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Human Monocyte-Derived Macrophages Infected with Virulent *Shigella flexneri* In Vitro Undergo a Rapid Cytolytic Event Similar to Oncosis but Not Apoptosis

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Infection of human monocyte-derived macrophages in vitro with virulent *Shigella flexneri* resulted in cell death which involved rupture of the plasma membrane, cell swelling, disintegration of ultrastructure, and generalized karyolysis. These features bore resemblance to oncosis and are in striking contrast to previously described observations of mouse macrophages, where a similar infection by virulent *Shigella* resulted in cell death by apoptosis. Cell death by oncosis in human macrophages was confirmed by lactate dehydrogenase release, light microscopy, electron microscopy, terminal deoxynucleotidyltransferase end labeling of DNA ends, DNA fragmentation assays, and fluorescence-activated cell sorter analysis of propidium-labeled nuclei. Thus, the phenomena of cell death induced by virulent *Shigella* in human and mouse macrophages reflect different biochemical pathways. Interleukin-1β (IL-1β) was released in culture supernatants of human macrophages infected with virulent bacteria. Inhibition with IL-1β-converting enzyme inhibitors indicated, however, that this release occurred as a passive event of cell lysis. The patterns of intracellular survival of *Shigella* strains within human and mouse macrophages reflect differences that exist not only between *Shigella* serotypes but also between the two different macrophage cell types.

In nature, shigellae, causing bacillary dysentery, are generally acquired by oral ingestion of contaminated food and water. A large invasion plasmid enables these facultative intracellular pathogens to enter the colonic epithelial cells, where they multiply, disseminate intra- and intercellularly, and cause inflammation with resulting necrosis (for a review, see references 11 and 32). Studies using guinea pig intestines (19) and chronically isolated rabbit intestinal loops (41, 44) have shown that the uptake of shigellae occurs primarily within the specialized surface epithelial cells (M cells) overlying the scattered collections of lymphoid tissues which form the Peyer's patches. These patches are located immediately below the rabbit intestinal epithelium (41). The M cells deliver the bacteria to the underlying mononuclear and dendritic cells and thence to gut-associated lymphoid tissues (16, 17). The mucosal lymphocytes, after local antigenic stimulation, migrate to the regional lymph nodes and home to submucosal sites, where they differentiate into plasma cells that secrete dimeric immunoglobulin A (IgA) antibodies. With the aid of the secretory component, these IgA antibodies, as secretory IgA (sIgA), are subsequently transported across epithelial cells to the lumen, where they constitute the major component of the mucosal immune response to oral antigens (17, 18). To obtain oral vaccines to mucosal pathogens that generate long and lasting immune responses, it is crucial to understand the interaction of orally introduced pathogens with immune cells of the lymphoid tissues and epithelial cells (16).

Recent studies in vitro have demonstrated that invasive *Shigella flexneri* is unable to enter the apical pole of human colonic T84 cells or Caco-2 cells (27). Analyses of *Shigella* infection of polarized epithelial cells have indicated that virulent bacteria enter through the basolateral pole of the epithelial cells (27). This entry is facilitated by *Shigella*-induced transepithelial migration of polymorphonuclear cells, which is considered responsible for acute inflammation of the colon and mucosal destruction in vivo (33). Macrophages play an important role in the generation, perpetuation, and resolution of this acute inflammatory process through their extensive repertoire of specific plasma membrane receptors and secretory products (35). Macrophage populations are found throughout the lamina propria of the gastrointestinal tract, often surrounding blood and lymph vessels (8, 22). The function of resident tissue macrophages is uncertain, and it is generally believed that during microbial infection, the major host defense depends on newly recruited blood monocytes that can be activated and induced to release cytotoxic molecules (8, 16, 22).

In vitro studies of infection of mouse peritoneal macrophages and macrophage cell line J774 indicate that virulent *Shigella* induces macrophage cell death by apoptosis (51). This phenomenon has been demonstrated by DNA fragmentation assays and electron microscopy (50). Recent studies have further demonstrated that the IpaB protein, expressed on the bacterial surface of virulent *Shigella* strains, is necessary to induce apoptosis in murine macrophages (50), releasing interleukin-1α (IL-1α) precursor and mature IL-1β (49). In a rabbit ileal loop model, animals treated intravenously with IL-1 receptor antagonist (IL-1Ra) at 2 mg/kg per h showed a striking decrease in inflammation, destruction, and invasion of the villous intestine and Peyer's patches (40). The existence of an apoptotic pathway for elimination of macrophages with intracellular organisms implies that such a suicide response is im-

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TABLE 1. *S. flexneri* strains used in this study

Strain	Characteristics	Source or reference[a]
M90T-W	Wild-type *S. flexneri* serotype 5	WRAIR
M90T-55	Plasmid-cured derivative of M90T-W	WRAIR
M90T-A3	Deletion of the *ipa/mxi/spa* region in M90T-W	WRAIR
SC403	*ipaB* deletion mutant in M90T-W	13
2457-T	Wild-type *S. flexneri* serotype 2a	WRAIR
M4243A	Plasmid-cured derivative of 2457-T	WRAIR
BS103	Plasmid-cured derivative of *S. flexneri* 2a	A. Maurelli, USUHS

[a] WRAIR, Walter Reed Army Institute of Research, Washington, D.C.; USUHS, Uniformed Services University of the Health Sciences, Bethesda, Md.

portant to the host. This, in turn, may have significance in the generation of the immune response during infection.

Macrophages are a heterogeneous population that varies depending on their species of origin, anatomic location, state of activation, and conditions of culture (8). Macrophages secrete many chemically reactive secretory products. The two most prominent in immunologically activated macrophages are the reactive oxygen intermediates and the reactive nitrogen intermediates (10, 42). Recent studies from several laboratories show that nitric oxide production is the principal inducible effector mechanism of macrophage-mediated killing in many systems, including mouse macrophages (3, 10, 42). The nitric oxide pathway is much less evident in human macrophages. Thus, biochemical events that operate during microbial infection in activated human macrophages may not be present in murine cells.

Since humans are the primary hosts for *Shigella* infection, the overall aim of this study was to characterize an in vitro model with which to analyze the interaction of *Shigella* with human macrophages. Peripheral blood monocytes are the precursors of tissue macrophages. Monocytes from peripheral blood, cultured in vitro, exhibit the morphologic appearance of tissue macrophages and are termed human monocyte-derived macrophages (22). In this study, we have demonstrated that the killing of human monocyte-derived macrophages by virulent *Shigella* in vitro occurs by a cytolytic event accompanied by cell swelling, plasma membrane disintegration, and karyolysis. The term oncosis has been recently reintroduced in the literature to describe this type of accidental cell death (2, 21). This phenomenon is distinct from programmed cell death, or apoptosis, which occurs during *Shigella* infection of murine macrophages (49–51).

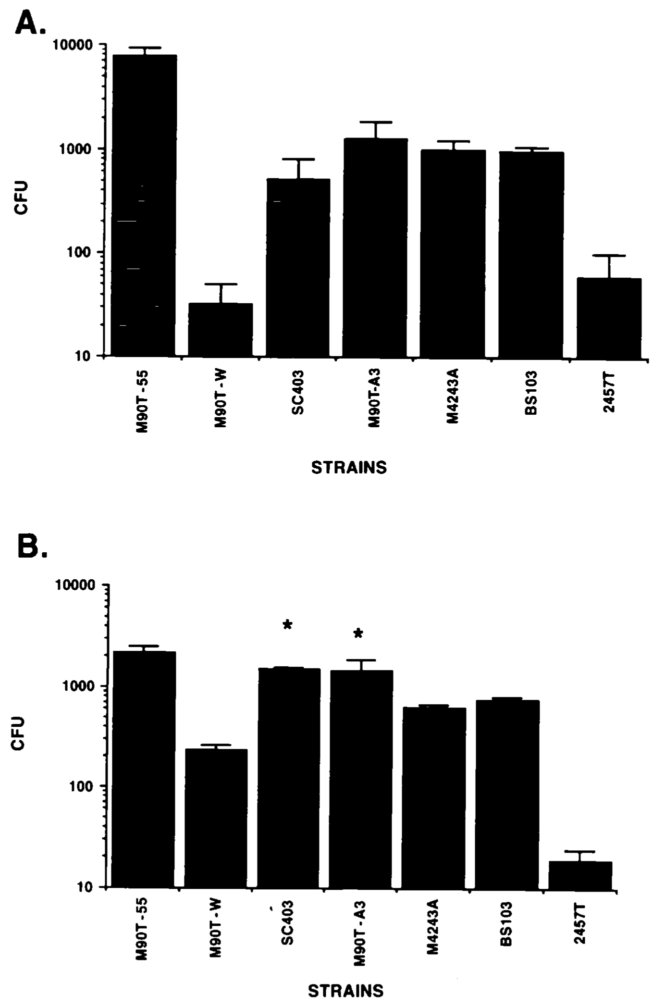

FIG. 1. Survival of *S. flexneri* strains in human monocyte-derived macrophages (A) and murine macrophage cell line J774 (B). Bacteria were left in contact with macrophages for 30 min and then treated with gentamicin-containing medium for another 50 min. CFU represents the total number of bacteria in macrophage cell lysates. The characteristics of the strains are listed in Table 1. *, not significant *P* values compared to M90T-55. Error bars show means ± standard deviations.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study to infect macrophages are listed in Table 1. Strain SC403 is an *S. flexneri* 5 *ipaB* mutant (13) and was obtained from P. J. Sansonetti, Institut Pasteur, Paris, France. For all macrophage infections, overnight cultures of the bacterial strains were diluted 50-fold in LB broth (Difco) and incubated at 37°C for 2 h until they reached the mid-log phase of growth. The bacteria were harvested and resuspended in Hanks balanced salt solution (HBSS; Gibco). Antibiotics were used at recommended concentrations as follows: ampicillin, 100 μg/ml; spectinomycin, 50 μg/ml; and streptomycin, 300 μg/ml.

Isolation and culture of human monocytes. Monocytes were isolated from citrated peripheral venous blood from healthy human volunteers by counterflow centrifugal elutriation and cultivated in RPMI 1640 medium containing 10% heat-inactivated human AB serum (Sigma), 10 ng of recombinant human macrophage colony-stimulating factor (MCSF) (a gift of Jay Stoudemire, Genetics Institute, Cambridge, Mass.) per ml, and 2 mM L-glutamine. Colony-stimulating factors such as MCSF are specific growth factors that mediate the survival, proliferation, differentiation, and functional modulation of blood cells, including monocytes (39). The monocytes were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Monocytes were cultivated in the presence of recombinant human MCSF for 7 to 10 days. The adherent population was >95% macrophages as determined morphologically by Wright's stain. The murine macrophage-like cell line J774 was grown in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco), 2 mM L-glutamine, and penicillin-streptomycin in a humidified 5% CO_2 incubator at 37°C. Twenty-four hours prior to infection, the monocyte-derived macrophages were resuspended in fresh medium in either 24-well culture plates or 100-mm-diameter tissue culture plates at a concentration of 10^6 cells/ml. The macrophages were infected with a multiplicity of infection (MOI) of 3 to 30 bacteria per eucaryotic cell, centrifuged at $70 \times g$ for 5 min, and incubated with the bacteria for different periods of time. The cells were washed with HBSS to remove extracellular bacteria and, when required, further incubated in medium containing 50 μg of gentamicin per ml to avoid reinfection of macrophages. Macrophage survival was assessed by light microscopy and trypan blue dye exclusion (33). At selected intervals following infection, between time zero and 24 h, the medium was removed and the macrophages were washed and lysed. The numbers of viable bacteria were obtained by plating dilutions of the lysates on tryptic soy agar (TSA) plates. CFU were counted after an overnight incubation of the plates at 37°C.

Light microscopy analysis of infected macrophages. Twenty-four hours prior to infection, human or murine macrophages were seeded in tissue chamber slides (Lab-Tek) and incubated at 37°C in a humidified 5% CO_2 incubator. At selected intervals after infection, the slides were washed and stained by using a LeukoStat

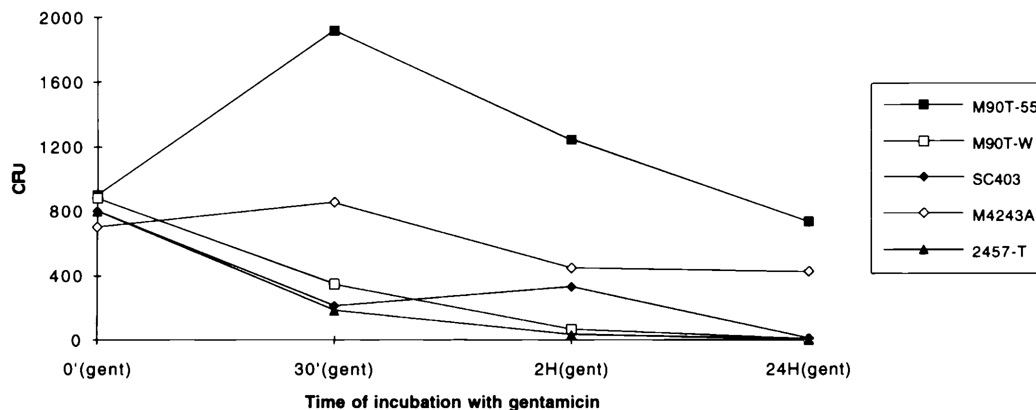

FIG. 2. Initial uptake of *S. flexneri* strains in human macrophages. *Shigella* strains were centrifuged for 5 min onto macrophages cultured in 24-well tissue culture plates. After centrifugation, some of the wells were washed immediately to remove unincorporated bacteria and then lysed (time zero). Gentamicin (gent)-containing medium was added to the other wells for different periods of time (30 min, 2 h, and 24 h), after which they were washed, lysed, and plated on TSA agar plates.

stain kit (Fisher); this is a modification of the Wright's stain technique. Alternatively, infected and uninfected macrophages were scraped from the plates, and an aliquot was fixed onto slides by using a cytospin machine. The slides were stained and observed under a light microscope.

Transmission electron microscopy. At selected intervals following infection, macrophages were washed with HBSS three times, fixed for 1 h at room temperature with either 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (SCB; pH 7.2) or 4% paraformaldehyde–1% glutaraldehyde in 0.2 M SCB, scraped off the tissue culture plate surfaces, and stored at 4°C until further processing. The samples were washed in SCB and postfixed with 1% OsO_4 in SCB for 2 h at room temperature. The postfixed material was washed again in SCB, dehydrated in a graded ethanol series (30 to 100%) plus propylene oxide, and embedded in Epon 812. Ultrathin sections were made by using a Leica Ultracut S ultramicrotome. Sections were stained with uranyl acetate and lead citrate as described by Reynolds (38) and examined under a Philips 400 transmission electron microscope operating at an acceleration voltage of 80 kV.

LDH assays for measuring cytotoxicity. Lactate dehydrogenase (LDH) is a cytoplasmic enzyme whose presence in the culture medium reflects the loss of plasma membrane integrity. LDH activity in the culture supernatants was measured by using a colorimetric Cytotox 96 kit (Promega Corp., Madison, Wis.) as instructed by the manufacturer. Spontaneous release is measured as the amount of LDH activity in supernatants of noninfected macrophages, while total release reflects the LDH activity released from uninfected macrophage subjected to lysis. Previous experiments have demonstrated that *S. flexneri* strains have no endogenous LDH activity when grown aerobically (50); for this reason, their values were not taken into account to calculate the percentage of cytotoxicity. The percentage of cytotoxicity was calculated as 100 × (experimental release − spontaneous release)/(total release − spontaneous release).

DNA fragmentation assay. Internucleosomal DNA fragmentation of infected human macrophages was measured by a previously described technique (24). The samples were electrophoresed on 1.2% agarose gels and stained with ethidium bromide.

TdT end labeling of DNA (TUNEL technique). To detect DNA fragmentation in individual cells, cytocentrifuge slides were prepared as described for light microscopic analysis and processed for in situ nick end labeling, using an Apoptag kit (Oncor Inc., Gaithersburg, Md.) according to the manufacturer's instructions. In this reaction, digoxigenin-labeled nucleotides are incorporated at sites of DNA strand breaks by terminal deoxynucleotidyltransferase (TdT). Mammary gland cells from weanling mouse were used as positive controls for apoptosis.

DNA analysis by flow cytometry. Morphology of DNA was also assessed by flow cytometry. Nuclei from infected and uninfected macrophages were released from cells by treatment with 1% Triton X-100 in 0.1 M citric acid, stained with 10 μg of propidium iodide (PI) per ml, and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.). PI intercalates into the DNA of cells, causing them to fluoresce red when illuminated with UV light.

Detection of IL-1β levels in the supernatant of infected macrophages. The release of IL-1β in the culture supernatants was measured at different times by using enzyme-linked immunosorbent assays (ELISA) for human IL-1β (R&D Systems, Minneapolis, Minn.). This immunoassay kit allows accurate measurement of active, or mature, IL-1β in tissue culture media without appreciable error due to small amounts of pro-IL-1β. IL-1β levels were also measured after pretreatment of macrophages with peptide inhibitors of IL-1β-converting enzyme (ICE) protease, ZVAD-FMK and its inactive analog, ZFA-FMK (kindly given by Apurva Sarin and Pierre Henkart, National Institutes of Health) (12). These inhibitors were added to the macrophages 30 min before the addition of the bacteria.

RT-PCR analysis of IL-1β mRNA. Total RNA was isolated from the macrophages by the single-step guanidinium thiocyanate procedure as described previously (4). cDNA was synthesized with a StrataScript reverse transcriptase PCR (RT-PCR) kit (Stratagene) essentially as described by the manufacturer. Briefly, 6 μg of total RNA in 32 μl of diethyl pyrocarbonate-treated water was mixed with 3 μl of oligo(dT) primer (100 ng/ml) and heated at 70°C for 10 min. The samples were chilled on ice for 1 min, and then the following reagents were added: 5 μl of 10× synthesis buffer, 3 μl of 0.1 M dithiothreitol, 1 μl (20 U/ml) of RNase Block Ribonuclease Inhibitor, and 1 μl of Moloney murine leukemia virus reverse transcriptase (50 U/ml). The mixture was incubated at 42°C for 1 h, heated at 90°C for 10 min, and stored at 4°C. PCR primer pairs specific for human IL-1β and β-actin were synthesized on an ABI DNA synthesizer. cDNA was amplified in the presence of 1 mM (final concentration) 5′ and 3′ primers, deoxynucleoside triphosphates (200 μM each), and 2.5 U of *Taq* DNA polymerase (Boehringer) in a final volume of 50 μl. PCR amplifications were performed with an initial denaturation step of 2 min at 95°C and then 30 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. The reaction products were visualized by electrophoresis of 15 μl of the reaction mixture at 100 V in a 2% agarose gel in 1× Tris-acetate-EDTA buffer containing 5 μl of ethidium bromide (10 mg/ml) per 100 ml of gel solution. The specificities of the amplified bands were confirmed by their predicted sizes (IL-1β, 810 bp; β-actin, 548 bp) by running a lane containing φX174.*Hae*III DNA as molecular weight markers (MWM IX; Boehringer).

Statistical analysis. Statistical analysis was done by Student's *t* test, using the INSTAT statistical analysis package (Graph Pad Software, Inc., San Diego, Calif.). Significance was taken as $P < 0.05$.

RESULTS

Survival of *Shigella* during in vitro infection of human and murine macrophages. Preliminary experiments were done by infecting murine macrophage cell line J774 and human monocyte-derived macrophages at a MOI of 30 bacteria per cell. The characteristics of the strains used in these studies are given in Table 1. Initially, several different times of infection and gentamicin treatment were evaluated for determining an optimum protocol. A 30-min infection followed by 50 min of gentamicin treatment appeared to be generally optimal for yielding the most CFU recovered for each strain, and this regimen was subsequently used in most of the experiments described here. The infected macrophages were lysed, and the intracellular bacteria were recovered by plating on TSA plates. The results depicted in Fig. 1 are representative of at least a dozen experiments that were carried out with this protocol.

Very few CFU were recovered from macrophages infected with virulent bacteria such as M90T-W and 2457T compared to avirulent strains such as M90T-55, SC403, M90T-A3, M4243-A, and BS103. This was true of both human monocyte-

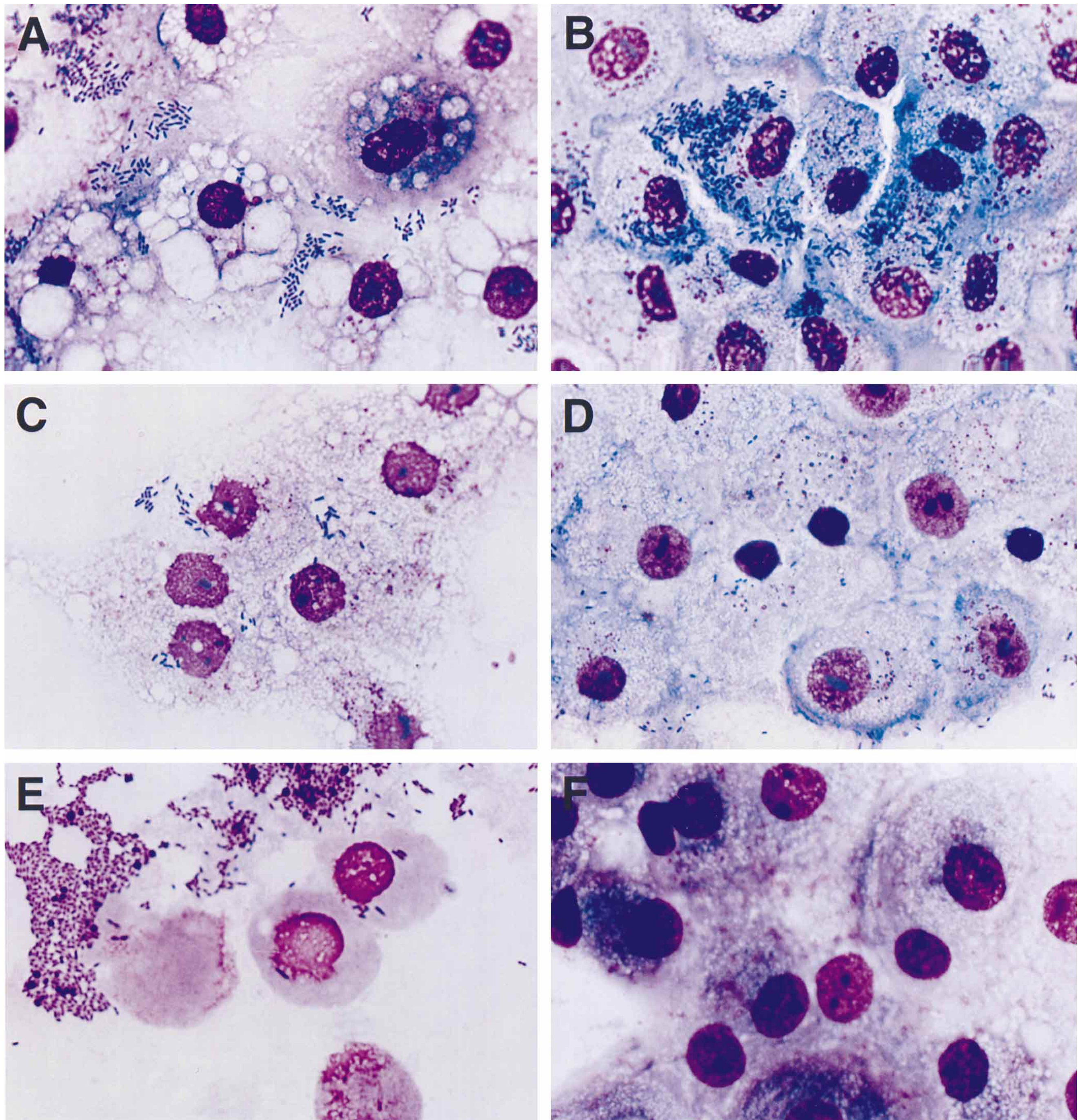

FIG. 3. Light microscopic analysis of human monocyte-derived macrophages infected with *S. flexneri* strains. Macrophages were stained with a modified Wright's stain after infection with M90T-W (A), M90T-55 (B), 2457-T (C), or M4243A (D). (E) Supernatant from 2457-T-infected macrophages; (F) noninfected macrophages. Magnification, ×89.

derived macrophages (Fig. 1A) and murine macrophage cell line J774 (Fig. 1B). However, the total CFU recovered from human macrophages after infection with avirulent strains was higher (two- to fourfold) than the amount recovered from the J774 cell line infected with the same strain at the same MOI.

The recovery of CFU with plasmid-cured avirulent strains is serotype specific. In J774 macrophages, M90T-55 infection yielded two- to threefold-higher CFU than infection with M4243A. Similarly, in human macrophages, there was a seven- to eightfold difference in yield between the two strains (Fig. 1). The serotypic difference in CFU recovery was confirmed when another plasmid-cured *S. flexneri* 2a strain, BS103, was used and compared with M90T-55 (Fig. 1). One difference between the two strains is the presence of a recently described chromosomal enterotoxin (ShET1) that is present in most *S. flexneri* 2a strains, including M4243, but absent in *S. flexneri* 5 strains such as M90T-55 (30). Whether this toxin plays a role in intracellular survival within macrophages is not known.

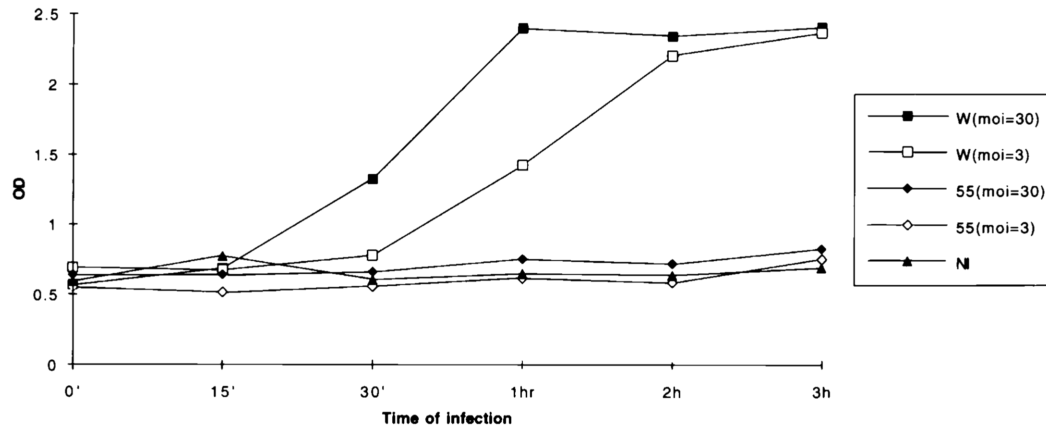

FIG. 4. Cytotoxicity measured by LDH release. M90T-W (W) and M90T-55 (55) were incubated with macrophages seeded in 24-well plates at two different MOIs (3 and 30 bacteria/macrophage). At different time periods of incubation, aliquots of the supernatant were collected and assayed for LDH release. NI, noninfected macrophages; OD, optical density.

Previous reports indicated that the *Shigella* IpaB protein, present in virulent strains, was critical for murine macrophage apoptosis (50). Infection of human macrophages with SC403, an *ipaB* mutant strain, and M90T-A3, an *S. flexneri* 5 strain containing a deletion of the *ipaBCDAR* region on the invasion plasmid, resulted in CFU recoveries that were a log higher than in M90T-W infection but a log lower than in plasmid-cured M90T-55 infection. In contrast, infection of J774 with SC403 or M90T-A3 resulted in CFU recovery that approached that obtained with M90T-55. This finding indicates that in human macrophages infected in vitro, the survival and recovery of bacteria are regulated by IpaB as well as other unidentified plasmid-encoded factors whereas in mouse macrophages, IpaB appears to play a dominant role in regulating bacterial survival. Thus, *Shigella* strains act differently in mouse and human macrophages. Since humans are the natural hosts for this pathogen, the studies described below were done with human macrophages.

To determine whether the differences in recovery of bacteria were due to differences in the initial uptake, bacteria were centrifuged onto macrophage monolayers, washed to remove unphagocytosed bacteria, and then either lysed (time zero) or further incubated with medium containing gentamicin (Fig. 2). At time zero, equal numbers of virulent and avirulent strains of *Shigella* were recovered from lysed macrophages, indicating that there was no difference in the initial uptake of strains by the macrophages (Fig. 2). However, M90T-55, and to a lesser extent M4243A, multiplied and survived within the macrophages and could be recovered even after 24 h, while M90T-W and 2457T showed a drastic reduction in recovery within 30 min of incubation (Fig. 2).

Light microscopic analysis *Shigella*-infected human macrophages. Morphological characteristics of human macrophages infected with *S. flexneri* strains were determined by staining and light microscopy (Fig. 3). Depending on the time of infection and gentamicin treatment, macrophages infected with virulent strains such as M90T-W and 2457T (Fig. 3A and C) showed an increasingly disturbed cell monolayer with highly vacuolated macrophages that appeared swollen. Many of the cells were lysed with accompanying plasma membrane disruption, and holes in the monolayer were evident. However, in the remaining cells, internalized bacteria were observed within vacuolar spaces. Additionally, the nuclei in these cells appeared intact and similar in structure to the nuclei of uninfected control cells (Fig. 3F). Although condensed nuclei were observed in a few cells in both specimens, there was no evidence of apoptosis. In contrast, macrophages infected with M90T-55 (Fig. 3B) appeared normal even though they were laden with large numbers of intravacuolated bacteria. Here too, some cells had condensed nuclei, but their appearance was otherwise normal and nonapoptotic. Differences between the two serotypes were observed. M90T-55 seemed to multiply very well inside the macrophages, and many bacteria could be seen within intact cells (Fig. 3B). In contrast, macrophages infected with M4243A contained fewer bacteria (Fig. 3D). These results correlated with the CFU recovered from lysed macrophages (Fig. 1).

To observe the morphology of the cells that were being released from the monolayers after infection, aliquots of the supernatant were taken and slides were prepared by a cytospin procedure (Fig. 3E). Supernatants from macrophages infected with 2457T showed lysed swollen cells with no clear cellular morphology. Cells had burst open, with disintegration of both cytoplasmic and nuclear contents. Large numbers of bacteria

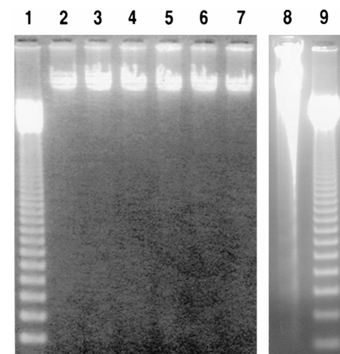

FIG. 5. DNA fragmentation assays on agarose gels. DNA was isolated from macrophages incubated with different *Shigella* strains. The DNA was electrophoresed on 1.2% agarose gels for 3 h at 100 V. DNA was isolated from macrophages infected with M90T-W (lane 2), M90T-55 (lane 3), SC403 (lane 4), 2457-T (lane 5), and M4243A (lane 6). Lane 7 represents DNA extracted from noninfected macrophages. DNA isolated from fresh human monocytes cultured for 24 h in a serum-deprived medium is represented in lane 8. Lanes 1 and 9 contain 123-bp DNA ladder molecular weight markers (Bethesda Research Laboratories, Gaithersburg, Md.).

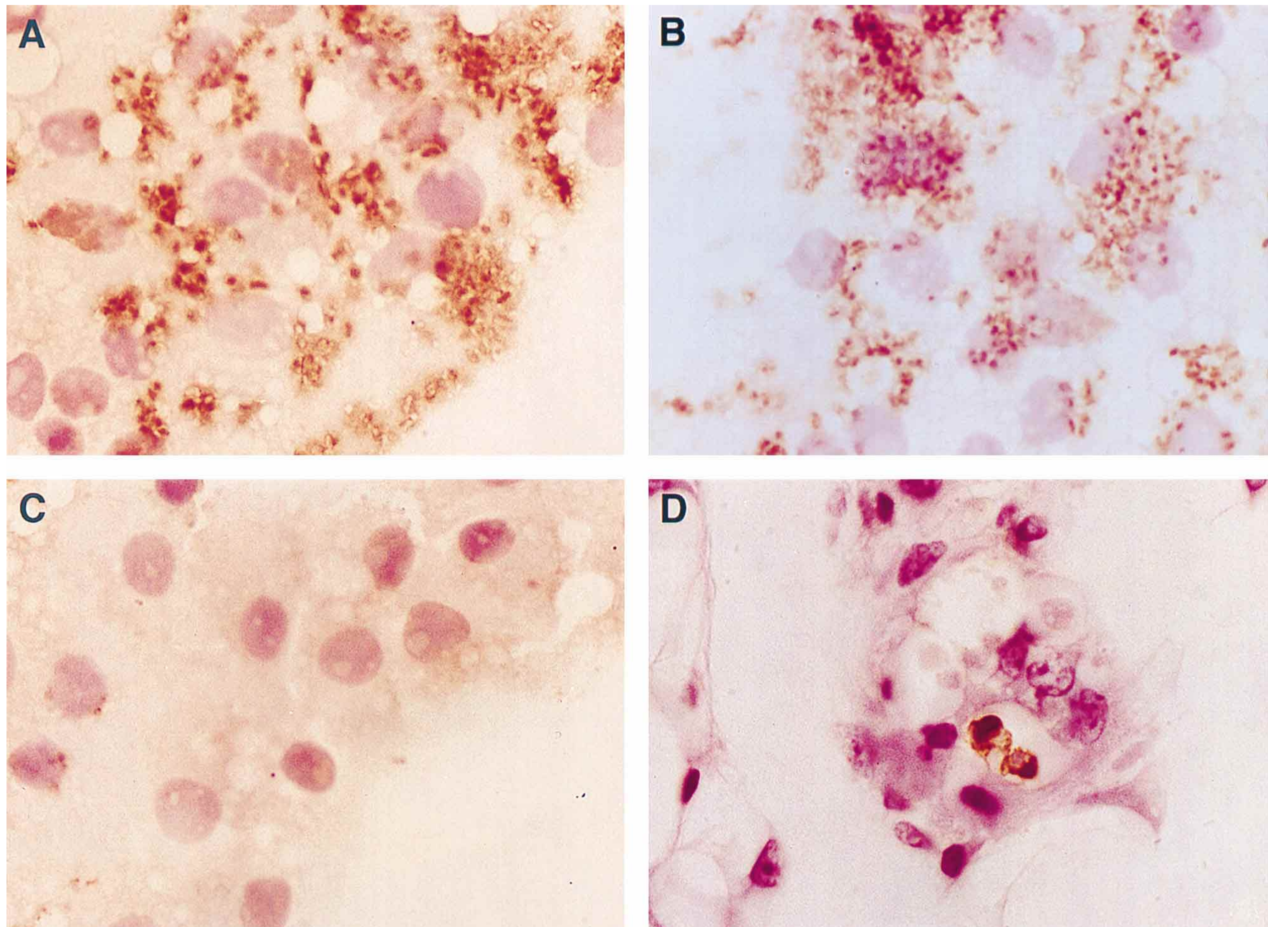

FIG. 6. In situ end labeling of DNA strand breaks (TUNEL labeling). Macrophages incubated with *Shigella* strains for 30 min were washed and subjected to TdT end labeling using an Apoptag peroxidase kit (Oncor). Macrophages were incubated with M90T-W (A) and M90T-55 (B). (C) Noninfected macrophages; (D) positive control containing mammary gland cells from a weanling mouse. The deep-brown-stained nuclei are indicative of apoptotic cells.

could be seen in the supernatant adjacent to the lysed cells, indicating their release from the macrophages (Fig. 3E). Here and there, a broken cell with intact nuclei could be observed (Fig. 3E). In such cells, no evidence of chromatin condensation and karyohexis, characteristic of apoptotic nuclei, was observed (Fig. 3E). In contrast, the supernatants of macrophages infected with M4243A showed very few cells or bacteria.

Evaluation of cytotoxicity by measurement of LDH activity. To quantitate macrophage cytolysis, cells were infected with bacterial strains at two different MOIs (3 and 30 bacteria/macrophage). At various times after infection, LDH activity was measured in the supernatant (Fig. 4). Cells infected with M90T-W showed a steady increase in LDH activity, reaching a maximal value within 1 to 2 h of infection, depending on the initial MOI. The values obtained at peak levels reflected the maximum release of LDH from lysed uninfected controls, indicating that at these time points after infection, most of the macrophages were dead. No LDH activity was detected in the supernatants of macrophages infected with M90T-55 (Fig. 4).

DNA fragmentation assays of human macrophages infected with *S. flexneri* strains. Cleavage of chromosomal DNA is a characteristic feature of apoptosis (14). Biochemically, the DNA is broken down into segments that are multiples of ~200 bp, due to specific cleavage between nucleosomes. Previous reports indicated that low-molecular-weight DNA isolated from J774 mouse macrophages infected with virulent *Shigella* generated a ladder-like nucleosomal pattern on agarose gels (51). Several different techniques to assess DNA fragmentation were used in the current study, and the results were similar (Fig. 5). DNA from human macrophages infected with virulent or avirulent *Shigella* showed no evidence of chromatin cleavage or ladder-like pattern on agarose gels (Fig. 5, lanes 2 to 7). As a positive control for the technique used, DNA isolated from fresh human monocytes cultured for 24 h in the absence of serum showed the characteristic ladder-like pattern indicative of apoptosis (Fig. 5, lane 8). The lowest bands appeared to be multiples of ~200 bp, which are characteristic of apoptosis (22). Under these conditions, human monocytes also clearly displayed the light microscopic features of apoptosis (data not shown) (22, 24).

It is now clear that some cells undergoing classic apoptosis do not show the nucleosomal ladder pattern of DNA fragmentation (43, 45, 47). Less extensive digestion of DNA, generating sizes of 50 to 300 kb, occurs in several examples of apoptosis without progressing to nucleosome-size fragments (31). In situ end labeling of DNA strand breaks is a more sensitive method to detect these limited DNA strand breaks (14). Fragmented DNA generates 3′-OH ends, which can be extended with digoxigenin-labeled nucleotides by the enzyme TdT. The incorporated label can be detected by using peroxidase-conjugated antidigoxigenin antibody (TUNEL technique). TdT labeling of human macrophage nuclei in cytospin sections after

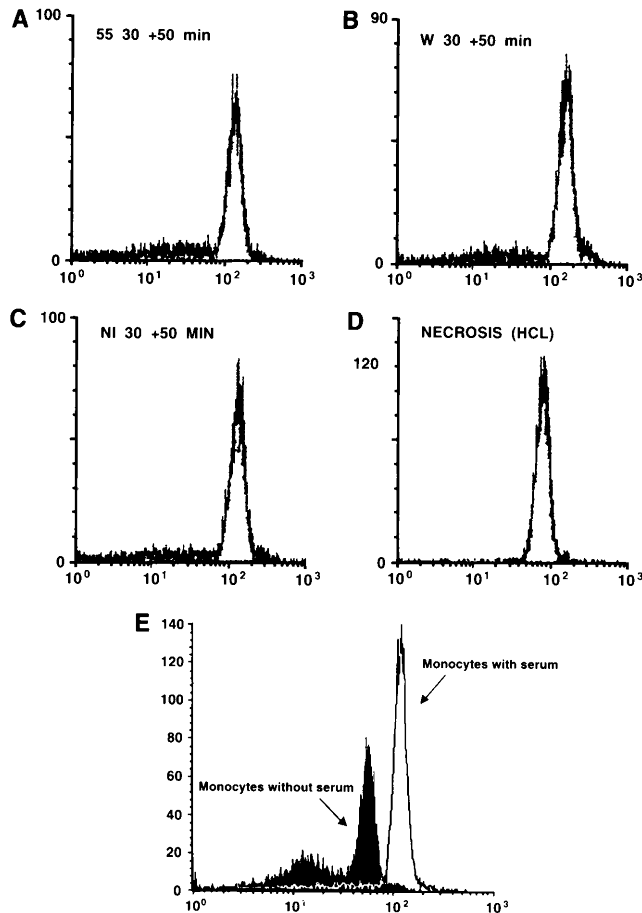

FIG. 7. FACS analysis of PI-labeled DNA. Macrophages incubated with *Shigella* strains were lysed, and the released nuclei were labeled with PI and subjected to flow cytometry. (A) M90T-55 (55); (B) M90T-W (W); (C) noninfected (NI) cells; (D) macrophages treated with HCl; (E) fresh monocytes incubated for 24 h with and without serum-containing medium.

TABLE 2. IL-1β release and effect of ICE inhibitor during *Shigella* infection

Strain	Addition (μg/ml)	IL-1β (pg/ml)	CFU[a]	% LDH release
M90T-W	None	40	520	100[b]
	ZVAD-FMK (10)	5	600	100
	ZVAK-FMK (50)	0	620	100
	ZFA-FMK (10)	48	460	100
	ZFA-FMK (50)	64	520	100
M90T-55	None	0	54×10^3	0
	ZVAD-FMK (10)	0	35×10^3	0
	ZVAK-FMK (50)	0	40×10^3	0

[a] Gentamicin-resistant CFU recovered 2 h after infection.
[b] The amount present in the supernatant of M90T-W-infected macrophages in the absence of inhibitors at the end of infection.

Shigella infection indicated no difference in the labeling patterns of cells infected with virulent and avirulent strains (Fig. 6A and B). In both cases, the nuclei remained unlabeled and showed a normal morphology similar to that of the nuclei seen in uninfected macrophages (Fig. 6C). The intracellular bacteria within the macrophages, on the other hand, stained prominently with the peroxidase label and could be easily detected in these sections. The origin or significance of this bacterial reaction is unclear at this time.

Changes in nuclear morphology were also assessed by the use of flow cytometry (fluorescence-activated cell sorter [FACS] analysis [28, 29]), using PI to stain the nuclei (Fig. 7). There was no difference in the FACS patterns of PI-labeled nuclei in cells infected with virulent strains, plasmid-cured strains, or uninfected controls (Fig. 7A to C). As a control, macrophages were treated with HCl to undergo necrosis; these cells retained normal nuclear morphology (Fig. 7D). In contrast, human monocytes induced to undergo apoptosis by culturing in a serum-deprived medium for 24 h displayed a prominent hypodiploid peak, characteristic of apoptotic nuclei (Fig. 7E).

Electron microscopy of human macrophages upon *Shigella* infection. Transmission electron microscopy of infected macrophages and serum-starved monocytes was carried out to observe detailed nuclear and cellular ultrastructure (Fig. 8). Human monocyte-derived macrophages infected with M90T-55 showed many bacteria enclosed within small and large phagocytic vacuoles. It is uncertain whether these vacuoles represent spacious phagosomes identified during *Salmonella* infection of bone marrow-derived mouse macrophages in tissue culture (1). It was clear that macrophages internalized more than one bacterium, suggesting that multiple uptake events occurred per macrophage. It was also evident that internalized cells were dividing, indicating either that the cells were internalized as such or that the bacteria after internalization were actively undergoing cell division within the confines of the phagosome (Fig. 8B). In contrast, internalized M90T-W cells were difficult to observe because few macrophages were adherent to the plate surface after infection. When available, infected cells possessed a nuclear ultrastructure indistinguishable from that of uninfected control cells (Fig. 8A and C). Lysosomal vacuoles were not readily seen, in marked contrast to mouse macrophages, where lysosomal vacuoles were often seen near phagocytic vacuoles with bacteria (unpublished observation). Occasionally, a cell infected with M90T-W was observed in an advanced stage of disintegration or necrosis (Fig. 8D). The nuclei were swollen, the chromatin was dispersed, cytoplasmic structures were obliterated, and the plasma membrane was ruptured. In contrast, freshly harvested human monocytes, starved for 24 h in a serum-free medium, underwent apoptosis as previously described (Fig. 8E and F) (22–24). In sharp contrast to nuclei in uninfected or M90T-W-infected macrophages, the nuclei contained in serum-starved monocytes were sharply condensed, the cells were highly vacuolated, yet the plasma membrane remained intact. Some of the cells showed secondary necrosis, where the cytoplasmic morphology was obliterated but the nuclei were still intact, highly condensed, and shrunken in size (Fig. 8F).

IL-1β release is not obligatory for cytolysis. Previous experiments with mouse peritoneal macrophages prestimulated with lipopolysaccharide (LPS) indicated a rapid and significant release of the biologically active precursor form of IL-1α and the precursor form of IL-1β into culture supernatants (49). Further infection of LPS-treated macrophages with M90T-W, but not its plasmid-cured derivative, resulted in the added secretion of mature IL-1β. In this study, ELISA of culture supernatants obtained from M90T-W-infected human macrophages indicated significant levels of mature IL-1β 1 to 2 h after infection (Table 2). Mature IL-1β was not detected in culture supernatants of uninfected or M90T-55-, M4243A- or SC403-infected human macrophages, confirming that the lytic phenomenon in both human and mouse macrophages, accompanied by release of mature IL-1β, was associated with the

presence of the IpaB protein on the bacterial surface. RT-PCRs were carried out to monitor the synthesis of IL-1β mRNAs. The results showed an increase in the synthesis of IL-1β mRNA upon infection of macrophages with M90T-W, M90T-55, and SC403 as well as in LPS-stimulated cells (Fig. 9). The presence of IL-1 mRNA in uninfected cells (Fig. 9, lane 4) reflects the sensitivity of IL-1 gene expression both to LPS, which is routinely found in low concentrations in tissue culture media, and to the adherence of monocytes to glass or polystyrene, which is known to trigger IL-1β gene expression (5, 42).

Results obtained with murine cells indicated that the release of IL-1 from infected macrophages is not a secondary effect of cell injury but is specific for *Shigella* infection (48). Precursor IL-1β (pro-IL-1β, 31 kDa) is cleaved proteolytically to the mature, biologically active, IL-1β (17.5 kDa) by ICE. Activation of ICE and ICE-like thiol proteases is involved in apoptosis of many neuronal and nonneuronal cells (12). We examined the effect of a specific peptide ICE inhibitor, ZVAD-FMK, on human macrophages infected with M90T-W. ZVAD-FMK has been shown to block apoptotic nuclear damage in thymocytes induced by etoposide, thapsigargin, and dexamethasone (7) and similar damage in monocytic cells induced by cycloheximide, thapsigargin, etoposide, and stauroporine (48). In cell-free systems, the concentrations of inhibitor used in these experiments blocked all apoptotic events, including morphological changes of added nuclei and internucleosomal DNA fragmentation (12). Incubation of human macrophages with ZVAD-FMK prior to infection with M90T-W blocked the subsequent appearance of IL-1β in the culture supernatants (Table 2) but did not prevent cytolysis and release of LDH into culture supernatants or improve the recovery of M90T-W after infection (Table 2). These results indicate that the cytolytic event induced by M90T-W occurred independently of IL-1β release. In other words, the release of mature IL-1β into the culture supernatant occurred as a consequence of passive leakage from lysed cells.

DISCUSSION

The activation of macrophages is essential not only for enhanced microbicidal activity during host resistance to infections but also for the regulation of inflammation and immunity during such infections (28). While the M cells of the Peyer's patch transport microorganisms, such as *Shigella*, from the intestinal lumen to the underlying lymphoid tissue, little is known about the mechanism of antigen processing and presentation within the cells of the lymphoid follicles lining the gut epithelium. Dendritic cells as well as tissue macrophages are important antigen-processing cells in this region and are believed to play major roles in the activation of T cells and in secretory IgA B-cell development (16). Which of these antigen-processing cells is critical for the presentation of *Shigella* antigens in humans is unknown.

In vitro, human monocytes can undergo two types of cell death, programmed cell death and accidental cell death, depending on the stimuli received. In medium lacking serum, cytokines, or other exogenous stimuli, human monocytes actively undergo programmed cell death or apoptosis (22–24). This is accompanied by decrease in cell size, condensation of the nucleus, and DNA fragmentation into oligosomal DNA of ~200 bp (14). When freshly harvested monocytes are allowed to adhere to tissue culture flasks in the presence of fresh or heated serum, the monocytes survive and differentiate into macrophages within several days (22). This process is further enhanced by the addition of MCSF, which is present in normal serum. Addition of LPS to serum-deprived medium greatly delays and reduces monocyte apoptosis (24, 25). Cytokines such as IL-1β and tumor necrosis factor alpha (TNF-α) are the most potent in inhibiting monocyte apoptosis, but other cytokines such as gamma interferon and certain hematopoietic growth factors such as granulocyte-macrophage colony-stimulating factor and MCSF are also able to substantially reduce monocyte cell death and DNA fragmentation (24, 25). IL-4, on the other hand, has been reported to enhance apoptosis of activated monocytes (23). Taken together, these results indicated that apoptosis in human monocytes is regulated by a complex interaction of cytokines and other stimuli. It has been suggested that in vivo, these stimuli may serve a homeostatic mechanism that regulates the level of circulating monocytes and their differentiation into tissue macrophages at sites of infection and inflammation (22–25).

In our studies, human monocytes were allowed to adhere to plastic in the presence of serum and MCSF and were exposed to bacterial LPS, producing IL-1, TNF-α, and other cytokines (this study and unpublished observations). There was no indication of IL-4 mRNA synthesis in these cells (unpublished observations). In this microenvironment, one would predict from earlier studies (22–25) that monocytes would survive, be prevented from undergoing apoptosis, become activated, and behave functionally as phagocytic macrophages. Results described here confirm this expectation, since monocyte-derived macrophages infected with *Shigella* in vitro did not reveal any of the characteristic morphological or biochemical markers that would indicate cell death due to apoptosis.

Monocytes subjected to nonphysiologic conditions such as exposure to acidic media, freeze-thaw conditions, heat, or other lethal injury undergo accidental cell death by a mechanism that occurs without concomitant DNA fragmentation (14, 21). This type of cell death is often referred to as necrosis, although some believe that necrosis does not indicate a form of cell death but refers to changes secondary to cell death (21). The term oncosis (from onkos, meaning swelling) was initially used to describe ischemic cell death in bone cells (21). In a recent review article on cell death, the authors reintroduced the term oncosis to describe cell death which did not show characteristics of apoptosis (21). Oncosis is defined in this review as a form of cell death accompanied by cellular swelling, organelle swelling, blebbing, vacuolization, increased membrane permeability, and karyolysis. The rapid killing of macrophages by virulent *Shigella* in vitro shares some of the features characteristic of oncosis (21). These features include cellular swelling accompanied by increased permeability of the plasma membrane, vacuolization, and nonspecific DNA breakdown or karyolysis. Other features of oncosis include loss of ion gradients and decreased cellular ATP levels (21), which were not measured in this study. The literature on cell death also seem to imply that "apoptosis and necrosis are part of a continuum" and that the same stimulus can cause either form of cell death under different conditions (2, 21). Both apoptosis and oncosis can lead to secondary necrosis (14, 21) as seen in this study with M90T-W-infected macrophages and starved human monocytes.

Why is it important to distinguish between these two types of cell death? If death occurred by apoptosis, as has been described for mouse macrophages, one would be inclined to study the interaction of *Shigella* proteins with host proteins such as ICE and ICE-like proteases which have been shown to initiate apoptosis in many cell systems. Activation of poly(ADP-ribose) polymerase, an endonuclease which when active leads to DNA fragmentation, would also be a process to study (20). Similarly, one would be inclined to ask whether *Shigella*-induced apoptosis in macrophages can be inhibited by the activ-

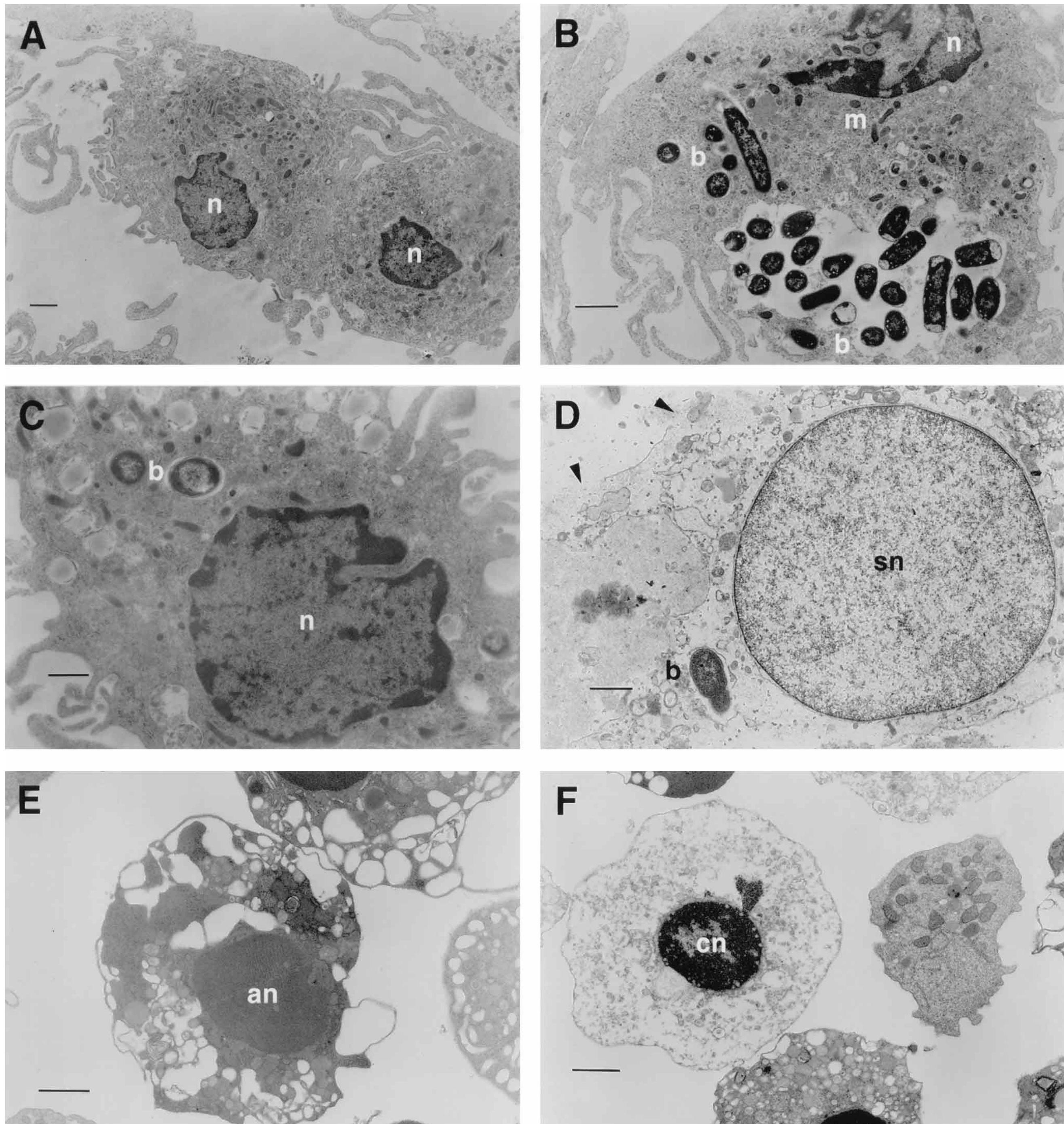

FIG. 8. Transmission electron photomicrographs of *Shigella*-infected human monocytes and macrophages. Shown are human monocyte-derived macrophages uninfected (A), infected with M90T-55 for 30 min (B), infected with M90T-W for 30 min (C), and M90T-W infected in a state of necrosis. Arrows point to ruptured plasma membrane. (E) Human monocytes cultured for 24 h in serum-free medium, showing an apoptotic cell with condensed nuclei and many vacuoles; (F) human apoptotic monocyte with condensed nuclei but undergoing secondary necrosis. Bar markers: 1 μm in panels A, B, D, E, and F; 0.5 μm in panel C. b, bacteria; n, nuclei; m, mitochondria; an, apoptotic nuclei; cn, condensed nuclei; sn, swollen nuclei.

ity of cell death-inhibiting genes such as the proto-oncogene *bcl-2*, cowpox virus *crmA*, and baculovirus *p35* (12). On the other hand, death by oncosis would mean that mechanisms distinct from those described above are in operation. For example, IpaB (and other bacterial proteins) could either act as a toxic agent leading to cellular disintegration by an unknown mechanism or interact with specific host proteins to activate cellular lysis. The mechanism of *Shigella*-induced macrophage oncosis is currently unknown and will be an important feature of future investigations. Physiologically, the rapid killing of macrophages by virulent strains of *Shigella* could be viewed as a bacterial survival strategy, permitting the pathogen to escape

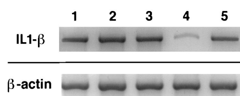

FIG. 9. RT-PCR analysis of IL-1β mRNA synthesis in human macrophages after *Shigella* infection. mRNAs specific for IL-1β and β-actin were detected after infection of macrophages with M90T-W (lane 1), M90T-55 (lane 2), SC403 (lane 3), and LPS (lane 5). Lane 4 represents noninfected macrophages.

and colonize the epithelial cells, thereby sequestering itself from host immune mechanisms. From the host point of view, the lysis of infected macrophages eliminates intracellular bacteria and/or exposes them to other bactericidal cells such as neutrophils, natural killer cells, and cytotoxic T lymphocytes (46).

Natural infection with *Shigella* is associated with the production of cytokines such as IL-1β, TNF-α, IL-1Ra, and IL-6, as seen in rectal tissues (36) and in stools (37), indicating local production in the gut. These cytokines are produced by a number of different cell types, including *Shigella*-infected epithelial cells (15). During inflammation, injury, or infection, IL-1 is a potent inducer of fever, hypotension, and shock (5), a feature that certainly contributes to safety considerations during *Shigella* vaccine trials. IL-1 enhances synthesis of IL-8, an important chemotactic cytokine whose local effects include neutrophil and macrophage emigration. In addition, it influences the activation of T cells and enhances B-cell proliferation, maturation, and immunoglobulin production (5, 42). Taken together, these effects of IL-1 play an important role physiologically and immunologically during infection and recovery from shigellosis.

IL-1Ra blocks the binding of IL-1α and IL-1β to IL-1 receptors blocking IL-1 activity (6, 9, 26). In *Shigella*-infected rabbit ileal loops, administration of IL-1Ra reduces the inflammatory response (40). Blocking IL-1 release by IL-1Ra, neutralizing antibodies, or specific inhibitors such as ZVAD-FMK might reduce the inflammatory and pyrogenic response to *Shigella* infection, desirable in a safe oral vaccine. However, the effect that this will have on the protective immune response is uncertain. Both animal and in vitro cell culture studies indicate that short-term blocking of IL-1 receptors by IL-1Ra does not appear to be immunosuppressive (6).

The physiological relevance of *Shigella*-induced macrophage lysis in vitro, however, is unclear. Maturation of human monocytes in vivo almost surely differs from their maturation in vitro. Moreover, in vivo, the expression of antigens and secretory products by macrophages within each microenvironment is normally influenced by interaction with cells of the lymphohemopoietic and neuroendocrine system as well as with fibroblasts, endothelium, and epithelium (8). These interactions give rise to the extensive heterogeneity and diversity within the macrophage population (8), influencing microbe-macrophage interaction in vitro and complicating the interpretation of observed differences in behavior between mouse and human macrophages. Nevertheless, the demonstration that virulent but not avirulent shigellae rapidly destroy human macrophages by a mechanism distinct from apoptosis should promote a mechanistic analysis of this phenomenon.

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