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The Interleukin 1β-Converting Enzyme, Caspase 1, Is Activated during *Shigella flexneri*-Induced Apoptosis in Human Monocyte-Derived Macrophages

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***Shigella*, the etiological agent of bacillary dysentery, rapidly kills human monocyte-derived macrophages in vitro. Wild-type *Shigella flexneri*, but not a nonvirulent derivative, induced human macrophage apoptosis as determined by morphology and terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL). *Shigella*-mediated macrophage cell death was blocked by the peptide inhibitors of caspases, acetyl-Tyr-Val-Ala-Asp-aldehyde (acetyl-YVAD-CHO) and acetyl-Tyr-Val-Ala-Asp-chloromethylketone (acetyl-YVAD-CMK). Protection from apoptosis by YVAD was observed in monocytes matured in the presence or absence of colony-stimulating factors (CSF) like macrophage-CSF or granulocyte-macrophage-CSF. Furthermore, lipopolysaccharide (LPS) or gamma interferon (IFN-γ) rendered human macrophages partially resistant to *Shigella* cytotoxicity. Macrophages stimulated with either LPS or IFN-γ were also protected by YVAD from *Shigella*-induced cell death. During *Shigella* infections of human macrophages, interleukin-1β (IL-1β) was cleaved to the mature form. IL-1β maturation was severely retarded by YVAD, indicating that IL-1β-converting enzyme (ICE; caspase 1) is activated in *Shigella*-induced apoptosis. The finding that *Shigella* induces apoptosis in human macrophages by activating ICE supports the hypothesis that the acute inflammation characteristic of shigellosis is initially triggered by apoptotic macrophages which release mature IL-1β during programmed cell death.**

Enterobacteria of the genus *Shigella* are the causative agents of bacillary dysentery, a devastating form of diarrhea. The disease is manifested by painful abdominal cramps, fever, and characteristically, blood and mucus in the stools. Although self-limiting in healthy adults, shigellosis can be fatal in young children because of dehydration. The clinical symptoms reflect the histopathological findings, which comprise a massive infiltration of neutrophils into the colonic mucosa, leading to profound damage of the colonic epithelium (14).

Shigella crosses the mucosal barrier through M cells (18, 23). Resident macrophages of the lymphoid follicles engulf *Shigella*, which escapes from the phagolysosome and kills the host cell by triggering its intrinsic cell death program. Macrophages infected with *Shigella flexneri* readily undergo apoptosis in vitro (28) and in vivo, as assessed in a rabbit animal model (30) and dysenteric patients (9). Apoptotic macrophages release interleukin-1 (IL-1) (26) and initiate an inflammatory cascade, leading to the recruitment of neutrophils at the site of infection (19, 30). Chemoattracted polymorphonuclear leukocytes transmigrate through the epithelium to the colonic lumen and boost the infection by compromising the integrity of the epithelial layer (18). Neutrophils are not susceptible to *Shigella* cytotoxicity, but they efficiently kill wild-type *Shigella* in vitro (10). Therefore, polymorphonuclear leukocytes are likely to contribute to the clearance and resolution of dysentery in vivo.

Shigella invasiveness and cytotoxicity are conferred by a 220-kb plasmid that is present in all clinical isolates (8) and encodes an array of virulence factors and a type III secretion apparatus (reviewed in reference [15]). Loss of this virulence plasmid renders *Shigella* completely nonpathogenic (20). *Shigella* must escape the phagolysosome to exert its cytotoxicity in macrophages (27). IpaB (invasion plasmid antigen), a 62-kDa secreted protein encoded by the virulence plasmid, is localized in the cytoplasm of infected macrophages (22). IpaB is sufficient to induce macrophage apoptosis by directly binding to a pivotal component of the cell death machinery, IL-1β-converting enzyme (ICE) (2). ICE is activated during *Shigella*-induced apoptosis in macrophages, and mature IL-1β is released during the infection. Hence, in shigellosis, the proapoptotic and proinflammatory abilities of ICE converge.

ICE belongs to a family of at least 10 homologous zymogens, which recently were termed caspases (cysteine protease cleaving after aspartic acid) (1). Caspase 1 (ICE) is synthesized as a 45-kDa precursor and, after it is processed at specific aspartate residues, forms the active $(p10)_2/(p20)_2$ heterotetramer (24). The substrates of ICE described thus far are proICE itself, proIL-1β, and proIGIF, the precursor of gamma interferon (IFN-γ)-inducing factor (6, 7). Thus, ICE is critically involved in the activation of multiple proinflammatory cytokines.

In this study we show that human monocyte-derived macrophages infected in vitro with *S. flexneri* display the hallmarks of a bona fide apoptosis. Upon infection with wild-type *Shigella*, the macrophages showed a typical apoptotic morphology, including marginalization of the chromatin at the nuclear boundary, and the cells were labeled by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL). Moreover, cytotoxicity was blocked by YVAD, a peptide inhibitor specific for caspases. In the course of the infection, IL-1β was cleaved to its biologically active form, and maturation was delayed by YVAD, thus demonstrating that ICE is a target of the inhibitor. Activation of the macrophages with

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lipopolysaccharide (LPS) or IFN-γ decreased sensitivity of the cells to *Shigella*. These results support the hypothesis that in shigellosis, programmed cell death of the macrophage is a proinflammatory event (29).

MATERIALS AND METHODS

Bacteria. The *S. flexneri* wild-type strain M90T is a virulent isolate of serotype 5. BS176 is a plasmid-cured, nonvirulent derivative of M90T (21). ΔIpaB is a nonpathogenic derivative of M90T wherein *ipaB* was disrupted by a nonpolar insertion of a kanamycin cassette (16). The bacteria were grown at 37°C in Trypticase soy broth in a shaking incubator. ΔIpaB was cultured in the presence of 50 μg of kanamycin per ml.

Isolation and maturation of human monocyte-derived macrophages. Peripheral blood monocytes (PBM) matured in vitro form cells resembling tissue macrophages and are termed monocyte-derived macrophages (11). PBM from citrated venous blood obtained from healthy donors were isolated by Ficoll-Paque and Percoll gradient centrifugations as described previously (4). Briefly, 38 ml of Buffy Coat diluted 1:5 with phosphate-buffered saline (PBS) was placed on 11.4 ml of Ficoll-Paque (Pharmacia, Piscataway, N.J.) and centrifuged at 900 × *g* for 30 min at room temperature. Washed leukocytes (2.5 ml) were placed on 7.5 ml of heat-inactivated fetal bovine serum (GibcoBRL, Gaithersburg, Md.) and centrifuged at 200 × *g* for 15 min. The leukocytes were resuspended in 7 ml of PBS (BioWhittaker, Walkersville, Md.), placed on the same volume of 46% Percoll (Pharmacia), and centrifuged at 600 × *g* for 30 min. Washed PBM were resuspended at a density of 10^6 cells/ml in Dulbecco's modified Eagle's medium (DMEM) containing 5% (vol/vol) decomplemented human AB serum (BioWhittaker), 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. The PBM were matured to macrophages for 7 to 14 days on 150-mm-diameter petri dishes or on 100-mm-diameter Teflon dishes in a humified atmosphere of 5% CO_2 at 37°C. During some maturations, granulocyte-macrophage colony-stimulating factor (GM-CSF; Genzyme, Cambridge, Mass.) or macrophage colony-stimulating factor (M-CSF; Genzyme) was added at 10 ng/ml.

TUNEL and confocal microscopy. Human monocyte-derived macrophages differentiated for 7 days were seeded on a sterile coverslip at a density of 2×10^6 cells per 35-mm-diameter dish. The cells were infected with *Shigella*, and after different times, the infected cells were fixed with 4% paraformaldehyde overnight at 4°C. DNA fragmentation was visualized with an apoptosis detection kit (Promega, Madison, Wis.) by TUNEL, with fluorescein isothiocyanate-conjugated dUTP as a substrate. The samples were counterstained with propidium iodide and analyzed with a confocal microscope (Molecular Dynamics) equipped with an argon laser. The optical sections were filtered with an alpha filter and reconstituted in three-dimensional projection.

Inhibition of cytotoxicity by YVAD. Human monocyte-derived macrophages were matured for 7 to 14 days and seeded on a 96-well plate at a density of 2×10^4 or 4.5×10^4 cells/well. In some experiments the macrophages were treated overnight with either 1 μg of LPS (*Shigella* serotype 1A; Sigma, St. Louis, Mo.) per ml, 100 U of human IFN-γ (Genzyme) per ml, or 100 U of human GM-CSF per ml. Prior to infection with the bacteria, the medium was replaced with serum-free DMEM and the cells were incubated for 1 h with 50 or 100 μM reversible peptide inhibitor acetyl-Tyr-Val-Ala-Asp-aldehyde (YVAD-CHO; Biomol, Plymouth Meeting, Pa.) or with the irreversible peptide inhibitor acetyl-Tyr-Val-Ala-Asp-chloromethylketone (YVAD-CMK; Bachem Bioscience, King of Prussia Pa.) of ICE-like proteases. Infection of the macrophages with *Shigella* was done as previously described (3), by spinning bacteria of different multiplicities of infection (MOI) onto the cells (600 × *g* for 10 min). After 30 min of incubation, gentamicin (90 μg/ml) was added to kill extracellular bacteria.

Cytotoxicity was quantified colorimetrically with the CytoTox96 lactate dehydrogenase (LDH)-release kit (Promega). The percentage of cytotoxicity was calculated with the formula 100× [(experimental release − spontaneous release)/(total release − spontaneous release)], where spontaneous release is the amount of LDH activity in the supernatant of noninfected cells and total release is the activity in macrophage lysates.

Inhibition of IL-1β maturation by YVAD. Human macrophages (0.75×10^6 cells/well in a 24-well plate) were activated overnight with 1 μg of LPS (*Shigella* serotype 1A) per ml. The cells were washed once with serum-free DMEM, and 100 μM YVAD-CMK was added 1 h prior to infection of the cells with bacteria (MOI, 150) as described above. After centrifugation, the supernatant was immediately replaced by 0.16 ml of serum-free DMEM. At given time points, the macrophages were lysed in situ by the addition of 40 μl of PBS containing 1% Triton X-100. The lysates were centrifuged (for 5 min at 15,000 rpm) and immediately frozen in liquid nitrogen. The presence and maturation of IL-1β in the samples were analyzed by separation on a 15% polyacrylamide gel, followed by Western blotting. Goat anti-human IL-1β polyclonal antibody was obtained from R&D Systems (Minneapolis, Minn.), peroxidase-labeled rabbit anti-goat immunoglobulin G was from Kirkegaard & Perry Laboratories (Gaithersburg, Md.), and the enhanced chemiluminescence kit was purchased from Amersham (Arlington Heights, Ill.).

Killing of *Shigella* by human macrophages. Human macrophages were seeded onto an 96-well plate (2.7×10^4 cells/well) or onto a 24-well plate (8×10^4 cells/well), and a portion of the cells was activated overnight with either 1 μg of LPS per ml or 100 U of human IFN-γ per ml. The cells were infected with bacteria at an MOI of 2 or 10 as described above. Immediately after centrifugation or after 10 min of incubation, the cells were washed two to three times with serum-free DMEM to remove extracellular bacteria, and at given time points, the infection was stopped by the addition of 20 to 50 μl of PBS containing 1% Triton X-100. Dilutions of the bacteria (1:100 to 1:1,000) were plated on Luria-Bertani plates and counted after incubation overnight at 37°C.

RESULTS

***Shigella flexneri* kills human monocyte-derived macrophages by induction of apoptosis.** To determine the cytotoxic strategy of *Shigella* against human macrophages, monocytes were isolated from human venous blood and matured to macrophages in vitro. Maturation of human monocytes to macrophages in vitro is independent of but is supported by the addition of growth factors such as GM-CSF or M-CSF (CSF-1) (11). Human macrophages matured in the absence of a CSF were rapidly killed by *Shigella* (40 to 80% cell death within 1 h at an MOI of 100 or 150 bacteria per cell). The addition of a CSF during maturation affected neither the sensitivity of the macrophages to *Shigella* nor the cytotoxic pathway (see below).

Upon infection with the wild-type *Shigella* strain M90T, human macrophages underwent morphological changes typical for apoptotic cells, including shrinkage, heavy cytoplasmic vacuolization, and chromatin condensation. Marginalization of the chromatin at the nuclear boundary was clearly visible after M90T-infected macrophages were stained with propidium iodide (Fig. 1C). Additionally, two of the three macrophages shown in Fig. 1C are labeled by TUNEL, indicating that their DNA was nicked and/or fragmented, a biochemical feature of apoptotic cells (Fig. 1D). In contrast, macrophages infected with the nonvirulent *Shigella* strain BS176 survived, showed normal morphology (Fig. 1A), and were not labeled by TUNEL (Fig. 1B).

Cytotoxicity of *S. flexneri* in human macrophages is mediated by an ICE-like protease. During *Shigella*-induced apoptosis of mouse peritoneal macrophages, ICE is activated, and in turn cell death can be inhibited by YVAD, a specific peptide inhibitor of ICE-like proteases (2). To test whether the same pathway is active in the apoptosis of human macrophages, cells were preincubated with YVAD prior to infection with *Shigella*. Regardless of whether monocytes were matured in the presence or absence of CSF (M-CSF or GM-CSF), the macrophages were protected from cell death by YVAD (data not shown). Furthermore, macrophages activated with either LPS (data not shown) or IFN-γ (see below) were also protected by YVAD from apoptosis. Therefore, the apoptotic pathway engaged during *Shigella* infection of human macrophages seems to be dependent on caspases, regardless of the maturation or activation state of the cells.

As expected, the cytotoxicity of *Shigella* on human macrophages was dependent on the number of bacteria per cell, and at MOIs ranging from 150 to 50, the protection of the cells from apoptosis by YVAD was dose dependent (Fig. 2). At an MOI of 150, preincubation of the macrophages with 100 μM YVAD reduced *Shigella*-induced cytotoxicity by 56% in GM-CSF-treated cells (Fig. 2A, striped bars) and by 74% in IFN-γ-activated cells (Fig. 2A, open bars). At an MOI of 100, inhibition of cell death by 100 μM YVAD was even more pronounced and reached 75 and 89%, respectively (Fig. 2B). The nonvirulent strain ΔIpaB killed less than 4% of the macrophages at an MOI of 150 (Fig. 2D). Additionally, activation with IFN-γ protected the human macrophages from *Shigella*-induced cell death in synergy with YVAD. Treatment with GM-CSF, on the other hand, had no protective effect (see below).

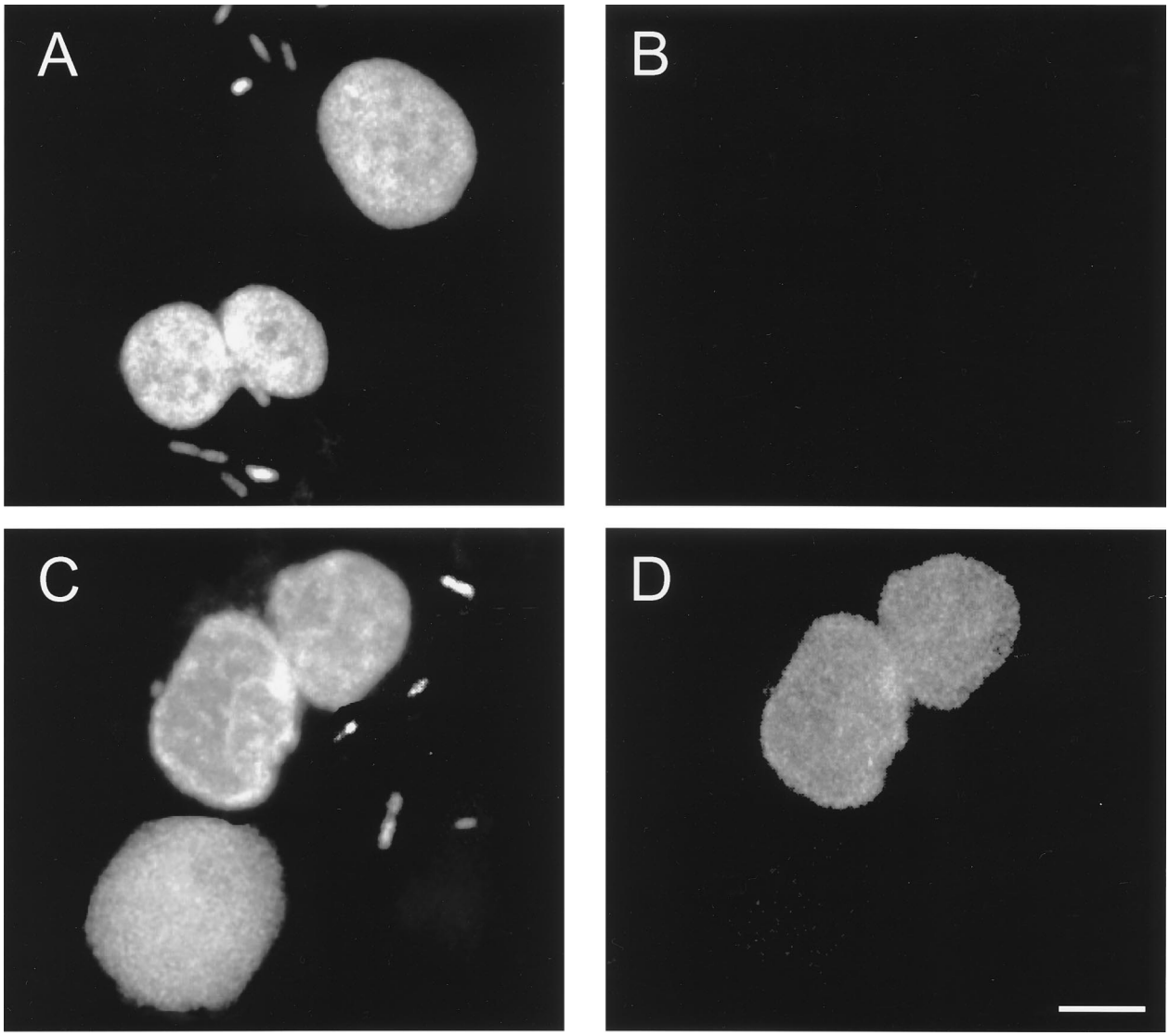

FIG. 1. Human monocyte-derived macrophages infected with wild-type *Shigella* show apoptotic morphology and are labeled by TUNEL. Confocal micrograph of human macrophages stained with propidium iodide (A) and TUNEL (B) after 60 min of infection with BS176, a nonvirulent *Shigella* strain. Human macrophages infected with the wild-type strain M90T for 60 min (C) are labeled by TUNEL (D), indicating that they undergo apoptosis. Some bacteria are seen in close association with the macrophages (A and C). Bar, 5 μm.

YVAD prevents the maturation of IL-1β during *Shigella*-induced apoptosis in human macrophages. Activation of ICE in a cell infected with *Shigella* should lead to the processing of proIL-1β the biologically inactive precursor of IL-1β (31 kDa) to its mature, biologically active 17.5-kDa form. Human macrophages were activated with LPS overnight to induce the expression of proIL-1β and were subsequently infected with wild-type *Shigella*. In lysates of infected macrophages, the mature form of IL-1β was detected as early as 10 min after the onset of the infection (Fig. 3A). No earlier time point could be measured, since shigellae are nonmotile and must be centrifuged onto the macrophages in order to synchronize infection. During the 3-h course of the infection, the amount of the mature protein increased. In contrast, preincubation of the macrophages with YVAD severely delayed the maturation of proIL-1β. The 17.5-kDa form appeared only after 25 min of infection and gradually increased in amount throughout the experiment (Fig. 3B). The fact that the maturation of IL-1β was only retarded but not blocked by YVAD is reflected by the finding that YVAD only partially inhibited *Shigella*-induced macrophage apoptosis. Nevertheless, since ICE is the only caspase known to cleave proIL-1β, the retardation by YVAD of the maturation of proIL-1β implies that ICE is a target of the inhibitor. BS176, a noncytotoxic *Shigella* strain, did not induce processing of proIL-1β within the 3-h period (Fig. 3A), and macrophages that were not activated with LPS expressed very little proIL-1β (Fig. 3A).

Human macrophages activated by LPS or IFN-γ are protected from killing by *Shigella*. Macrophages treated with LPS and IFN-γ, but not those treated with GM-CSF, were significantly protected from being killed by *Shigella* compared to untreated macrophages (Fig. 4). The two compounds decreased *Shigella* cytotoxicity by 52% (LPS) and 41% (IFN-γ), respectively. Similar results were obtained at an MOI of 150 and in the presence of YVAD (data not shown). Interestingly, at a low MOI (10 bacteria per macrophage) the protection of

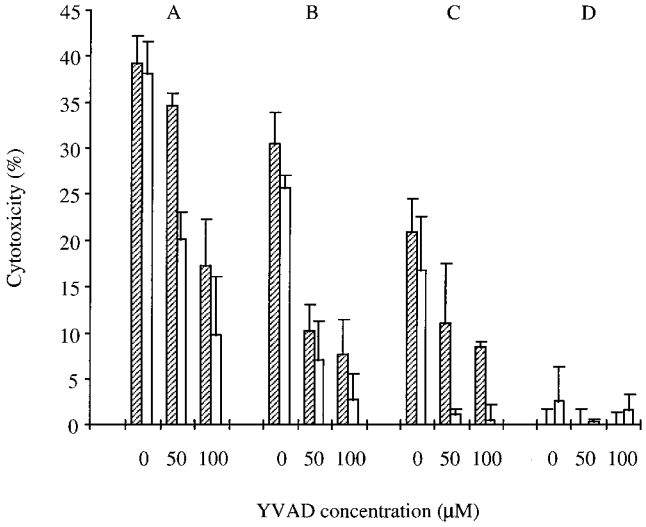

FIG. 2. Dose-dependent protection by YVAD of human macrophages from *Shigella*-induced cell death at different MOIs. Human macrophages were treated overnight with either GM-CSF (striped bars) or IFN-γ (open bars) and preincubated with 50 or 100 μM YVAD 1 h prior to infection. The virulent *Shigella* strain M90T was incubated at an MOI of 150 (A), 100 (B), or 50 (C). As a control, macrophages were infected with ΔIpaB, a nonvirulent mutant at an MOI of 150 (D). The means and standard deviations of three experiments are shown.

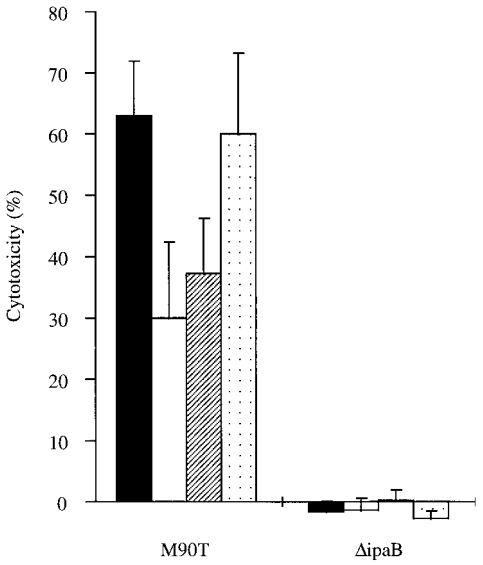

FIG. 4. LPS and IFN-γ protect human macrophages from *Shigella* cytotoxicity. Prior to an LDH release experiment, human macrophages (solid bars, no addition) were treated with LPS (open bars), IFN-γ (striped bars), or GM-CSF (dotted bars). The means and standard deviations of three experiments performed with the wild-type strain M90T or the noncytotoxic strain ΔipaB are shown.

cells from *Shigella*-mediated cytotoxicity by LPS and IFN-γ was even more pronounced and amounted to 82 and 47%, respectively, after 90 min of infection (Fig. 5).

The activation state of the macrophage could influence its ability to kill intracellular *Shigella*, possibly explaining the observed differences in cytotoxicity. Therefore, we tested whether untreated macrophages or macrophages pretreated with LPS or IFN-γ exhibit different bactericidal efficiencies. However, we detected no significant differences in the killing of *Shigella* by the phagocytes at MOIs of 2 and 10 bacteria per cell, regardless of which compound was used to activate macrophages (data not shown).

DISCUSSION

In this study we present evidence that human monocyte-derived macrophages undergo apoptosis upon infection with *S. flexneri*. The experimental findings presented in this study can be summarized as follows: (i) human macrophages infected with virulent *Shigella* show an apoptotic morphology, including chromatin condensation at the nuclear boundary; (ii) infected macrophages are labeled by TUNEL; (iii) YVAD, a specific inhibitor of caspases, protects macrophages from *Shigella* cy-

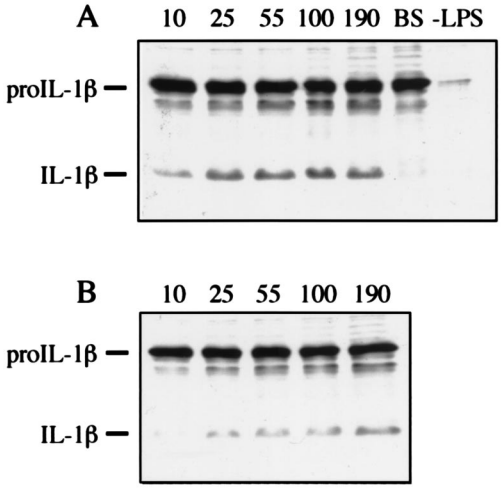

FIG. 3. Western blot of anti-human IL-1β of lysates from human macrophages infected with *Shigella* in the absence (A) or the presence (B) of YVAD. The macrophages were infected with wild-type *Shigella* strain M90T for 10, 25, 55, 100, and 190 min, respectively, or with the nonpathogenic strain BS176 for 190 min (BS). Macrophages not activated with LPS were lysed after 190 min (−LPS).

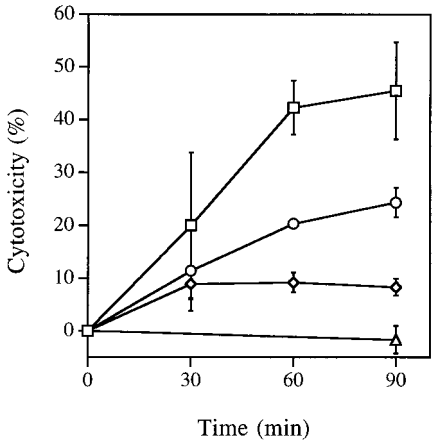

FIG. 5. Kinetics of cytotoxicity and protection of macrophages by LPS and IFN-γ. The cytotoxicity of *Shigella* against unactivated human macrophages (□) or cells previously activated with LPS (◇) or IFN-γ (○) was determined by LDH release. The cells were infected at an MOI of 10 and, as a control, the nonvirulent strain BS176 was used (△). Means and standard deviations of three experiments are shown.

totoxicity; (iv) the maturation of proIL-1β during the infection of macrophages with *Shigella* is delayed by YVAD, implying that ICE is a target of the inhibitor; and (v) LPS and IFN-γ decrease *Shigella* cytotoxicity.

Our data obtained from TUNEL experiments indicate that during the infection of human macrophages with virulent *Shigella*, the cell's DNA is nicked and/or fragmented, a hallmark of apoptosis. This finding contrasts with the results described in a recent study by Fernandez-Prada et al. in which DNA fragmentation in *Shigella*-infected human monocyte-derived macrophages was not detected (5). In our TUNEL assay, the difference in labeling between the virulent strain M90T and the nonpathogenic strain BS176 was most prominent at 60 min postinfection. Fernandez-Prada et al., on the other hand, did the TUNEL labeling at 30 min postinfection and did not provide information about the time point(s) at which they assessed DNA fragmentation on agarose gels. These researchers concluded that human macrophages infected with virulent *Shigella* do not undergo apoptosis but, rather, die by a cytolytic event termed oncosis. However, experiments designed to detect DNA fragmentation are hampered by the narrowness of the time slot during which the fragmentation can be detected and, therefore, the informative time point might have been missed. Still, the possibility that *Shigella* triggers other types of cell death in human macrophages in addition to apoptosis remains interesting.

YVAD, a specific peptide inhibitor of caspases, inhibits cell death and the maturation of IL-1β in both human and mouse peritoneal macrophages infected with virulent *S. flexneri* (Fig. 2 and 3) (2). Remarkably, however, YVAD was less effective as a protective agent in human monocyte-derived macrophages than in mouse peritoneal macrophages, regardless of whether reversible acetyl-YVAD-CHO or irreversible acetyl-YVAD-CMK was used. Whereas in unactivated human macrophages, 100 μM YVAD decreased cytotoxicity 75% at maximum (Fig. 2B), mouse peritoneal macrophages obtained over 80% protection from apoptosis with only 25 μM YVAD (2). Possible explanations for this discrepancy include (i) variations in the K_i values of YVAD for human and mouse ICE, (ii) differences in the membrane permeability of the peptide inhibitor between human and mouse macrophages, and (iii) the possibility that caspases other than ICE are activated in *Shigella*-infected human macrophages. ICE is the only caspase known to process IL-1β, and YVAD delayed the maturation of proIL-1β in the course of a *Shigella* infection in both human macrophages (Fig. 3) and mouse peritoneal macrophages (2). Therefore, ICE is unequivocally involved in *Shigella*-induced macrophage apoptosis.

In accordance with our results, Fernandez-Prada et al. reported that another irreversible peptide inhibitor of caspases, Z-Val-Ala-Asp-fluoromethylketone (ZVAD-FMK), abolished the maturation of IL-1β in *Shigella*-infected human macrophages (5). Cytotoxicity, however, was not decreased by 20 μM ZVAD-FMK. Using YVAD-CMK, we found a significant inhibition of *Shigella*-induced cytotoxicity only at concentrations above 50 μM (Fig. 2, data not shown). Since ZVAD-FMK is less specific for ICE than YVAD-CMK (25), one would expect at least 50 μM ZVAD-FMK to be necessary to protect human macrophages from *Shigella*-mediated cell death. We clearly show that human monocyte-derived macrophages matured under a variety of conditions (including those described by Fernandez-Prada et al.) are protected by YVAD from *Shigella* cytotoxicity. This finding indicates that *Shigella*-induced cell death is initiated by the activation of a member(s) of the caspase enzyme family (24).

Activation of human macrophages by LPS, and to a lesser extent by IFN-γ, rescued the cells from *Shigella*-mediated cytotoxicity (Fig. 4 and 5). Similarly, human peripheral blood monocytes are known to be protected from serum deprivation-induced apoptosis by LPS, the proinflammatory cytokines IFN-γ, tumor necrosis factor alpha, IL-1β, and GM-CSF (12, 13). Mature, long-lived tissue macrophages, on the other hand, lose their sensitivity to undergo apoptosis induced by cytokine withdrawal but undergo apoptosis triggered by certain stimuli only after exposure to IFN-γ (17). Hence, depending on the stimulus, IFN-γ either increases or decreases sensitivity to apoptosis in monocytes and macrophages.

The mechanisms by which LPS and IFN-γ protect macrophages from *Shigella*-induced apoptosis are unclear. Enhanced microbicidal activity of phagocytes stimulated by LPS and IFN-γ could account for the protective effect. However, we did not observe differences in the killing of *Shigella* by activated or nonstimulated human macrophages. Additionally, LPS and IFN-γ might directly trigger an antiapoptotic intracellular signaling cascade or induce cytokines that act in an autocrine manner. Furthermore, differences in phagocytotic efficiency might occur, or target molecules of the bacterial toxin(s) might be regulated differentially. It is interesting to speculate that the protection of macrophages from cell death by LPS applies not only to macrophages matured in vitro but also to the macrophage population present in *Shigella* infection. Thus, LPS and IFN-γ might contribute to limit the spread of the pathogen in the colonic mucosa. In cooperation with inflammatory neutrophils, which are not susceptible to *Shigella* cytotoxicity (10), macrophages protected from *Shigella* could participate in the clearance of the pathogen.

Human macrophages were protected by YVAD from apoptosis at MOIs between 50 and 150. These high experimental MOIs may exceed the in vivo ratio of bacteria per macrophage. It is noteworthy in this context that in clinical trials an inoculum of less than 100 *Shigella* cells is sufficient to cause dysentery. Furthermore, at lower MOIs the protection by YVAD was even more pronounced (Fig. 2), suggesting that ICE-mediated apoptosis of *Shigella*-infected macrophages is also relevant in vivo.

Recently, further evidence that Shigella-induced macrophage apoptosis is relevant in vivo was provided by Islam et al. (9). In their study, rectal mucosal biopsies from patients suffering from shigellosis were assessed for apoptotic cells and apoptotic bodies by in situ end labeling of nicked DNA fragments (TUNEL). Macrophages were identified by immunostaining with an anti-CD68 antibody, a macrophage-associated granular protein. In acute-phase shigellosis, which is characterized by severe inflammation, Islam et al. found a massive increase of apoptotic macrophages in the lamina propria.

In summary, the results presented here and those presented previously indicate that human and mouse macrophages infected with virulent *Shigella* both die by engaging the intrinsic cell death program and release mature IL-1β. These findings support our model of *Shigella* pathogenesis, in which the activation of ICE in macrophages is considered to be a crucial event for the initiation of both apoptosis, and via the secretion of mature IL-1β, the massive inflammation characteristic of the disease. Additionally, human macrophages are protected from *Shigella* by LPS and IFN-γ. Thus, macrophages contribute in multiple ways to the limitation of shigellosis as follows: (i) an invasive pathogen's conceivable host cell is destroyed by apoptosis, (ii) inflammation is triggered by the release of mature IL-1β, and (iii) protection by LPS and IFN-γ may lead to the enhanced clearance of *Shigella*.

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