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Apoptosis-Inducing Factor Participation in Bovine Macrophage *Mycobacterium bovis*-Induced Caspase-Independent Cell Death▽

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***Mycobacterium tuberculosis* complex species survive and replicate in phagosomes of the host cell. Cell death (CD) has been highlighted as one of the probable outcomes in this host-pathogen interaction. Previously, our group demonstrated macrophage apoptosis as a consequence of *Mycobacterium bovis* infection. In this study, we aimed to identify the contribution of apoptotic effector elements in *M. bovis*-induced CD. Bovine macrophages were either infected with *M. bovis* (multiplicity of infection, 10:1) or treated with an *M. bovis* cell extract (CFE). Structural changes compatible with CD were evaluated. Chromatin condensation was increased three times by the CFE. On the other hand, a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay demonstrated that levels of DNA fragmentation induced by *M. bovis* and CFE were 53.7% ± 24% and 38.9% ± 14%, respectively, whereas control cells had a basal proportion of 8.9% ± 4.1%. Rates of DNA fragmentation were unaffected by the presence of the pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp (z-VAD). Cells treated with 100 μg of CFE for 12 h had a fivefold decrease in the level of mitochondrial outer membrane permeabilization compared to that of untreated cells. Neither *M. bovis* infection nor CFE treatment induced activation of caspase 3, 8, or 9. Translocation of apoptosis-inducing factor (AIF) to the nucleus was identified in 32% ± 3.5% and 26.3% ± 4.9% of *M. bovis*-infected and CFE-treated cells, respectively. Incubation of macrophages with z-VAD prior to infection did not alter the percentage of cells showing AIF translocation. Our data suggest that *M. bovis*-induced CD in bovine macrophages is caspase independent with AIF participation.**

Cell death (CD) is a very important process in the development and conservation of multicellular organisms maintaining the equilibrium between life and death (10). Disorders in CD lead to (i) embryogenic problems after exposure to a wide variety of teratogens, (ii) neurodegenerative diseases, and (iii) cancer (17, 28). Apoptosis is a type of CD that is morphologically defined by cellular and nuclear shrinkage, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies. It is generally accepted that execution of the apoptotic pathway relies on the activation of a family of cysteine proteases, also known as caspases (21). However, activation of caspases is not a hallmark of every cell undergoing a death process. Several authors have shown that cell death occurs even in the presence of caspase inhibitors, giving rise to the concept of caspase-independent cell death (CICD). Cells experiencing CICD feature morphological changes resembling apoptosis but do not engage caspases to disassemble the cell (1, 5, 21).

In one of the first CICD reports, Xiang et al. (40) proved that *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-Vad-fmk), a general caspase inhibitor, blocked caspase proteolytic activity but did not prevent the suicide of Jurkat cells overexpressing BAX. In addition, data from a different study demonstrated that BH3 domain-only molecules kill cells deficient in the downstream effectors Apaf-1, caspase 9, and caspase 3 by a caspase-independent process of mitochondrial dysfunction (9). CICD has also been associated with host-pathogen interactions; for instance, it has been reported that soluble toxins of *Escherichia coli* activated CD via extracellular signal-regulated kinase (ERK) signaling in renal proximal tubular epithelial cells without caspase involvement (8). Additionally, Salmond et al. (36) demonstrated that the B subunit of *E. coli* heat-labile enterotoxin (EtxB) induced a rapid loss of mitochondrial membrane potential and cell viability, which were not affected by caspase inhibitors, in $CD8^+$ T cells. Moreover, Carrero et al. (7) proved that listeriolysin O induced loss of mitochondrial membrane potential and that exposure of phosphatidylserine on the cell surface was not abrogated by the incubation of a pan-caspase inhibitor with murine T cells. Finally, pneumococci and the major cytotoxins H_2O_2 and pneumolysin have been shown to induce apoptosis-like programmed cell death of cerebral endothelial cells in the presence of rapid mitochondrial damage that was followed by translocation of mitochondrial apoptosis-inducing factor (AIF) into the cytoplasm (3).

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Mycobacterium species have been associated with cell death induction. Fratazzi et al. (16) found that human macrophages infected with *Mycobacterium avium* serovar 4 suffered a CD process that helped to prevent dissemination of the pathogen. Moreover, addition of fresh uninfected macrophages contributed to 90% inhibition of mycobacterial growth. On the other hand, Placido et al. (32) demonstrated that apoptosis of human alveolar and monocyte-derived macrophages infected with *Mycobacterium tuberculosis* required viable bacteria and was dose dependent. The results in these reports have illustrated the importance of CD in the pathogenesis of tuberculosis, providing partial evidence to postulate CD as an innate immune mechanism. In the bovine model, chromatin condensation and DNA fragmentation of *Mycobacterium bovis*-infected monocyte-derived macrophages were dependent on the multiplicity of infection (MOI) and time (18). In addition, H. Esquivel-Solís et al. (unpublished data) proved that the host resistance phenotype and the virulence of the strain influenced the degree of CD. These observations, taken together, point to CD as a mechanism that may make a substantial contribution to the final outcome of this host-pathogen interaction. In order to identify the roles of macrophage apoptosis effectors, we used live *M. bovis* and its cell extract (CFE) in the presence of bovine macrophages to induce CD. We found that structural changes compatible with CD in macrophages that had been either infected or incubated with CFE occurred in a caspase-independent fashion with AIF translocation to the nuclei. Our data suggest that *M. bovis*-induced CD in bovine macrophages is caspase independent and is associated with AIF participation.

MATERIALS AND METHODS

Bacteria. We used the virulent *Mycobacterium bovis* field strain 9926, which was isolated from tubercular lung lesions of a tuberculin skin test-positive cow. Bacteria were grown at 37°C with shaking in Middlebrook 7H9 broth (Becton Dickinson, Cockeysville, MD) with 0.05% Tween 80 and 10% oleic acid-albumin-dextrose-catalase enrichment (Becton Dickinson, Sparks, MD). The bacterial suspension was centrifuged at 2,500 × *g* for 10 min, and the pellet was suspended in RPMI 1640 plus 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 20 mM sodium bicarbonate (Gibco BRL, Life Technologies, Grand Island, NY) (CRPMI). Bacteria were passed twice through a 27-gauge needle in order to disrupt the clumps. Aliquots of 1 ml were stored at −80°C for at least 1 day, and the inoculum was titrated by plating serial dilutions on Middlebrook 7H11 medium plus 10% oleic acid-albumin-dextrose-catalase (both from Becton Dickinson Co. Sparks, MD).

Protein extraction. A protocol by Parish and Wheeler (30) was used to obtain the *M. bovis* CFE. Briefly, bacteria were inactivated at 60°C for 90 min, suspended in 10 ml of phosphate-buffered saline (PBS), and disrupted by using a French press (SLM Aminco; Spectronic Instruments, Rochester, NY). After bacterial rupture, the suspension was centrifuged for 20 min at 3,000 × *g*, the cell pellet discarded, and the CFE-containing supernatant collected and kept at −70°C until further use. The CFE protein concentration was evaluated using the Micro BCA protein assay reagent kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Protein aliquots were electrophoresed using a 12% polyacrylamide gel and visualized with Coomassie and silver stains.

Culture of bovine macrophages. Peripheral venous blood was obtained from healthy adult cattle from a tuberculosis-free herd (not vaccinated, exposed, or challenged) housed at the facilities of the Research and Teaching Center (CEPIPSA) of the Universidad Nacional Autónoma de México (UNAM). Macrophages were obtained from peripheral blood mononuclear cells (PBMC) by the method of Stich et al. (38) with slight modifications. Blood was collected from the jugular vein into 60-ml syringes containing acid-citrate-dextrose solution and was centrifuged at 1,000 × *g* for 30 min. Buffy coats were diluted in 30 ml of citrated PBS, layered onto 15 ml of Percoll (Pharmacia, Uppsala, Sweden) at a specific density of 1.077, and centrifuged at 1,200 × *g* for 25 min. PBMC were then removed from the interface between the plasma and Percoll solution, pooled, diluted in 50 ml of citrated PBS, and centrifuged at 500 × *g* for 15 min. The cell pellets were then washed three times with citrated PBS at 500 × *g* for 10 min, suspended in CRPMI containing 4% autologous serum to facilitate adherence, placed at 5×10^6 PBMC in 50-ml Teflon flasks (6), and cultured 4 h at 37°C under 5% CO_2. Nonadherent cells were then removed by three washes with prewarmed PBS, and adherent monocytes were cultured just as described previously in CRPMI plus 12% autologous serum for 10 to 12 days until they differentiated to macrophages.

Infection of bovine macrophages. Macrophage monolayers containing 1×10^6 cells were infected with *M. bovis* (MOI, 10:1) and incubated for 4 h at 37°C under 5% CO_2. After the time allowed for phagocytosis, cells were washed four times with 5 ml of fresh RPMI medium to remove extracellular bacteria and then incubated again with CRPMI plus 12% autologous serum for 16 h. Cultures of uninfected macrophages were maintained under the same conditions during the whole time of the assays. Alternatively, macrophages were incubated either with 20 μg of camptothecin (Sigma-Aldrich Co., St. Louis, MO)/ml for 16 h to generate a positive control for the CD studies or with 50 μM z-VAD (Sigma-Aldrich Co., St. Louis, MO), a pan-caspase inhibitor, as described by Santos et al. (37). After incubation, the cells were fixed in 1% paraformaldehyde (Electron Microscopy Sciences, Washington, PA) for 30 min on ice, centrifuged at 300 × *g* for 10 min, and resuspended in 70% ethanol. Cells were kept at −20°C until DNA fragmentation analysis by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL).

Chromatin condensation analysis. Round coverslips containing 2×10^5 macrophages in CRPMI were prepared in 24-well tissue culture plates (Nalgene Nunc International, Rochester, NY). Cells received different treatments, such as CFE (100 μg at 37°C for 24 h) or camptothecin (20 μg/ml). After incubation, the cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Washington, PA) for 40 min at 4°C, washed, and stained with propidium iodide (5 μg/ml with 100 μg of RNase) (Sigma, St. Louis, MO). Chromatin condensation was analyzed by counting at least 100 cells per sample, using a fluorescence microscope. Results represent means ± standard deviations from three independent experiments.

DNA fragmentation analysis. DNA fragmentation was detected in macrophage populations treated with either *M. bovis* (MOI, 10:1), z-VAD (50 μM), CFE (100 μg at 37°C for 24 h), camptothecin (20 μg/ml), or z-VAD followed by *M. bovis* by using the TUNEL assay. The APO-BRDU kit (Pharmingen, San Diego, CA) was used according to the manufacturer's instructions. Flow cytometry analysis was performed using an Altra Beckman cytometer (Beckman Coulter, Miami, FL). At least 10,000 cells were analyzed per sample. Results are presented as means ± standard deviations from three independent experiments.

Caspase activity. A set of fluorogenic caspase substrates was used to identify caspase activation. Substrates Ac-YVAD-AMC, Ac-IETD-AMC, and Ac-LEHD-AMC (Peptide Institute Inc., Osaka, Japan) identified the activities of caspases 3, 8, and 9, respectively. Treated cells were scraped off in a standard buffer containing 100 mM HEPES, 10% (wt/vol) sucrose, 0.1% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 5 μg/ml leupeptin. After harvesting, caspase activity was detected by a fluorogenic assay as described by Thornberry (39). Cell homogenates (50 μg/ml) were suspended in standard buffer and supplemented with 25 μM each substrate, and dye absorbance was measured at 450 nm in an RF-5301PC spectrofluorophotometer (Shimadzu Corp., Kyoto, Japan). Caspase activities are expressed as fluorescence produced per hour per milligram of protein and presented as means ± standard deviations from three independent experiments.

Detection of MOMP. Macrophages were incubated for 12, 24, or 48 h in the presence of 100 μg of *M. bovis* CFE, followed by centrifugation at 300 × *g*. Cells were resuspended in PBS containing 0.1 μM dihexyloxacarbocyanine iodide ($DiOC_6$; Molecular Probes, Eugene, OR) and incubated for 30 min in the dark. Mitochondrial outer membrane permeabilization (MOMP) was determined by flow cytometry using an Altra Beckman cytometer (Beckman Coulter, Miami, FL). At least 10,000 cells were analyzed per sample. Results are means ± standard deviations from three independent experiments.

Immunohistochemistry. Macrophage monolayers were fixed for 40 min with 4% paraformaldehyde after infection or treatment. Cells were incubated 2 h with 2% normal porcine serum to block nonspecific binding, followed by washes and an overnight incubation using a 1:100 dilution of the horseradish peroxidase-conjugated anti-AIF monoclonal antibody D-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Samples were washed, and the enzyme was visualized with 0.05% diaminobenzidine as a substrate (Dako Cytomation, Carpinteria, CA). Cells were counterstained with hematoxylin. AIF translocation to the nucleus

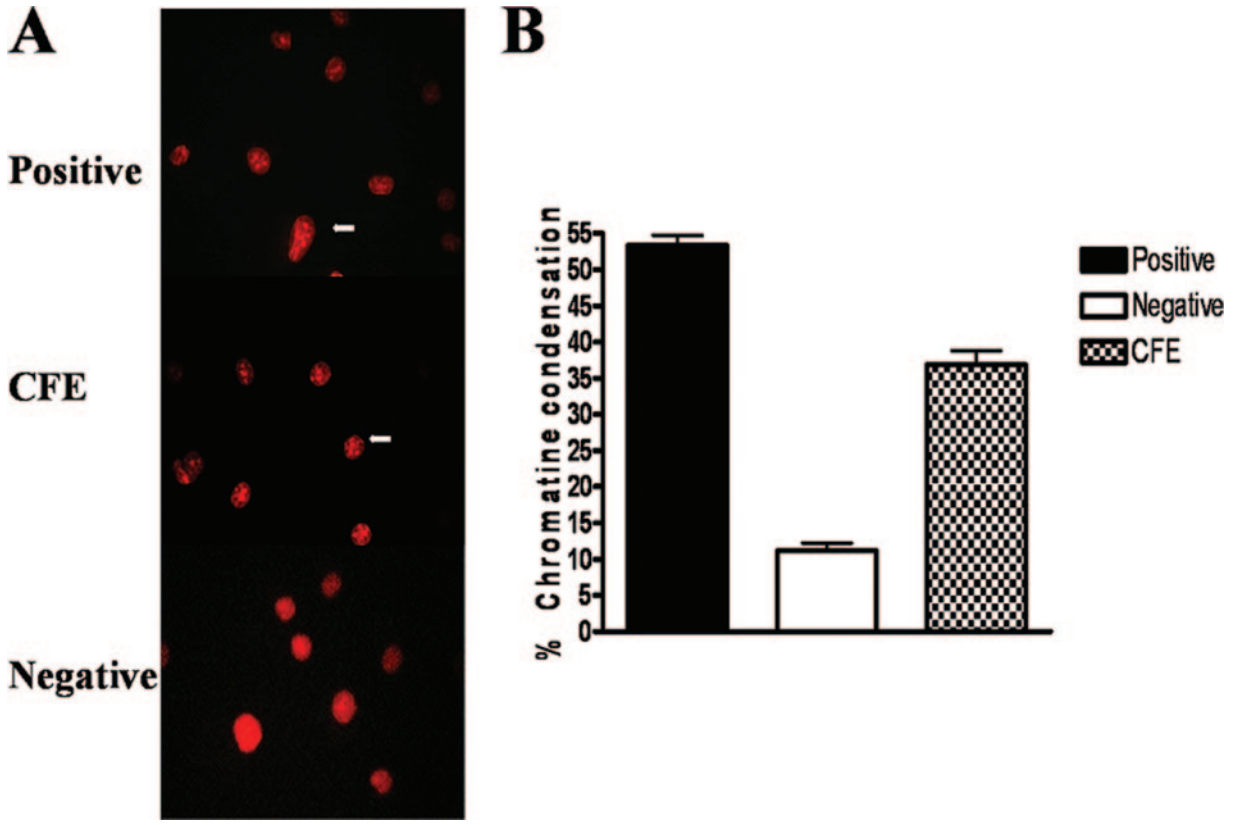

FIG. 1. Chromatin condensation of CFE-treated macrophages. Cells were incubated in the presence of 100 μg of *M. bovis* CFE. (A) Macrophage nuclei stained with propidium iodide showing chromatin condensation (arrows). (B) Percentages of cells showing chromatin condensation. A positive control was generated by incubating cells with 20 μg of camptothecin/ml. Chromatin condensation was analyzed by counting at least 100 cells per sample, using a fluorescence microscope. Results are means ± standard deviations from three independent experiments.

was visualized under the microscope (magnification, ×100) by counting at least 100 cells per sample. Negative controls consisted of sections incubated in the absence of the antibody. Results are means ± standard deviations from three independent experiments.

RESULTS

***M. bovis* and its CFE increased macrophage chromatin condensation and DNA fragmentation in a caspase-independent fashion.** Chromatin condensation was increased three times by CFE compared to that for untreated cells (Fig. 1). On the other hand, a TUNEL assay demonstrated that the levels of DNA fragmentation induced by *M. bovis* and CFE were 53.7% ± 24% and 38.9% ± 14%, respectively, whereas control cells had a basal proportion of 8.9% ± 4.1% (Fig. 2). Rates of DNA fragmentation were not affected by the presence of the pancaspase inhibitor z-VAD (Fig. 2).

***M. bovis* CFE decreases macrophage MOMP.** In order to determine the role of the mitochondrial membrane potential in the CD process observed, the lipophilic cation $DiOC_6$ was used with treated and control macrophages. Cells treated with 100 μg of *M. bovis* CFE for 12 h had a fivefold decrease in the macrophage MOMP relative to that of untreated cells. MOMP was maintained over the incubation time (24 h); however, at 48 h of treatment, the proportion of cells showing MOMP further increased, revealing a final divergence of 15 times from that of control cells (Fig. 3).

Macrophage caspase activation was not induced by *M. bovis* infection or CFE treatment. Induction of caspase activity in bovine macrophages was explored by a fluorogenic technique. Neither *M. bovis* infection nor CFE treatment induced caspase activation. Cells evaluated at 16 and 24 h showed no evidence of activation of caspase 3, 8, or 9 (Fig. 4A and B), whereas control cells incubated with the apoptotic inducer camptothecin had 0.044 ± 0.018, 0.016 ± 0.011, and 0.016 ± 0.016 units

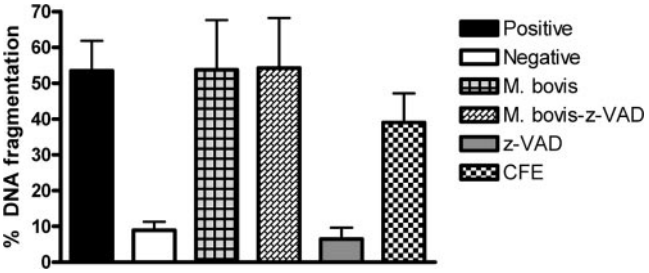

FIG. 2. DNA fragmentation of bovine macrophages after *M. bovis* infection or CFE treatment. Macrophages were incubated under each of the following conditions: camptothecin (20 μg/ml) (positive control), CRPMI medium (negative control), *M. bovis* (MOI, 10:1), z-VAD (50 μM) followed by *M. bovis*, z-VAD alone, and CFE (100 μg). DNA fragmentation was detected using the TUNEL assay. Results are means ± standard deviations from three independent experiments.

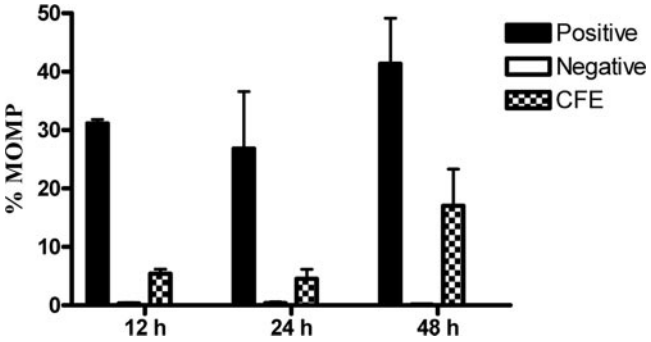

FIG. 3. Determination of mitochondrial outer membrane permeabilization in bovine macrophages. MOMP was measured in cells treated with 100 μg of *M. bovis* CFE by using $DiOC_6$. At least 10,000 cells were evaluated by flow cytometry. Results are means ± standard deviations from three independent experiments.

of fluorescence/h/mg of protein for the same caspases, respectively.

AIF translocation to nuclei is linked to *M. bovis* infection. An immunohistochemistry technique was used to detect the presence of AIF in macrophages. Monoclonal antibodies recognized AIF translocation to the nucleus for 32% ± 3.5%, 26.3% ± 4.9%, and 41% ± 5.69% of *M. bovis*-infected, CFE-treated, and camptothecin-treated cells, respectively. Incubation of macrophages with z-VAD before infection did not alter the percentage of positive cells. Cells that were not infected or that were incubated only in the presence of z-VAD showed 8% ± 2.4% AIF translocation (Fig. 5A and B).

DISCUSSION

Caspase activation via the intrinsic or extrinsic pathways has been demonstrated in *Mycobacterium*-induced apoptosis (4, 11, 15, 31, 33). Most of the reports described structural changes and participation of CD effectors associated with caspase 3 activity and MOMP (35). CD induced by *Mycobacterium* spp. has been linked to the presence of whole bacteria (16, 20, 22, 32) or protein extracts (12, 19, 25). Our group previously reported macrophage chromatin condensation and DNA fragmentation as a consequence of *M. bovis* infection (18). In the present study, we have identified the contribution of additional apoptotic effector elements in *M. bovis*-induced CD. Our experiments were conducted using a virulent *M. bovis* field isolate that had not been tested before as an apoptosis inducer. In our hands, 53.7% of cells underwent DNA fragmentation (Fig. 2). Similar results have been reported previously (18), confirming viable *M. bovis* as a CD activator; moreover, bovine macrophages incubated with an *M. bovis* extract (CFE) also developed structural changes compatible with CD (Fig. 1).

The method used to extract the CFE separated cytosolic and cell membrane soluble proteins. CD induction by the CFE suggests that proteins from the cytoplasm are partially responsible for the changes observed. On the other hand, we cannot ignore the presence of small amounts of lipoarabinomannan (LAM) contaminating the CFE (data not shown); however, a contribution of LAM to CD is very unlikely. Results from different studies indicate that LAM inhibits CD by stimulating phosphorylation of the proapoptotic protein Bad in a phosphatidylinositol 3-kinase-dependent manner (26, 29, 34, 35).

The first evidence indicating that a macrophage caspase-independent CD pathway was linked to *M. bovis* infection became apparent when macrophages were incubated with the pan-caspase inhibitor z-VAD prior to infection. Cell DNA fragmentation was not abrogated by the presence of the inhibitor (Fig. 2), whereas control cells incubated with the apoptotic inducer camptothecin had a reduction in the percentage of DNA fragmentation (data not shown). In order to confirm our observation, we evaluated the activation of individual caspases using a fluorogenic technique. No activation of caspase 3, 8, or 9 was detected in infected macrophages, in contrast to camptothecin-treated cells, where the activities of the three proteins were substantiated (Fig. 4). The potential of CFE to activate caspases was also investigated. As with *M. bovis* infection, incubation with z-VAD did not affect the DNA fragmentation rates (data not shown). When the fluorogenic technique was applied, none of the caspases examined was activated (Fig. 4). Collectively, these data strongly suggest that *M. bovis* and its CFE induced macrophage CD in a caspase-independent fashion.

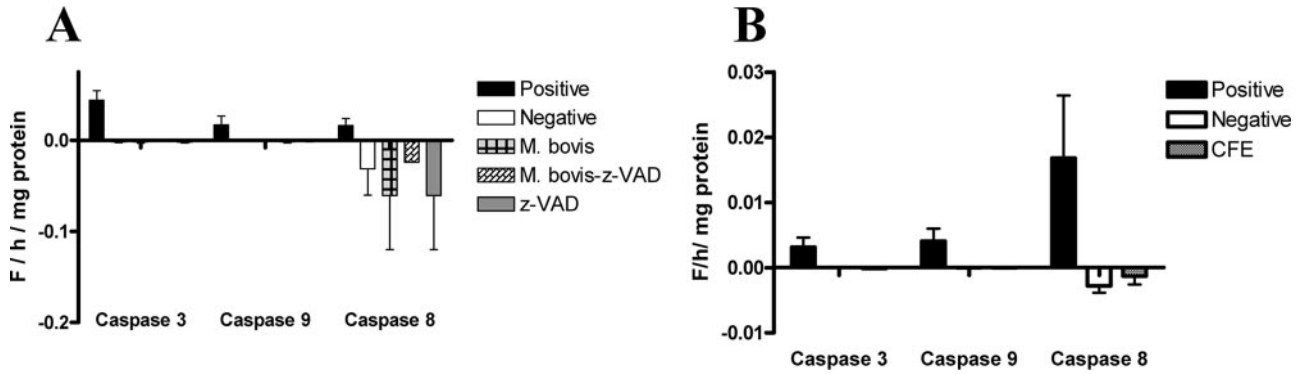

FIG. 4. Caspase activation of bovine macrophages after *M. bovis* infection or CFE treatment. Macrophages were incubated under each of the following conditions: camptothecin (20 μg/ml) (positive control), CRPMI medium (negative control), *M. bovis* (MOI, 10:1), z-VAD (50 μM) followed by *M. bovis*, z-VAD alone, and CFE (100 μg). The fluorometric substrates Ac-YVAD-AMC, Ac-IETD-AMC, and Ac-LEHD-AMC detected the activities of caspases 3, 8, and 9, respectively. (A) *M. bovis*-infected cells; (B) CFE-treated cells. Results are expressed as fluorescence per hour per milligram of protein (F/h/mg protein) and are means ± standard deviations from three independent experiments.

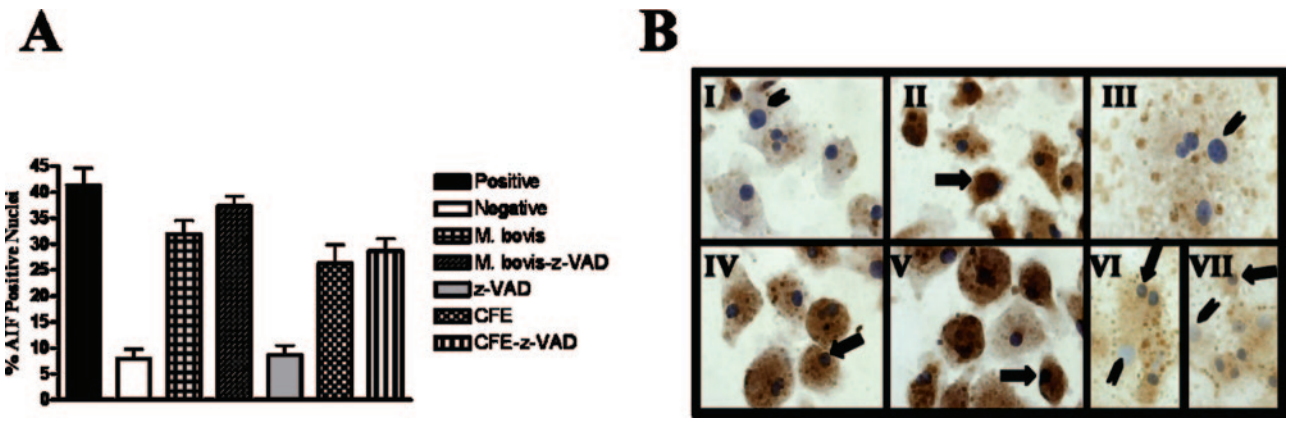

FIG. 5. AIF translocation to the nuclei. Macrophages were incubated under each of the following conditions: camptothecin (20 μg/ml) (positive control), CRPMI medium (negative control), *M. bovis* (MOI, 10:1), z-VAD (50 μM) followed by *M. bovis*, z-VAD alone, and CFE (100 μg). (A) Percentage of cells showing a positive dye in the nucleus. (B) Immunohistochemistry of infected cells showing a brown dye corresponding to the presence of AIF in the nuclei (arrows) or a blue color (arrowheads) indicating the lack of a positive signal. (I) Negative control; (II) positive control; (III) z-VAD-treated cells; (IV) *M. bovis*-infected cells; (V) z-VAD-treated and *M. bovis*-infected cells; (VI) CFE-treated cells; (VII) z-VAD- and CFE-treated cells.

M. tuberculosis-infected cells are known to undergo CD with caspase activation (4, 11, 15, 31, 33). However, a recent publication (23) demonstrated that CICD in *M. tuberculosis*-infected mouse macrophages is restricted to cells with a high intracellular bacterial burden (MOI, 25:1). Preliminary results from our laboratory indicate that in the bovine model, *M. tuberculosis* H37Rv infection at different MOIs was not sufficient to activate caspase 3 (data not shown), suggesting that bovine macrophages do not respond the same way as murine or human cells when infected by *Mycobacterium* species.

In an attempt to gain additional insights into the CD process, MOMP was measured in CFE-treated macrophages. A 12-h treatment yielded a MOMP 5-fold greater than that of untreated cells, increasing to 15 times greater when the incubation period was extended to 48 h (Fig. 3). Our data further suggest that *M. bovis* induced structural changes characteristic of macrophage CD occurring in the presence of MOMP.

The AIF is one of the proteins released into the cytosol as a result of MOMP. AIF is a mitochondrial flavoprotein that translocates to the nucleus during apoptosis and causes chromatin condensation and large-scale DNA fragmentation (10, 24, 27, 41). Additionally, AIF has been proposed to be a key element in CICD, because changes induced by AIF occurred in the absence of caspase activation (14). We used specific monoclonal antibodies to identify the location of AIF in *M. bovis*-infected cells. AIF translocation to the nucleus was identified in 32% of macrophages; however, the percentage of positive nuclei may be higher, because detached cells were not considered. Based on our results, we hypothesize that *M. bovis* infection drives the release of AIF into the cytosol and its translocation to the nucleus, where it participates in chromatin condensation and DNA fragmentation in a caspase-independent pathway. However, it remains to be determined whether AIF is the only molecule being released from the mitochondria. Other proteins are discharged during MOMP, although it has been pointed out that AIF is released from the mitochondria by a distinct mechanism (13). In addition, it is not clear from our studies why caspases are not activated and whether other proteins participate in AIF translocation to the nucleus. It is known that Bax, a Bcl-2 family member, has been associated with AIF release from the mitochondria (2, 13), where cytosolic Ca^{2+} released from the endoplasmic reticulum seems to play a role (5).

In conclusion, we report for the first time macrophage CICD associated with *M. bovis* infection. CD is considered to be an innate immune mechanism that plays a role in decreasing bacterial viability and preventing dissemination of the pathogen; however, it may also contribute to bacterial dissemination in individuals with compromised microbicidal activity.

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