes with this pathway by regulating the interaction between bacteria and host cells during uptake of *Brucella*. The resulting interference appears instrumental in determining the eventual fate of the bacteria. The slight MAPK activation associated with virulent smooth *Brucella* infections suggests a novel point of immune intervention by *Brucella*.

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