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Subversion of Monocyte Functions by *Coxiella burnetii*: Impairment of the Cross-Talk Between $\alpha_v\beta_3$ Integrin and CR3[1]

Christian Capo,* Frederik P. Lindberg,† Sonia Meconi,* Yona Zaffran,* Gratiela Tardei,* Eric J. Brown,† Didier Raoult,* and Jean-Louis Mege[2]*

Several intracellular pathogens exploit macrophages as a niche for survival and replication. The success of this strategy requires the subversion or the avoidance of microbicidal functions of macrophages. *Coxiella burnetii*, the agent of Q fever, is a strictly intracellular bacterium that multiplies in myeloid cells. The survival of *C. burnetii* may depend on the selective use of macrophage receptors. Virulent *C. burnetii* organisms were poorly internalized but survived successfully in human monocytes, whereas avirulent variants were efficiently phagocytosed but were also rapidly eliminated. The uptake of avirulent organisms was mediated by leukocyte response integrin ($\alpha_v\beta_3$ integrin) and CR3 ($\alpha_M\beta_2$ integrin), as demonstrated by using specific Abs and RGD sequence-containing peptides. The phagocytic efficiency of CR3 depends on its activation via $\alpha_v\beta_3$ integrin and integrin-associated protein. Indeed, CR3-mediated phagocytosis of avirulent *C. burnetii* was abrogated in macrophages from integrin-associated protein$^{-/-}$ mice. In contrast, the internalization of virulent *C. burnetii* organisms involved the engagement of $\alpha_v\beta_3$ integrin but not that of CR3. The pretreatment of monocytes with virulent *C. burnetii* organisms prevented the CR3-mediated phagocytosis of zymosan particles and CR3 activation assessed by the expression of the 24 neo-epitope. We conclude that the virulence of *C. burnetii* is associated with the engagement of $\alpha_v\beta_3$ integrin and the impairment of CR3 activity, which probably results from uncoupling $\alpha_v\beta_3$ integrin from integrin-associated protein. This study describes a strategy not previously reported of phagocytosis modulation by intracellular pathogens. *The Journal of Immunology*, 1999, 163: 6078–6085.

Coxiella burnetii is the etiologic agent of Q fever, a disease that typically manifests either as an acute polymorphic form with usually favorable outcome or a chronic form, usually endocarditis with severe prognosis, occurring in the context of defective cell-mediated immunity (1, 2). *C. burnetii* organisms are strictly intracellular Gram-negative bacteria classified in the γ subdivision of Proteobacteria (3). Their survival strategy is based on multiplication in mature phagosomes of monocytes/macrophages (4). *C. burnetii* virulence is mainly related to the expression of LPS. Upon serial passage in culture, *C. burnetii* undergoes an irreversible transition from virulent to avirulent form, which is similar to smooth-to-rough LPS transition of enterobacteria. Hence, phase I bacteria expressing a smooth-type LPS are virulent, whereas phase II variants exhibit a truncated rough-type LPS and are avirulent (5).

The entry of *C. burnetii* into monocytes and macrophages is likely to be critical for its adaptation to host cells and the development of Q fever. So far, no convincing evidence about the type of receptors engaged by *C. burnetii* has been provided (6). During the past few years, it has become increasingly clear that some receptors of eukaryotic cells including macrophages are especially important in bacterial recognition and probably determine the fate of internalized pathogens (7, 8). Numerous microorganisms use integrins, heterodimeric glycoproteins comprised of various combinations of several α and β subunits (9), as specific receptors to invade host cells (10). A leukocyte β_2 integrin, CR3 ($\alpha_M\beta_2$, CD11b/CD18), is involved in the recognition of iC3b-coated particles and β glucan (11), as well as pathogens such as *Escherichia coli*, *Mycobacteria* sp., *Leishmania* sp., *Legionella pneumophila*, *Bordetella pertussis*, *Rhodococcus equi*, and *Histoplasma capsulatum* (12). Nevertheless, CR3-mediated uptake of targets by macrophages usually requires an additional activation signal (13). β_1 and β_3 integrins, which are involved in the interaction of leukocytes with extracellular matrix, can also recognize microorganisms such as *B. pertussis*, *Mycobacteria avium/intracellulare*, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, and *Borrelia burgdorferi* (10, 14–16). Leukocyte response integrin (LRI;[3] $\alpha_v\beta_3$ integrin) and integrin-associated protein (IAP; CD47), a member of the Ig family, can be functionally associated (17) and are involved in the binding of filamentous hemagglutinin of *B. pertussis* by monocytes (18). Recently, it has been reported that IAP-deficient mice exhibited decreased resistance to bacterial infection (19). Nevertheless, the way used by intracellular pathogens to govern leukocyte integrin activity and their survival inside host macrophages remains largely unknown.

In this report, we demonstrated that virulent *C. burnetii* organisms entered into monocytes through $\alpha_v\beta_3$ integrin and survived

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[3] Abbreviations used in this paper: LRI, leukocyte response integrin; BMDM, bone marrow-derived macrophage; IF, immunofluorescence; IAP, integrin-associated protein; LPC, lysophosphatidylcholine; HEL, human embryonic lung.

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inside the cells. Avirulent variants were more easily ingested than virulent bacteria but were eliminated by monocytes. Their phagocytosis was mediated by $\alpha_v\beta_3$ integrin-IAP complex and CR3, suggesting that the efficiency of *C. burnetii* phagocytosis mainly results from the activation of CR3 and the availability of IAP. Virulent bacteria interfered with CR3 activation and CR3-dependent phagocytosis, thus preventing CR3 engagement. We suggest that *C. burnetii*-induced impairment of CR3 function results from uncoupling $\alpha_v\beta_3$ integrin from IAP.

Materials and Methods

Reagents and Ab

mAbs directed to monocyte surface Ags were purchased from Immunotech (Marseille, France). They included: the common β_1 integrin chain (CD29, IgG1), the common β_2 integrin chain (CD18, IgG1), $\alpha_L\beta_2$ (CD11a, IgG1), $\alpha_M\beta_2$ (CD11b, IgG1), $\alpha_X\beta_2$ (CD11c, IgG1), and IgG1 controls. OKM1 mAb and BM104 mAb directed against $\alpha_M\beta_2$ (CD11b) were IgG1, obtained from Ortho Diagnostic Systems (New Jersey) and Bender Medsystem (Austria), respectively. mAbs against LRI (7G2, IgG1) and IAP (B6H12, 2D3, IgG1), and $F(ab')_2$ of anti-LRI and anti-IAP mAb were obtained as previously described (17). The mAb 24 was IgG1 specific for the leukocyte integrin α subunit (20). Rabbit Ab directed to *C. burnetii* recognized virulent and avirulent bacteria. FITC-conjugated $F(ab')_2$ anti-rabbit IgG were obtained from Immunotech. SRBC and specific immune serum were provided by BioMérieux (Marcy l'Etoile, France). Synthetic hexapeptides KGAGDV, KGRGDV, and KGALEV were prepared as described (21). Lysophosphatidylcholine (LPC), zymosan and cytochalasin D were purchased from Sigma (St. Louis, MO).

Monocytes and macrophages

PBMCs were isolated from healthy volunteers on Ficoll-Hypaque (Eurobio, Les Ulis, France) and suspended in RPMI 1640 containing 20 mM HEPES (Life Technologies, Eragny, France), 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies) as previously described (22). Monocytes were purified by incubating 5×10^5 PBMCs in a glass Labtek chamber/slide (Miles, Naperville, IL) for 60 min at 37°C. Nonadherent cells were removed by washing, and remaining cells were designated as monocytes because >90% of them were $CD14^+$ and had phagocytic characteristics. In some experiments, monocytes were incubated with mAb or peptides for 60 min at 37°C before infection.

Bone marrow-derived macrophages (BMDMs) were isolated from wild-type mice ($IAP^{+/+}$) and gene-targeted knockout mice deficient in IAP expression ($IAP^{-/-}$) and incubated in a medium consisting of high-glucose DMEM with 15% L929 cell supernatant (19). BMDMs were pipetted off after addition of EDTA, and suspended cells were plated on glass Labtek chamber/slides before infection.

Bacteria

Virulent *C. burnetii* (Nine Mile strain, VR-615; American Type Culture Collection, Manassas, VA) were injected into mice and 10 days later were recovered from spleens, then cultured in mouse L929 fibroblasts maintained in antibiotic-free MEM (Life Technologies) supplemented with 4% FCS and 2 mM L-glutamine for two passages. Avirulent variants were cultured in L929 cells by repeated passages of Nine Mile strain as previously described (23). After 1 wk, L929 cells were sonicated, and the homogenates were centrifuged at 10,000 rpm for 10 min. Bacteria were layered on 25–45% linear Renograffin gradient, the gradients were spun down, then the bacteria were collected, washed, and suspended in serum-free medium before being stored at −80°C. The concentration of *C. burnetii* was determined by Gimenez staining (24).

Bacterial phagocytosis

Adherent monocytes (5×10^4 cells/assay) were incubated with *C. burnetii* at different bacterium-to-cell ratios in 0.2 ml HBSS. After different periods of time, the cells were washed and fixed with 1% formaldehyde, and bacteria were revealed by immunofluorescence (IF). Bacterial labeling was obtained by incubating cell preparations with rabbit Ab directed against *C. burnetii* or control serum at 1:250 in PBS containing 1% BSA for 30 min at room temperature. After washing, 1:200 dilution of FITC-conjugated $F(ab')_2$ anti-rabbit IgG was added to cells for 30 min. The number of bacteria associated with monocytes was determined by fluorescence microscopy with a Zeiss microscope. To discriminate between extra and intracellular bacteria, monocytes were permeabilized with 0.1 mg/ml LPC. In the absence of LPC, only monocyte-bound organisms were detected (Fig. 1*A*), whereas LPC allowed the detection of attached and internalized bacteria (Fig. 1*B*). Phagocytosis was quantified as follows. The product of the number of bacteria per positive monocyte in the presence of LPC and the percentage of positive cells was designated as the association index. The difference between indexes in the presence or the absence of LPC quantified the uptake of *C. burnetii* and was named as the phagocytosis index. This parameter is a specific measurement of phagocytosis because cytochalasin D dramatically decreased the phagocytosis index but not the association index (Fig. 1, *C* and *D*). The phagocytosis of *C. burnetii* was also expressed as the phagocytosis efficiency, which was defined as the ratio of phagocytosis index/association index (25).

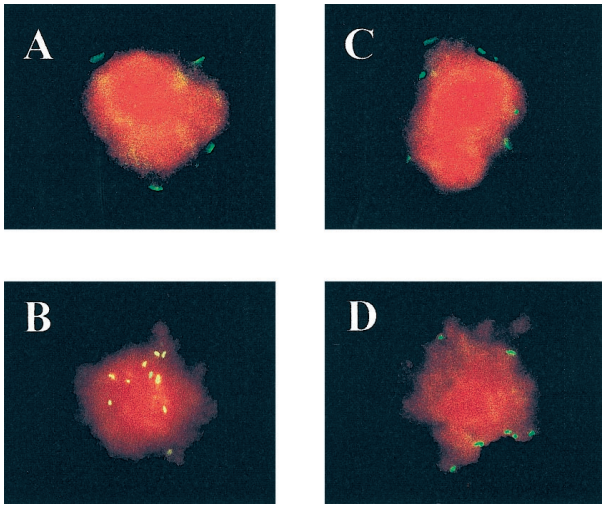

FIGURE 1. Phagocytosis of *C. burnetii*. Monocytes were pretreated with 5 μg/ml cytochalasin D (*C* and *D*) or vehicle (*A* and *B*) and incubated with *C. burnetii* at a 200:1 bacterium-to-cell ratio for 2 h. Then cells were incubated in the presence (*B* and *D*) or the absence (*A* and *C*) of LPC. Extra- and intracellular bacteria were determined by IF. Phagocytozed bacteria were only detected when LPC was used in the absence of cytochalasin D (*B*). The figure is representative of at least five experiments.

Infection procedure

Monocytes (5×10^4 cells/assay) were incubated with *C. burnetii* at a 200:1 bacterium-to-cell ratio for 24 h at 37°C in RPMI 1640. Cells were then washed to remove free bacteria (corresponding to day 0) and cultured for 12 days in RPMI 1640 supplemented with 10% FCS. Cellular infection was quantified by IF every 3 days. Results were expressed as a relative infection index compared with day 0. The viability of bacteria was determined as previously described (24). Briefly, infected monocytes were sonicated and the homogenates were added to human embryonic lung (HEL) cell monolayers in shell vials. After 7 days, *C. burnetii* replication was studied by indirect IF as described above.

Particle phagocytosis

In some experiments, monocytes (5×10^4 cells/assay) were pretreated with *C. burnetii* at a 200:1 bacterium-to-cell ratio for 15 min at 37°C, washed, and incubated with different particles. Unopsonized zymosan and zymosan opsonized by 50% human serum were used to assess the function of CR3 lectin sites and iC3b-binding sites, respectively. SRBC were opsonized by 1:250 dilution of specific immune serum. IgG-SRBC were used to assess FcγR activity. Monocytes were incubated with 10^7 IgG-opsonized SRBC, unopsonized zymosan or iC3b-coated zymosan (1 mg/ml) in RPMI 1640 containing 10% heat-inactivated FCS for 60 min at 37°C. The cells were washed to remove unbound particles, and extracellular IgG-coated SRBC were lyzed by distilled water. Monocytes were then fixed with 1% formaldehyde and examined microscopically. Phagocytosis results were expressed as the product of the percentage of cells having phagocytozed at least one particle and the number of phagocytozed particles per cell × 100 (phagocytosis index).

Flow cytometry

Adherent monocytes were treated with *C. burnetii* for 15 min at 37°C, washed, and then gently scraped with a rubber policeman. In some experiments, suspended PBMC were incubated with *C. burnetii* for 15 min at

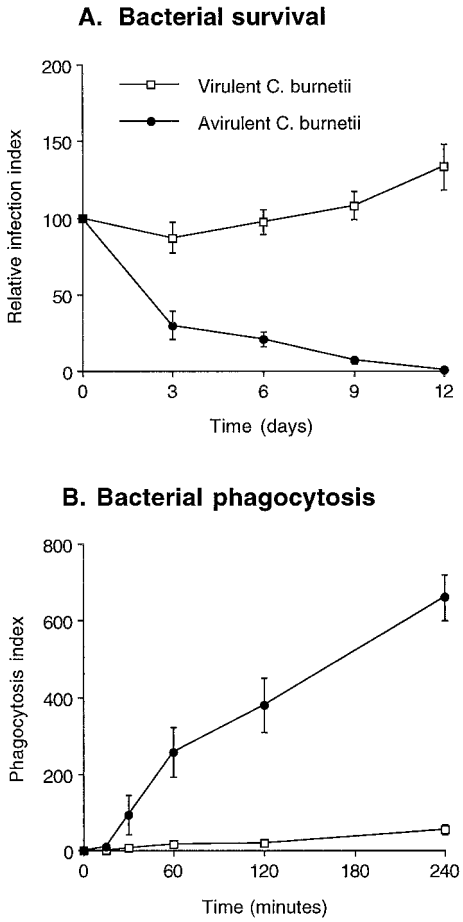

FIGURE 2. Survival and uptake of *C. burnetii*. *A,* Monocytes were incubated with *C. burnetii* at a 200:1 bacterium-to-cell ratio for 24 h, washed, and incubated in RPMI 1640 containing 10% FCS for 12 days. Cellular infection was quantified by IF, and results of the relative infection index are expressed as a mean ± SE of four experiments. They represent the comparison of infection index values after 3, 6, 9, or 12 days with values at day 0 (110 ± 24 for virulent *C. burnetii* and 681 ± 79 for avirulent bacteria). *B, C. burnetii* organisms were incubated with monocytes in HBSS for different periods at 37°C. Phagocytozed bacteria were detected by IF. Results of the phagocytosis index are expressed as a mean ± SE of five experiments.

37°C. Then, cells were incubated with anti-CD18, anti-CD11b, anti-LRI mAb, mAb 24, or isotypic controls at 1/100 dilution for 30 min at 4°C and stained with FITC-tagged $F(ab')_2$ anti-mouse Igs. The cell fluorescence was analyzed by an EPICS XL (Coulter Electronics, Hialeah, FL). Gating was established using forward and side scatters and fluorescence recorded on the log scale.

Statistical analysis

Results are given as the mean ± SE and compared with Mann Whitney U test. Differences were considered as significant if $p < 0.05$.

Results

Survival and uptake of C. burnetii

Monocyte infection by virulent *C. burnetii* organisms steadily increased from 3 days to 12 days postinfection as determined by IF (Fig. 2*A*). After 12 days, 70% of infected cells contained each three to four bacteria. In contrast, the number of avirulent organisms rapidly decreased after 3 days, and they were undetectable after 9 days. The decrease in the number of avirulent organisms resulted from monocyte-mediated bacterial killing. The viability of intracellular organisms was assessed by culture of infected cell homogenates on HEL cells. Virulent bacteria were viable (152 ± 20 at day 12 vs 104 ± 24 vacuoles per shell vial at day 0), whereas avirulent variants were completely eliminated by monocytes (24 ± 12 at day 12 vs 450 ± 80 vacuoles per shell vial at day 0). The distinct intracellular behavior of virulent and avirulent *C. burnetii* may result from different mechanisms of entry into monocytes. The uptake of virulent organisms (bacterium-to-cell ratio of 200:1) required 60 min to become detectable whereas intracellular avirulent bacteria were detected after 30 min (Fig. 2*B*). After 2 h, the phagocytosis of virulent bacteria was 10 times lower than that of avirulent organisms and phagocytosis efficiency did not exceed 33 ± 4%. In contrast, the phagocytosis efficiency of avirulent *C. burnetii* was 84 ± 8%, demonstrating that almost all the bacteria that bound to monocytes were ingested. A prolonged incubation of monocytes (>4 h) with a bacterium-to-cell ratio of 500:1 did increase the phagocytosis of virulent *C. burnetii*, but again not to a level close to that seen with avirulent variants (data not shown). We also excluded the hypothesis that virulent organisms settled less well than avirulent variants during the course of phagocytosis assay. Bacteria were added to monocytes and spun down at 1500 rpm for 10 min before a 2-h incubation at 37°C. This procedure increased the phagocytosis index of virulent *C. burnetii* (113 ± 31 vs 41 ± 5 in the absence of centrifugation). But, it remained markedly lower than the phagocytosis index of avirulent bacteria (569 ± 81). The restriction of phagocytosis is a property of *C. burnetii* virulence.

Role of $\alpha_v\beta_3$ integrin and CR3 in C. burnetii *phagocytosis*

The difference between the phagocytosis of virulent and avirulent *C. burnetii* may be related to the engagement of specific receptors on monocytes. Chelation of divalent cations completely inhibited the interaction of *C. burnetii* with monocytes (83 ± 8% inhibition), as did lowering of the incubation temperature to 4°C (86 ± 7% inhibition), indicating a role for monocyte integrins. Therefore, we investigated the role of β_1, β_2, and β_3 integrins in the uptake of *C. burnetii* using specific mAbs. Phagocytosis of virulent bacteria was not impaired by mAbs directed against β_1 chain (CD29), β_2 chain (CD18), and α-chains of β_2 integrins (CD11a, CD11b, CD11c) (Fig. 3*A*), whatever the concentration of mAbs used (Fig. 4, *A* and *B*, and data not shown). Blocking $\alpha_v\beta_3$ integrin with mAb 7G2 markedly decreased the phagocytosis index in a dose-dependent manner (Fig. 3*A* and Fig. 4*C*) with a maximum inhibition at a mAb concentration of 5 μg/ml (75 ± 11%). $F(ab')_2$ of mAb 7G2 (5 μg/ml) also decreased the phagocytosis by 73 ± 6%. Taken together, these results suggest that the uptake of virulent *C. burnetii* by monocytes depends on the engagement of $\alpha_v\beta_3$ integrin.

The internalization of avirulent *C. burnetii* by monocytes requires additional receptors. As with virulent bacteria, mAbs directed against CD29, CD11a, and CD11c did not modify the uptake of avirulent variants (Fig. 3*B*). The concentrations of $\alpha_v\beta_3$ integrin-blocking mAb 7G2 necessary to inhibit the uptake of avirulent *C. burnetii* were similar to those required to prevent the phagocytosis of virulent bacteria (Fig. 3*B* and Fig. 4*C*). The use of $F(ab')_2$ of mAb 7G2 yielded the same results as those obtained with the complete mAb. In contrast to virulent organisms, CR3 (CD11b/CD18) was involved in the phagocytosis of avirulent bacteria by monocytes. First, a mAb directed against CD18 (used at 5 μg/ml) decreased the phagocytosis index by 61 ± 6% (Fig. 3*B*). Second, the OKM1 mAb directed against the lectin domain of CD11b inhibited the uptake of avirulent *C. burnetii* by 55 ± 7% (Fig. 3*B*). The BM104 mAb, directed against the I domain of CD11b, inhibited the phagocytosis of avirulent bacteria to the same extent (data not shown). It is noteworthy that mAbs against

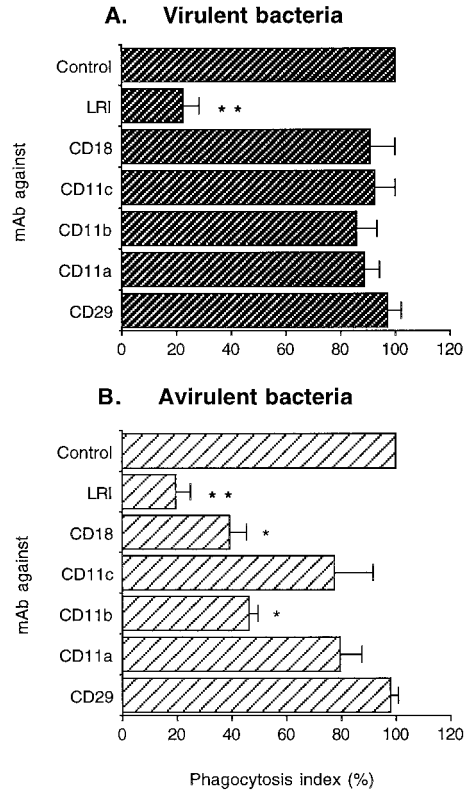

FIGURE 3. Effect of mAb against monocyte receptors on uptake of *C. burnetii.* Virulent (*A*) or avirulent (*B*) *C. burnetii* organisms at a 200:1 bacterium-to-cell ratio were incubated with monocytes in HBSS at 37°C in the presence of mAbs directed to monocyte surface Ags or IgG controls (at 5 μg/ml). Internalized bacteria were detected by IF. Results of the phagocytosis index (in the presence of mAbs) are expressed as a percentage of controls and represent the means ± SE of four experiments. *, $p < 0.03$; **, $p < 0.001$.

CD18 and CD11b exhibited a superimposable pattern of dose-dependent inhibition (Fig. 4, *A* and *B*). Clearly, our results demonstrate that the phagocytosis of avirulent *C. burnetii* depends on both $\alpha_v\beta_3$ integrin and CR3.

KGAGDV and KGRGDV peptides and C. burnetii *phagocytosis*

To confirm the role of integrins in the uptake of *C. burnetii*, we used peptides containing RGD-related sequences. KGRGDV inhibits $\alpha_v\beta_3$ integrin function independently of its activation state, whereas KGAGDV is selective for its activated state and the control peptide KGALEV is without effect (21). KGALEV did not affect the phagocytosis of *C. burnetii* whatever the peptide concentration used (Fig. 5). KGAGDV inhibited the uptake of virulent (Fig. 5*A*) and avirulent (Fig. 5*B*) *C. burnetii* in a dose-dependent manner. The inhibition was evident with 1 μM peptide (Fig. 5, *A* and *B, insets*) and reached maximum value with 20 μM (80% inhibition). KGRGDV displayed a distinct pattern of inhibition. Although as potent as KGAGDV at inhibiting the phagocytosis of avirulent bacteria (Fig. 5*B*), it was less potent at inhibiting the internalization of virulent *C. burnetii* (Fig. 5*A, inset*). Maximum inhibition required 10–20 times higher concentrations of KGRGDV than those of KGAGDV. Hence, the phagocytosis of virulent *C. burnetii* is particularly sensitive to KGAGDV peptide.

CR3 is functionally impaired by virulent C. burnetii

The difference between phagocytosis of virulent and avirulent organisms could be due to *C. burnetii*-mediated effect on CR3 function. First, adherent monocytes were treated with virulent or avirulent bacteria for 15 min (this time enabled *C. burnetii* to induce F-actin reorganization (23)). After scraping of monocytes, the expression of CR3 was assessed by flow cytometry. The levels of CD18 and CD11b expression remained similar in the presence or the absence of *C. burnetii* (Fig. 6). PBMC were also incubated with *C. burnetii* for 15 min, and the CR3 expression was studied in suspended monocytes. This expression was similar in control monocytes and monocytes treated by virulent or avirulent organisms (data not shown). The increase in the incubation time with *C. burnetii* up to 2 h did not change the expression of CR3 (data not shown). Second, we investigated the ability of *C. burnetii* to activate CR3 by measuring the expression of activation neo-epitopes, such as the 24 epitope, on β_2 integrins. While no detectable binding of mAb 24 to monocytes was observed in the absence of divalent cations, the addition of 200 μM Mn^{2+} dramatically increased its binding to monocytes (Fig. 7). Virulent *C. burnetii* organisms were unable to induce the expression of the 24 epitope (Fig. 7*A*), whereas avirulent bacteria stimulated its expression after 15 min (Fig. 7*B*). The 24 epitope was not expressed by monocytes even after 2 h of incubation with virulent bacteria (data not shown). It is noteworthy that the preincubation of monocytes for

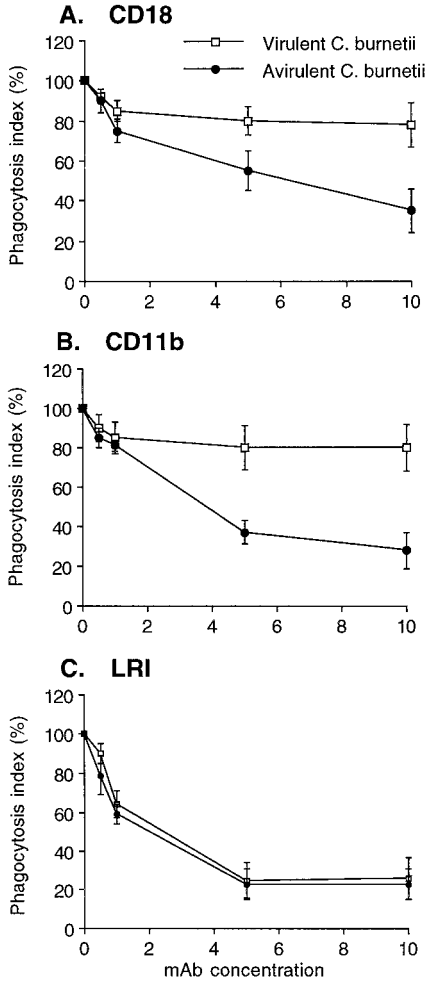

FIGURE 4. Effect of mAb concentration on *C. burnetii* phagocytosis. Virulent or avirulent *C. burnetii* organisms were incubated with monocytes in the presence of different concentrations of mAb (in μg/ml) directed to CD18, CD11b, and LRI. Phagocytozed bacteria were revealed by IF. Results of phagocytosis are expressed as described in Fig. 3 and presented as mean ± SE of three experiments.

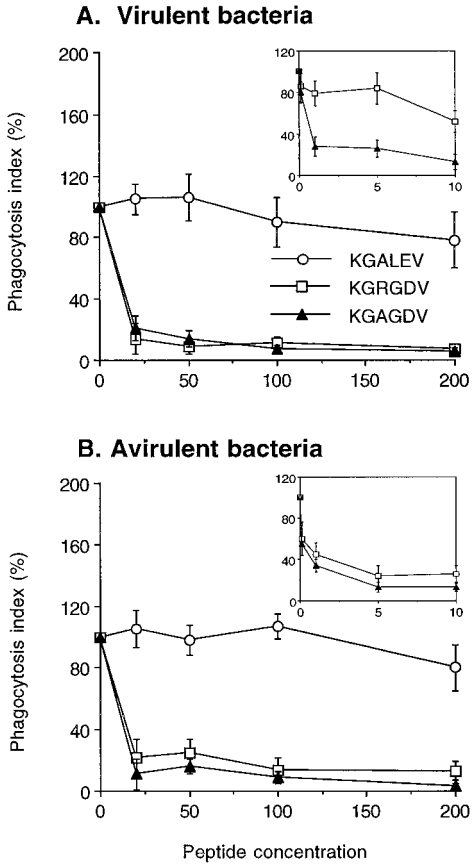

FIGURE 5. Effect of RGD peptides on uptake of *C. burnetii*. Virulent (*A*) or avirulent (*B*) *C. burnetii* organisms were incubated with monocytes in the presence of 0–200 μM RGD-related peptides. Insets show the effect of 0–10 μM KGRGDG and KGAGDV on bacterial phagocytosis. Results of relative phagocytosis index are expressed as a mean ± SE of three experiments.

15 min or 2 h with virulent organisms did not interfere with Mn^{2+}-stimulated expression of 24 epitope (data not shown). Third, we assessed the effect of bacterium-monocyte interaction on CR3-dependent phagocytosis (Table I). In monocytes treated with virulent *C. burnetii* for 15 min, the phagocytosis of unopsonized zymosan (which binds lectin sites on CR3) and avirulent bacteria was down-modulated, whereas the uptake of serum-opsonized zymosan (which binds iC3b sites on CR3) and virulent bacteria was not affected. The effect of *C. burnetii* on CR3-mediated phagocytosis was sustained because CR3-dependent uptake was not corrected after 2 h of monocyte pretreatment with virulent organisms (data not shown). The pretreatment of monocytes with avirulent *C. burnetii* for 15 min (Table I) or 2 h (data not shown) did not modify their phagocytic ability. Indeed, virulent *C. burnetii* organisms specifically prevented the activation of CR3 and reduced the phagocytic activity of CR3 dependent on its lectin sites.

IAP is involved in the uptake of avirulent C. burnetii

As CR3 activation requires a cosignal provided by $\alpha_v\beta_3$ integrin and its transductional unit, IAP (17), we investigated the role of IAP in monocyte uptake of *C. burnetii* using two mAb: mAb B6H12 recognizes a site on the extracellular domain of IAP necessary for functional interaction with $\alpha_v\beta_3$ integrin, whereas mAb 2D3 binds to a distinct IAP site (26). mAb B6H12 significantly depressed the phagocytosis index of virulent and avirulent *C. burnetii* (Fig. 8*A*). The inhibition was evident with 1 μg/ml of B6H12 (36 ± 7%) and reached a maximum value with 5 μg/ml (65 ± 10% inhibition). Similar data were obtained with $F(ab')_2$ of the anti-IAP mAb B6H12 used at 5 μg/ml (60 ± 10% inhibition). mAb 2D3 was inactive whatever the concentrations of mAb used (Fig. 8*A* and data not shown). Taken together, these results suggest that the phagocytosis of *C. burnetii* is sensitive to mAb directed to a site of IAP involved in the interaction with $\alpha_v\beta_3$ integrin.

The inhibitory effect of mAb B6H12 may be steric, result from a IAP-mediated inhibition of $\alpha_v\beta_3$ integrin function, or be due to a requirement for IAP per se. To discriminate between these hypotheses, we used BMDMs from normal and IAP-deficient mice (19). In $IAP^{+/+}$ BMDMs, avirulent variants of *C. burnetii* were more efficiently internalized than virulent bacteria (Fig. 8*B*), thus supporting the results we obtained using human monocytes. In $IAP^{-/-}$ BMDMs, the uptake of avirulent *C. burnetii* was reduced to the level of that of virulent bacteria. These results emphasize an essential role for IAP in the phagocytosis of avirulent bacteria.

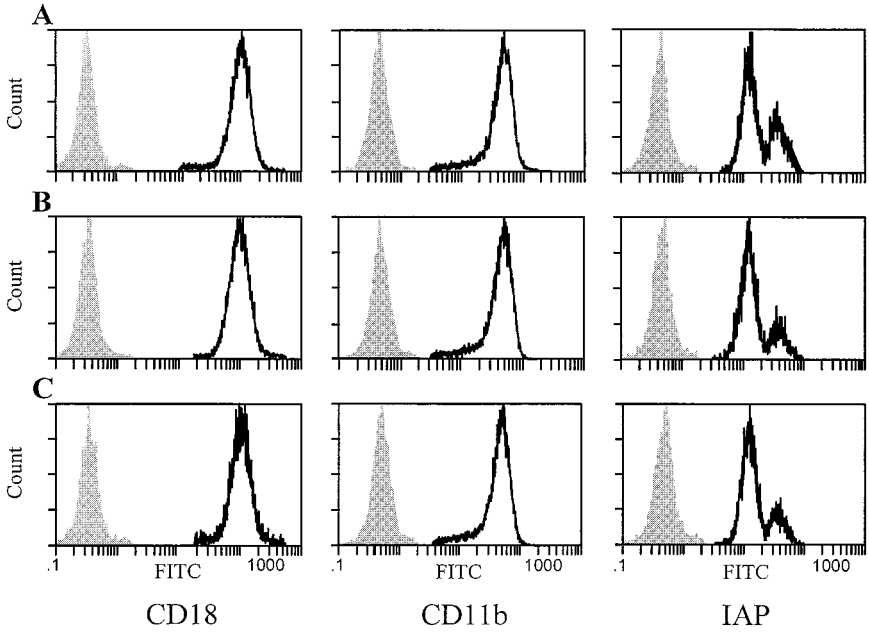

FIGURE 6. Effect of *C. burnetii* on the expression of monocyte receptors. Monocytes were incubated in the absence of bacteria (*A*) or in the presence of virulent (*B*) or avirulent (*C*) *C. burnetii* for 15 min at 37°C. mAbs directed against CD18, CD11b, or IAP were then added at 1/100 dilution followed by FITC-tagged $F(ab')_2$ anti-mouse Igs. Isotypic controls were included (gray area). The receptor expression was determined by flow cytometry. Representative histograms of three experiments are shown.

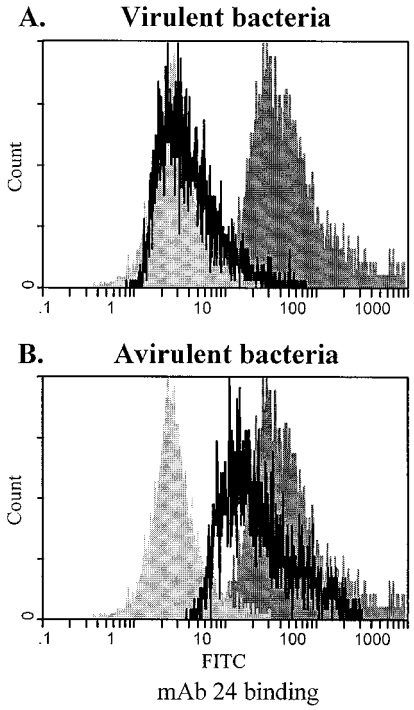

FIGURE 7. Effect of *C. burnetii* on the expression of 24 epitope. Adherent monocytes were treated with virulent (*A*) or avirulent (*B*) *C. burnetii* for 15 min at 37°C. mAb 24 was then added at 1/100 dilution followed by FITC-tagged $F(ab')_2$ anti-mouse Igs. As controls, monocytes were incubated with (dark area) or without (clear area) Mn^{2+} (200 μM). The expression of the epitope in response to *C. burnetii* (in black) was determined by flow cytometry. Representative histograms of three experiments are shown.

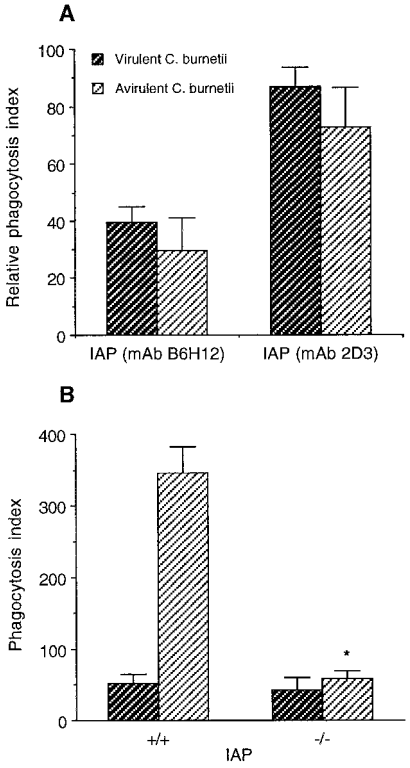

FIGURE 8. Role of IAP in *C. burnetii* phagocytosis. *A*, *C. burnetii* organisms were incubated with monocytes in the presence of 5 μg/ml mAbs directed to IAP (B6H12, 2D3) or IgG controls as described in Fig. 3. The results of phagocytosis index (in the presence of mAb) are expressed as a percentage of controls and represent the means ± SE of four experiments. *B*, BMDMs from $IAP^{+/+}$ and $IAP^{-/-}$ mice were incubated with *C. burnetii* at a bacterium-to-cell ratio of 200:1 for 2 h at 37°C. Bacterial phagocytosis was detected by IF as described for human monocytes. Results of the phagocytosis index are the means ± SE of three experiments. *, $p < 0.001$.

Thus, both IAP and CR3 are necessary for the high-level phagocytosis of avirulent *C. burnetii*, but both are dispensable for the low-level phagocytosis of virulent organisms.

Discussion

In this report, we show that virulent *C. burnetii* organisms survived inside human monocytes, whereas avirulent bacteria were eliminated. In addition, they were phagocytozed by host cells at markedly lower efficiency than avirulent variants. The inverse relationship between phagocytosis and survival of *C. burnetii* is not cell-type specific, because it was observed with circulating human monocytes, THP-1 cell line (data not shown), and murine BMDMs. It is not a unique property of *C. burnetii*. For instance, pathogenic strains of *Mycobacterium avium* are ingested by macrophages at a lower level than attenuated variants but they replicate more efficiently inside the cells (27). The difference of phagocytosis between virulent and avirulent *C. burnetii* was not due to lack or differential binding of serum opsonins. Indeed, *C. burnetii* phagocytosis was increased by the addition of nonimmune serum but phagocytosis level of virulent bacteria remained lower than that of avirulent variants (data not shown). In addition, the low phagocytosis of virulent organisms was not overcome by initial centrifugation of bacteria, suggesting that the probability for virulent or avirulent bacteria to encounter monocytes was similar. The relationship between bacterial uptake and bacterial survival inside monocytes/macrophages suggests the involvement of monocyte receptors specific for infection. This concept has been a source of debate. Zimmerli et al. showed that the intracellular survival and replication of *M. tuberculosis* are equivalent regardless of the receptors used for binding and phagocytosis (28). C receptors are involved in the phagocytosis of both virulent and attenuated strains of *M. tuberculosis* by human macrophages but only the mannose receptor plays an important role in the phagocytosis of virulent strains (29). Alveolar macrophages limit intracellular growth of *M. tuberculosis* more efficiently than monocytes: CR1 and CR3 are the major receptors mediating uptake of *M. tuberculosis* by monocytes, while CR4 is the major receptor for alveolar macrophages (30).

We show here a selective use of monocyte receptors according to the virulence of *C. burnetii*. The phagocytosis of virulent

Table I. *Effect of* C. burnetii *on particle phagocytosis by monocytes*[a]

	Pretreatment of Monocytes with	
	Avirulent *C. burnetii*	Virulent *C. burnetii*
Zymosan	110 ± 15	55 ± 6*
iC3b-zymosan	95 ± 7	103 ± 8
IgG-SRBC	103 ± 20	95 ± 10
Avirulent *C. burnetii*	85 ± 8	45 ± 6*
Virulent *C. burnetii*	105 ± 11	83 ± 10

[a] Monocytes were pretreated with virulent or avirulent *C. burnetii* at a bacterium-to-cell ratio of 200:1 for 15 min at 37°C. Cells were washed and incubated with unopsonized zymosan, iC3b-opsonized zymosan (at 1 mg/ml), 10^7 IgG-opsonized SRBC as a control for 60 minutes, or *C. burnetii* for 2 h at 37°C. Bacteria were detected by IF, and zymosan and SRBC were detected by light microscopy. Results of the relative phagocytosis index (as compared to untreated monocytes) are expressed as a mean ± SE of three experiments.

*, $p < 0.01$.

organisms was independent of β_1 and β_2 integrins but could be largely inhibited by mAb directed against $\alpha_v\beta_3$ integrin and peptides KGAGDV and KGRGDV known to inhibit $\alpha_v\beta_3$ integrin-dependent phagocytosis. Thus, independent of other integrins, the activated form of $\alpha_v\beta_3$ integrin allowed phagocytosis of virulent *C. burnetii*, as demonstrated by the potent inhibitory effect of KGAGDV. The use of $\alpha_v\beta_3$ integrin as a receptor for viral infection has been previously reported. The infection with pathogenic hantavirus is inhibited by Abs to β_3 integrin and by vitronectin, the ligand of $\alpha_v\beta_3$ integrin, whereas the infection with the nonpathogenic virus was prevented by β_1 integrin-specific Abs and fibronectin (31). Virulent foot-and-mouth virus uses $\alpha_v\beta_3$ integrin as a primary receptor for infection. The adaptation of the virus to cell culture results in its ability to use heparan sulfate instead of $\alpha_v\beta_3$ integrin and a concomitant loss of virulence (32). In contrast to that of virulent *C. burnetii*, phagocytosis of avirulent *C. burnetii* by monocytes involves both $\alpha_v\beta_3$ integrin and CR3. Again, anti-$\alpha_v\beta_3$ integrin mAbs as well as KGAGDV and KGRGDV induced a significant inhibition of the phagocytosis of avirulent organisms, suggesting that it requires $\alpha_v\beta_3$ integrin. The uptake of avirulent bacteria was also inhibited by anti-CD11b or anti-CD18 Abs, demonstrating an essential role for CR3. The mAbs directed against the I domain or the lectin sites of CD11b inhibited the uptake of avirulent *C. burnetii* to the same extent. These domains exhibit distinct specificities of recognition. I domain ligands include ICAM-1, fibrinogen, and neutrophil-inhibiting factor, while lectin domain binds β-glucan and mannan polysaccharides (33, 34). Recently, it has been reported that *Candida albicans*, a fungal pathogen, uses I domain and lectin domain to efficiently interact with CR3 (35). It is likely that both regions of CD11b are involved in the internalization of avirulent *C. burnetii* by monocytes.

The virulence of *C. burnetii* is associated with the inhibition of cross-talk between $\alpha_v\beta_3$ integrin and CR3. The efficient phagocytosis of avirulent organisms requires the activation of CR3, as demonstrated by using mAb 24 initially described to be specific for the leukocyte integrin α subunits. Manganese is known to directly alter the conformation of β_2 integrins and to strongly promote the expression of the 24 epitope (36). This mAb inhibits monocyte-dependent T cell proliferation and CR3-mediated neutrophil chemotaxis to FMLP, each event being dependent on the activation of β_2 integrins (20). Avirulent *C. burnetii* organisms caused an increase in mAb 24 expression, which seems to be associated with high affinity of CR3 (N. Hogg, unpublished observation). The activated state of CR3 is regulated by several cosignals including the engagement of other integrins (12, 13). Hence, the ligation of $\alpha_v\beta_3$ integrin increases the avidity of CR3 (37). Similarly, the binding of *B. pertussis* filamentous hemagglutinin to $\alpha_v\beta_3$ integrin leads to the activation of CR3 (18). As the phagocytosis of avirulent *C. burnetii* is associated with the engagement of both $\alpha_v\beta_3$ integrin and CR3, the integrin cross-talk should result in the efficient internalization of avirulent bacteria via activated CR3. In contrast, the interaction of virulent *C. burnetii* with monocytes actively inhibits this integrin cross-talk. Virulent organisms did not induce the expression of 24 epitope on CR3, suggesting that they did not enable CR3 to become activated and competent for phagocytosis. In addition, bacterium-monocyte interaction affected CR3-mediated phagocytosis. The preincubation of monocytes with virulent *C. burnetii* inhibited the phagocytosis mediated by lectin sites of CR3 (unopsonized zymosan and avirulent *C. burnetii*) whereas the uptake involving iC3b recognition site (serum-opsonized zymosan) was preserved. It is likely that the impairment of lectin site activity of CD11b prevents both conformational changes of I domain and exposure of activation epitopes required for CR3 activation (38). Thus, virulent *C. burnetii* organisms appear to actively prevent CR3 activation by interfering with its lectin sites. As the impairment of CR3 activation occurred when the interaction of virulent *C. burnetii* with monocytes was very low, it is likely that some fluid phase bacterial products play a role in this impairment. We found that LPS from virulent organisms was able to interfere with CR3 (data not shown), suggesting that the interaction of LPS expressed or released by virulent *C. burnetii* is involved in the inhibition of CR3-dependent functions.

As the ability of $\alpha_v\beta_3$ integrin to mediate CR3 activation depends on IAP, the inhibitory mechanism mediated by virulent bacteria may be directed to IAP. IAP is physically and functionally associated with β_3 integrins. The mAbs that recognize IAP inhibit some β_3 integrin-mediated functions such as binding of vitronectin-coated beads to cells, neutrophil activation by RGD-containing ligands, and calcium increase during adhesion of endothelial cells to fibronectin or vitronectin (17, 39). We demonstrated that IAP-blocking mAb inhibited the phagocytosis of virulent and avirulent *C. burnetii*. The inhibitory effect on virulent *C. burnetii* uptake was likely steric because only the phagocytosis of avirulent bacteria was down-modulated in macrophages from $IAP^{-/-}$ mice. As the uptake of virulent and avirulent *C. burnetii* was similar in $IAP^{-/-}$ macrophages, it is likely that CR3-mediated phagocytosis of avirulent bacteria is under the control of IAP. Conversely, virulent bacteria prevent CR3-dependent phagocytosis by interfering with the cosignal activity of IAP but not by down-modulating its expression (Fig. 6).

In conclusion, *C. burnetii* interacts with the monocyte $\alpha_v\beta_3$ integrin. Normally, this would result in IAP-dependent activation of CR3, rapid phagocytosis, and death of the bacterium. However, virulent organisms via a novel mechanism interfere with this host defense signal, allowing them to be taken up via a nonmicrobicidal pathway allowing intracellular survival.

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