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The Mechanism of Cell Death in *Listeria monocytogenes*-Infected Murine Macrophages Is Distinct from Apoptosis

JOHANNES BARSIG[1] AND STEFAN H. E. KAUFMANN[1,2]*

Department of Immunology, University of Ulm, Ulm,[1] and Max-Planck Institute for Infection Biology, Berlin,[2] Germany

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Various pathogenic bacteria with the capacity to live within eukaryotic cells activate an apoptotic program in infected host cells. Induction of apoptosis by *Listeria monocytogenes* in murine dendritic cells and hepatocytes has been described. Here we address the questions of whether and how the pathogen kills macrophages, its most important habitat. Employing several complementary techniques aimed at discriminating between apoptosis and necrosis, we show that murine bone marrow-derived macrophages (BMM) undergo delayed necrosis but not apoptosis when infected with listeriolysin (Hly)-producing *L. monocytogenes*. This pathogen failed to elicit apoptotic morphology, DNA fragmentation, and surface annexin V binding of macrophages, in contrast to *Shigella flexneri* infection or gliotoxin treatment, which were used as positive controls. Furthermore, macrophages infected with *L. monocytogenes* released lower quantities of interleukin-1β (IL-1β) than did *Shigella flexneri*-infected ones, indicating diminished or even absent activation of IL-1-converting enzyme in macrophages harboring *L. monocytogenes*. We conclude that murine BMM die by necrosis after several hours of cytoplasmic replication of *L. monocytogenes*. The pathogen may benefit from this feature by the possibility of taking advantage of cells of "pseudo-healthy" appearance, thus avoiding rapid elimination by other phagocytes.

Many pathogenic bacteria have exploited the intracellular milieu as their permanent or transient habitat (5). These include *Listeria monocytogenes*, *Salmonella enterica* serovars, some mycobacteria, and *Shigella flexneri*, which have been increasingly recognized as communicating in a complex and specific way with their respective host cells, especially mononuclear phagocytes (14). *S. flexneri* and *S. enterica* serovars have been shown to induce apoptosis in murine macrophage cell lines or bone marrow-derived macrophages (BMM) (3, 5, 17, 35). Induction of apoptosis is dependent on the expression of virulence factors, e.g., IpaB in the case of *S. flexneri* (34). This protein directly interacts with the apoptotic machinery of the host cell by binding and activating interleukin-1β (IL-1β)-converting enzyme (ICE) (4, 29), an effector of both cell death and IL-1β maturation (19). Interestingly, induction of apoptotic cell death by *S. flexneri* is not seen in all cell types; e.g., epithelial-like HeLa cells infected with the pathogen do not show typical signs of apoptosis (15). Also, differences between mice and humans exist, since human macrophages seem to die by necrosis rather than apoptosis after infection with *S. flexneri* (9). It is not yet clear which features of the cells, of the bacteria, or of both are responsible for these specificities.

In contrast to the case for *S. flexneri*, for which induction of apoptosis is well documented and mechanistically elucidated, research on *L. monocytogenes* has focused on bacterial survival mechanisms rather than on the fate of the infected cell (21). To date, only a few reports have described in detail the death of *L. monocytogenes*-infected host cells, and the findings do not yet allow a consistent view. Rogers et al. observed DNA fragmentation, which is characteristic of apoptotic cell death, in primary murine hepatocytes infected with *L. monocytogenes* in vivo and in vitro (22). Another study described apoptosis caused by *L. monocytogenes* in murine dendritic cells in vitro (11). However, in contrast to *S. flexneri*, the intracellular pathogen did not induce apoptosis of macrophage-like J774 cells (35). Significantly, a mechanistic cognate of IpaB in *S. flexneri*, specifically interacting with the apoptotic machinery of the host cell, has not yet been identified in *L. monocytogenes.*

This study attempted to gain deeper insight into whether and how *L. monocytogenes* kills murine macrophages, the cells that represent the predominent compartment of bacterial multiplication during experimental listeriosis (6, 10). By applying several complementary methods, we show that murine BMM infected with *L. monocytogenes* die by necrosis without the characteristic hallmarks of apoptosis.

MATERIALS AND METHODS

BMM culture. BMM were grown from bone marrow cells of male C57BL/6 mice bred at the Animal Facilities of the University of Ulm. Femurs of hind legs were flushed with cold Dulbecco modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, glutamine and sodium pyruvate (all from Gibco, Eggenstein, Germany). This complete medium was supplemented with 30% macrophage colony-stimulating factor-containing L929 supernatant and 5% horse serum. Bone marrow cells were cultured at an initial density of 10^5/ml in Petriperm dishes (Heraeus, Hanau, Germany) at 37°C in a humidified 10% CO_2 atmosphere for 7 to 9 days. Cells were then harvested with cold phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} but with 0.5 mM EDTA. BMM were washed, resuspended in complete medium, and used at a density of 5×10^5/ml in the experiments. Cells were left untreated for at least 4 h at 37°C in 10% CO_2 prior to further handling.

Bacteria. *L. monocytogenes* EGD, the listeriolysin (Hly)-deficient *L. monocytogenes* mutant M3 harboring a Tn*916* insert in the promoter region of the *hly* gene (13) (kindly provided by W. Goebel), and *S. flexneri* (wild-type strain M90T, kindly provided by A. Zychlinsky) were grown overnight in tryptic soy broth at 37°C on a horizontal shaker. Aliquots of the bacterial suspensions were kept at −70°C.

Infection of BMM with intracellular bacteria and treatment with gliotoxin. Shortly before infection, aliquots of bacterial suspensions were thawed and adjusted to the desired density in complete medium. BMM were then infected at various multiplicities of infection (MOI) by adding the bacteria in 1/10 of the total volume to the cells. Alternatively, gliotoxin (Sigma, Deisenhofen, Germany) was diluted in complete medium from a stock in dimethyl sulfoxide and added to BMM cultures to a final concentration of 5 μM. After 1 h, complete medium containing gentamicin was added to the cultures (final concentration, 10 μg/ml) to restrict extracellular bacterial growth. BMM were further cultured at 37°C in humidified 10% CO_2.

* Corresponding author. Mailing address: Dept. of Immunology, Albert-Einstein-Allee 11, 89081 Ulm, Germany. Phone: 49-0731/5023360. Fax: 49-0731/5023367. E-mail: stefan.kaufmann@medizin.uni-ulm.de.

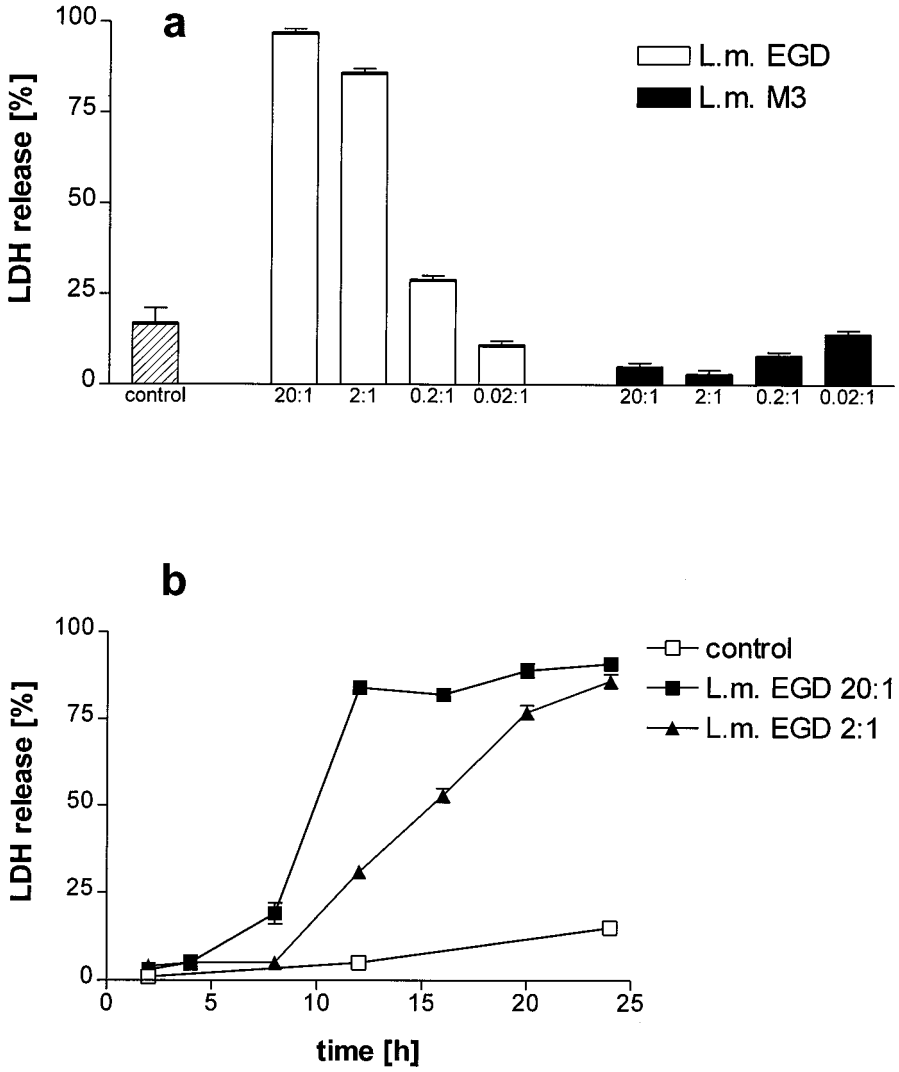

FIG. 1. Cytotoxicity of *L. monocytogenes* EGD and the Hly-deficient mutant M3 for murine BMM. BMM were seeded at a density of 5×10^4/well in 96-well plates and infected with *L. monocytogenes* (L.m.) EGD or the Hly-deficient mutant M3 at the indicated MOI. After 24 h (a) or at the indicated time points (b), the LDH contents in culture supernatants and the remaining cell layers were determined, and the relative LDH release was calculated. Data are means ± standard deviations ($n = 3$) from one representative experiment of two. Missing error bars are within symbols.

Macrophage cytotoxicity assay. Cytotoxicity was determined by measuring the relative release of the cytosolic enzyme lactate dehydrogenase (LDH) from macrophages into culture supernatants. At various time points, the supernatants of BMM were harvested and substituted for the same volume of fresh complete medium supplemented with 0.1% Triton X-100. After 30 min, cell lysates and supernatants were analyzed for their LDH content by using a kit purchased from Promega (Madison, Wis.) according to the protocol of the manufacturer. Absorbance values at 490 nm were determined photometrically with a 96-well plate reader. The relative LDH release for each well was calculated as follows, where OD is optical density at 490 nm: $[OD_{supernatant}/(OD_{supernatant} + OD_{cell\ lysate})] \times 100\%$.

Morphologic analysis of BMM. For visualizing the nuclear morphology, BMM grown on glass chamber slides (Nunc, Wiesbaden-Biebrich, Germany) were incubated with 1 μg of Hoechst 33342 dye (Sigma) per ml in PBS at 37°C for 15 min. Cells were then washed once with PBS and fixed with 1% paraformaldehyde in PBS for at least 15 min. Nuclei were visualized and photographed with an Olympus BH-2 fluorescence microscope with a UV filter.

Detection of histone-associated cytoplasmic DNA fragments in dying BMM. The cytoplasmic appearance of fragmented DNA was assessed by using the ELISA^{plus} (Boehringer, Mannheim, Germany) cell death detection enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer. Briefly, BMM seeded in 96-well tissue culture dishes (5×10^4/well) were lysed at various time points with 200 μl of the lysis buffer supplied with the kit. After 30 min, lysates were cleared by centrifugation at 14,000 × *g* for 10 min, and supernatants (150 μl) were stored at −20°C until determination of histone-associated DNA fragments with the cell death detection ELISA. An aliquot corresponding to 5×10^3 cells was used for one determination by ELISA.

Annexin V binding and propidium iodide staining of BMM. BMM were seeded in 24-well low-attachment tissue culture plates (Costar, Bodenheim, Germany) at a density of 2.5×10^5/ml with 500 μl per well. At various time points after infection or after treatment with gliotoxin, cells were harvested and stained with fluorescein isothiocyanate (FITC)-labeled annexin V and propidium iodide (Clontech Laboratories, Palo Alto, Calif.) according to the supplier's protocol. Fluorescence was analyzed with a Becton Dickinson three-color fluorescence-activated cell sorter (FACS).

IL-1 release from BMM infected with *L. monocytogenes* EGD or *S. flexneri*. BMM were seeded on 96-well tissue culture dishes. At various time points after infection with *L. monocytogenes* EGD or *S. flexneri*, supernatants were collected and stored at −20°C. Concentrations of IL-1α and IL-1β in these samples were determined by using ELISA kits purchased from Endogen (Woburn, Mass.).

Statistics. Data are given as means ± standard deviations, and differences were tested by using the two-sided Student *t* test. Means were considered significantly different from each other when the *P* value was <0.05.

RESULTS

Hly-producing *L. monocytogenes* EGD efficiently kills infected BMM. Infection of murine BMM in vitro with wild-type

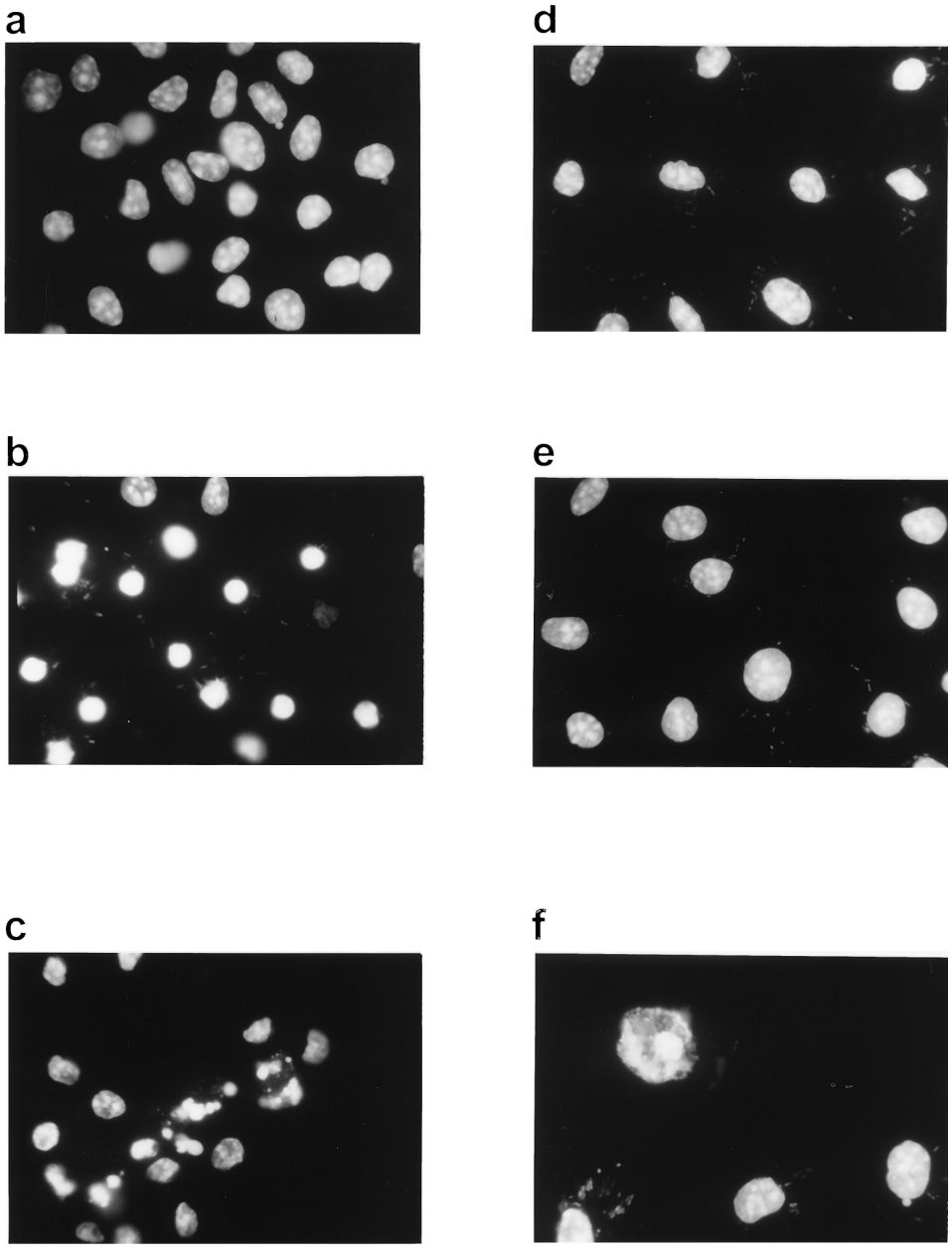

FIG. 2. Nuclei of BMM after infection with *L. monocytogenes* EGD or *S. flexneri* or after treatment with gliotoxin. At different time points after infection with *L. monocytogenes* EGD or *S. flexneri* (MOI, 20:1) or after treatment with gliotoxin (5 μM), BMM grown on glass chamber slides were stained with Hoechst 33342 dye. Nuclei were visualized with a UV fluorescence microscope (magnification, ×740). (a) Untreated BMM; (b) BMM infected with *S. flexneri* (20:1) for 3 h; (c) BMM treated with gliotoxin (5 μM) for 3 h; (d to f) BMM at 1 (d), 3 (e), and 8 (f) h p.i. with *L. monocytogenes* (20:1).

L. monocytogenes EGD even at an MOI as low as 2:1 resulted in almost complete destruction of the cells within 24 h as assessed by LDH release into culture supernatants (Fig. 1a). In contrast, the Hly-deficient mutant *L. monocytogenes* M3 was totally noncytotoxic to BMM under identical conditions (Fig. 1a). At higher MOI (20:1 and 2:1) these mutants even reduced the spontaneous LDH release ($P < 0.05$) observed in cultures of untreated control cells. Lysis of *L. monocytogenes* EGD-infected macrophages (MOI, 20:1) started between 4 and 8 h postinfection (p.i.) and was maximal from 12 h on (Fig. 1b). These data demonstrate a strictly Hly-dependent cytotoxicity of *L. monocytogenes* EGD towards murine BMM.

***L. monocytogenes* EGD does not induce apoptotic nuclear morphology in infected BMM, in contrast to known apoptotic stimuli.** In order to unravel the mode of macrophage death induced by wild-type *L. monocytogenes* EGD, we further investigated the cellular destruction of BMM by several methods aimed to discriminate between apoptosis and necrosis. Previously reported apoptotic stimuli for murine macrophages, i.e., infection with wild-type *S. flexneri* (strain M90T) (35) and the NF-κB inhibitor gliotoxin (20, 32), were used as positive controls.

Photomicrographic examination of Hoechst 33342-stained nuclei of BMM at different time points after infection with *L.*

monocytogenes EGD (MOI, 20:1) did not reveal a higher percentage of chromatin condensation or nuclear fragmentation than that in uninfected controls (Fig. 2d to f versus a). Cells remained attached to the surfaces of the culture wells but were obviously destroyed by intracellular listeriae at later time points (≥8 h) (Fig. 2f), in line with the results of LDH determinations in a parallel experiment (Fig. 1b). In contrast, nuclei of *S. flexneri*-infected (MOI, 20:1) and (even more pronounced) gliotoxin-treated BMM rapidly underwent shrinkage and/or fragmentation (Fig. 2b and c). Fragmentation of nuclei was more often observed in gliotoxin-treated cultures, while condensed and shrunk nuclei prevailed after infection with *S. flexneri*. Cells massively detached from the surfaces of the chamber slides (particularly during the staining process), so that pictures of stained BMM could not be taken later than 3 h p.i. with *S. flexneri* or after addition of gliotoxin.

***L. monocytogenes* EGD does not induce DNA fragmentation of infected BMM.** One of the biochemical hallmarks of apoptosis is fragmentation of chromatin in the nucleus and the appearance of nucleosomal fragments in the cytosol of the dying cell. We used an ELISA to examine the characteristic DNA fragmentation in BMM after infection with either *L. monocytogenes* EGD or *S. flexneri* or after treatment with gliotoxin. Both known inducers of apoptosis, *S. flexneri* and gliotoxin, elicited early DNA fragmentation prior to (gliotoxin) or concomitant with (*S. flexneri*) the LDH release. In contrast, *L. monocytogenes* EGD infection failed to induce the appearance of cytoplasmic DNA fragments throughout the observation period (Fig. 3). Additionally, we did not detect any DNA laddering when DNA from *L. monocytogenes* EGD-infected BMM was analyzed on agarose gels (data not shown). These results further verify that *L. monocytogenes* EGD efficiently kills murine BMM without inducing apoptosis.

***L. monocytogenes* EGD-infected macrophages do not express phosphatidylserine on the cell surface.** Recently, it has been recognized that apoptotic but not necrotic cells redistribute phosphatidylserine from the inside to the outside of the cell membrane independently of the stimuli used (16). This is an early process during apoptosis that may physiologically serve as a signal for phagocytes to remove the dying cell before it lyses and elicits potentially harmful inflammatory reactions (26). We used FITC-conjugated annexin V, with high affinity for phosphatidylserine, to detect by FACS analysis apoptotic BMM after infection with *L. monocytogenes* EGD or *S. flexneri* or after treatment with gliotoxin. Within <8 h, infection with *S. flexneri* and (more pronounced) treatment with gliotoxin induced redistribution of phosphatidylserine to the cell surface of BMM (Fig. 4). This effect was not seen when cells had been infected with *L. monocytogenes* EGD. Furthermore, 8 h after addition of listeriae to macrophage cultures, a significant proportion of the cells appeared to be propidium iodide accessible, which is indicative of necrotic ruptures of plasma membranes. This finding corroborates our suggestion that infection with *L. monocytogenes* EGD kills murine BMM in a necrotic rather than an apoptotic manner.

Differential release of IL-1α and IL-1β by BMM infected with *L. monocytogenes* EGD and *S. flexneri*. It has been shown by Zychlinsky and coworkers (4, 33) that *S. flexneri* organisms secrete IpaB as a virulence factor within infected macrophages; IpaB binds and activates ICE, a protease implicated in apoptosis as well as release of mature IL-1β. Moreover, it has been demonstrated that production of IL-1 and the subsequent inflammatory response at the site of infection are central steps in the initiation of the histopathological symptoms of experimental shigellosis (23). Since in the case of *S. flexneri*, initiation of apoptosis is associated with IL-1β production by infected macrophages, we compared the IL-1 release by *L. monocytogenes* EGD- and *S. flexneri*-infected BMM. *S. flexneri* and *L. monocytogenes* EGD induced similar levels of IL-1α (Fig. 5a), which is not a substrate of ICE, while *S. flexneri* elicited significantly higher levels of IL-1β ($P < 0.01$), the substrate of ICE (Fig.

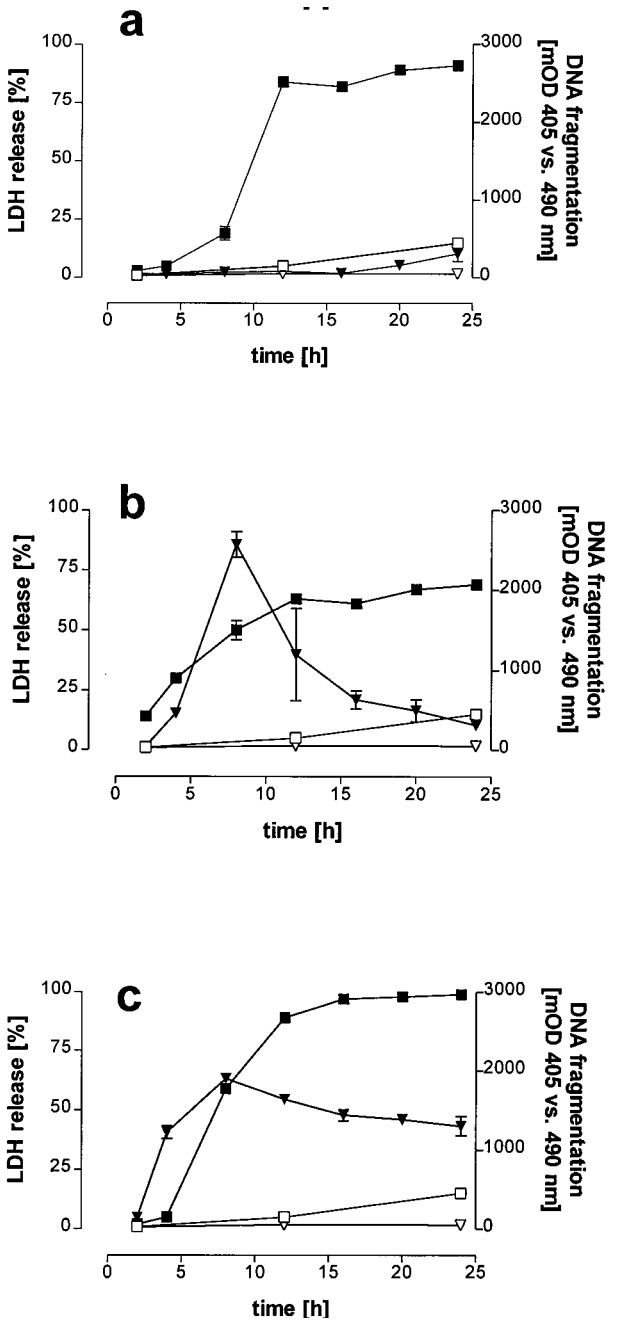

FIG. 3. LDH release from and DNA fragmentation of BMM infected with *L. monocytogenes* EGD or *S. flexneri* or treated with gliotoxin. BMM were seeded at a density of 5×10^4/well in 96-well plates. At 0 h, cells were infected with bacteria (*L. monocytogenes* [a] or *S. flexneri* [b]) (MOI, 20:1) or treated with gliotoxin (5 μM) (c). At the indicated time points, the LDH contents in culture supernatants and the remaining cell layers were determined, and the relative LDH release was calculated (squares). Parallel cultures were harvested for determination of DNA fragmentation with the Boehringer cell death detection ELISA (triangles). Closed symbols, infected or gliotoxin-treated cultures; open symbols, control cultures. Data are means ± standard deviations ($n = 3$) from one representative experiment of two. Missing error bars are within symbols.

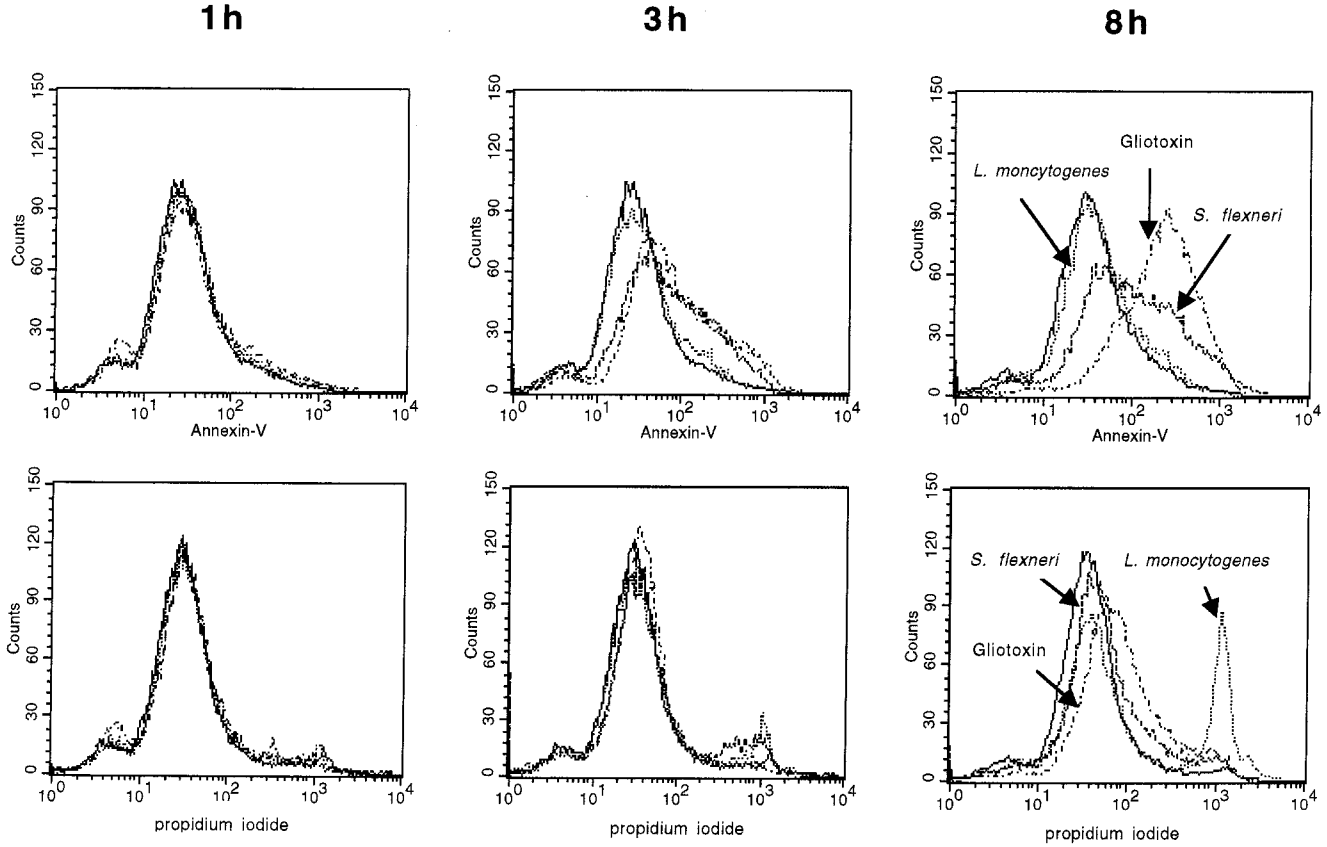

FIG. 4. Annexin V and propidium iodide staining of BMM infected with *L. monocytogenes* EGD or *S. flexneri* or treated with gliotoxin. BMM were seeded at a density of 2.5×10^5/ml in 500 μl per well in 24-well low-attachment plates and infected with bacteria or treated with gliotoxin. At the indicated time points, cells were stained with propidium iodide or FITC-conjugated annexin V. Staining was analyzed by FACS on a Becton Dickinson three-color flow cytometer. Data from one of two similar experiments are shown. Solid lines, untreated control cells; dotted lines, BMM infected with *L. monocytogenes* EGD (MOI, 20:1); dashed lines, BMM infected with *S. flexneri* (MOI, 20:1); dashed and dotted lines, BMM treated with gliotoxin (5 μM).

5b). The time courses of IL-1α production after infection with *L. monocytogenes* EGD and *S. flexneri* generally paralleled that of LDH release (after membrane rupture) from the cells (cf. Fig. 2a and b), indicating the possible liberation of membrane-bound IL-1α from lysed cells (2). The differential IL-1β-releasing capacities of *S. flexneri* and *L. monocytogenes* EGD was most prominent at early time points after infection (between 4 and 8 h p.i.), before massive cell membrane destruction (Fig. 1b and 3). Since the IL-1β ELISA used may detect premature IL-1β along with the processed form, it is possible that the IL-1β induced by *L. monocytogenes* at later time points (12 h) was of the unprocessed 26-kDa form derived from storage of destroyed cells. This differential capacity of *S. flexneri* and *L. monocytogenes* EGD to induce IL-1β release from murine BMM argues against actively induced apoptosis in these cells via ICE activation by listeriae.

DISCUSSION

Our data argue against induction of apoptosis in murine BMM by *L. monocytogenes*. In contrast, this pathogen apparently kills macrophages by delayed necrosis after multiplication in the cytosol (8, 28). Destruction of infected macrophages is strictly dependent on expression of Hly (Fig. 1), the major virulence factor of *L. monocytogenes* (21). This finding is in line with previous observations for *L. monocytogenes*-infected murine dendritic cells (11), although there are apparent differences in the mode of cell death induced (necrosis here versus apoptosis in the previous report [11]).

Although most of our experiments were conducted at an *L. monocytogenes* MOI of 20:1, signs of apoptosis were also not observed at a lower but still cytotoxic MOI, i.e., 2:1 (not shown). This is of particular relevance, since it has been shown that high MOI of *S. enterica* prompted more macrophages to undergo rapid necrosis, which might mask apoptosis (3).

Future work will determine whether *L. monocytogenes* specifically blocks or circumvents apoptosis in macrophages to reach its goal. There are several, not mutually exclusive, explanations for the occurrence of necrosis but not apoptosis of *L. monocytogenes*-infected BMM. (i) Although this is unlikely, *L. monocytogenes* might produce one or more factors that specifically block apoptosis. In preliminary experiments, however, we did not observe blocking of gliotoxin- or *S. flexneri*-induced apoptosis by preinfection with listeriae (data not shown). (ii) *L. monocytogenes* might lack a specific apoptosis inducer, such as IpaB expressed by virulent *S. flexneri* (4), and the mere presence of listeriae in the cytosol might be an insufficient trigger for induction of apoptosis. This is plausible, because hemolysin-producing cytosolic *S. flexneri* lacking IpaB is incapable of prompting murine macrophages to undergo apoptosis (34). (iii) Infection by *L. monocytogenes* might stimulate the macrophage to produce mediators that promote survival of the cell. This last assumption is favored by several observations. BMM infected with *Leishmania donovani* are

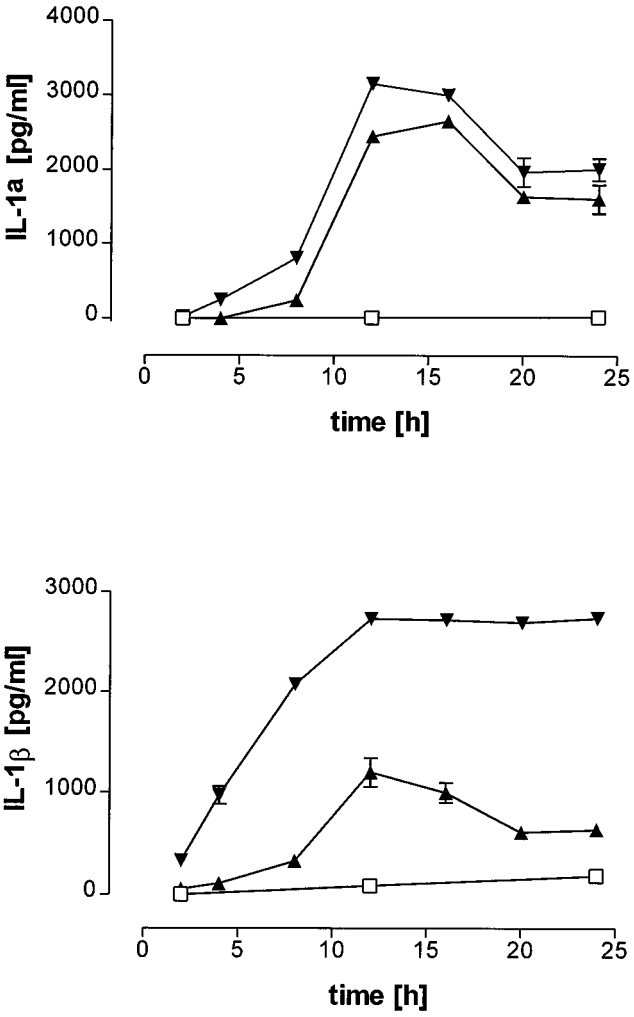

FIG. 5. IL-1α and IL-1β release from BMM infected with *L. monocytogenes* EGD or *S. flexneri*. BMM were seeded at a density of 5×10^4/well in 96-well plates. At 0 h, cells were infected with bacteria. At the indicated time points, supernatants were analyzed for their IL-1α (a) and IL-1β (b) contents by ELISA. □, control; ▲, *L. monocytogenes* EGD (MOI, 20:1); ▼, *S. flexneri* (MOI, 20:1). Data are means ± standard deviations ($n = 3$) from one experiment. Missing error bars are within symbols.

protected from apoptosis caused by removal of growth factor (18). Granulocyte-macrophage colony-stimulating factor and tumor necrosis factor alpha (TNF-α) produced by the macrophage itself upon infection have been thought to be partly responsible for this effect. Moreover, we observed that the nonhemolytic mutant *L. monocytogenes* M3 protected BMM from the eventual cell death seen in control cultures deprived of macrophage colony-stimulating factor for 24 h (Fig. 1a). This could be caused by the initial production by BMM of a cytokine, e.g., TNF-α, that is induced by Hly-producing as well as non-Hly-producing strains of *L. monocytogenes* (31). A critical role for TNF-α in this context is suggestive, since this cytokine has antiapoptotic properties in murine macrophages via activation of NF-κB (1).

In any case, differences exist between murine BMM on the one hand and murine dendritic cells (11) and hepatocytes (22) on the other, where apoptosis after infection with *L. monocytogenes* was observed. Given that *L. monocytogenes*-induced necrotic cell death is not restricted to BMM but is also a characteristic feature of other murine macrophage populations, e.g., Kupffer cells or splenic macrophages, different host cells thus seem to die in unique ways. At present we can only speculate on the reasons for these differences. Time course analysis of the LDH release from *L. monocytogenes*-infected BMM revealed that the cells remained intact for several hours (Fig. 1b and 2d to f). In contrast, infection with *S. flexneri* causes more rapid destruction of macrophages (Fig. 2b and 3b) (35). Considering that either the host cell or the pathogen may profit from the respective form of cell death, it is tempting to assume that *L. monocytogenes* does not rapidly induce apoptosis in BMM because the pathogen depends on the intact macrophage to efficiently multiply in its cytoplasm, undetectable by other phagocytes. This suggestion is further supported by the existence of another virulence mechanism of *L. monocytogenes* in macrophages, which depends on intact cells, i.e., intercellular spreading (30). *S. flexneri* also has the capacity to spread between epithelial cells (24), but, unlike macrophages, the pathogen does not kill these cells by apoptosis (15). The survival strategies of *S. flexneri* thus seem to be different in macrophages and epithelial cells: the latter cell type is used as permanent habitat, whereas infected macrophages are instantly eliminated by IpaB-induced apoptosis, thereby making use of the concomitant IL-1-induced inflammatory response in the intestinal epithelium, which allows the pathogen to cross this barrier (23). *S. flexneri*, therefore, appears to infect murine macrophages only transiently and hence is less dependent on the integrity of this host cell than *L. monocytogenes*, which uses macrophages as a more permanent niche of intracellular living.

It remains to be established whether the microbe or the host preferentially benefits from differential sensitivity to cell death. *L. monocytogenes* is highly susceptible to killing by activated macrophages but can survive inside hepatocytes. It has been suggested that apoptosis of heavily infected hepatocytes with simultaneous production of chemoattractants may benefit the host by promoting the influx of inflammatory phagocytes which rapidly kill the bacilli in the liver (22, 36). In this view, selective apoptosis of hepatocytes and resistance to early apoptosis of macrophages would represent a successful host defense strategy.

The different fates of macrophages infected with *L. monocytogenes* or *S. flexneri* may also provide an explanation as to why the risk of developing listeriosis is low for humans (7, 12, 27). In contrast to shigellae, which frequently cause bacillary dysentery (25), *L. monocytogenes* generally induces only transient colonization in the immunocompetent host, with eradication of the bacteria after several days of mild disease or even clinically inapparent infection.

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