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Research Article

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Rapid Publication

Interleukin 1 Is Released by Murine Macrophages during Apoptosis Induced by *Shigella flexneri*

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Abstract

Peritoneal macrophages undergoing apoptosis induced by *Shigella flexneri* infection release the inflammatory cytokine interleukin 1 (IL-1), but not IL-6 or tumor necrosis factor α (TNFα). Wild type shigella causes a very fast and significant release of IL-1 from prestimulated peritoneal macrophages, before the cell's integrity is compromised. Both IL-1α and IL-1β are released, IL-1β in its mature processed form. IL-1 is released from presynthesized cytoplasmic pools. These results demonstrate that bacteria-induced apoptosis of macrophages may play an active role in vivo by releasing IL-1, which in turn mediates an early inflammatory response in epithelial tissues. (*J. Clin. Invest.* 1994. 94:1328–1332.) Key words: inflammation • cell death • enterobacteria • colon • infection

Introduction

Shigella flexneri, a gram-negative enterobacterium, is the etiological agent of bacillary dysentery. It causes disease by invading the epithelial layer and the lamina propria of the colon, generating a very severe inflammation (1). Shigella invades the colon by translocating through M cells (2) from the lumen of the intestine directly into lymphoid follicles and subsequently to the submucosa. Once in the lymphoid follicles and in the lamina propria, shigella infects tissue macrophages. We have recently shown that invasive strains of shigella induce macrophage apoptosis (3). Here we address the question whether *S. flexneri*–infected macrophages that are undergoing apoptosis can liberate cytokines capable of mediating an inflammatory process.

Macrophages produce, in response to a variety of stimuli, three proinflammatory cytokines: IL-1, IL-6, and TNFα. IL-6 and TNFα are secreted soon after synthesis. In contrast, both the precursor forms of IL-1α and IL-1β are accumulated in the cell's cytosol (4). Their proteolytically processed forms are found in culture supernatants, but not intracellularly. Since IL-1 does not have a signal peptide and is not secreted via a classical pathway, it has been suggested that this cytokine is released during apoptosis (5).

Colonic macrophages contain IL-1 (6–8). As a model system to study the release of cytokines after *S. flexneri* infection, we used LPS-stimulated murine peritoneal macrophages, which contain pools of IL-1. We showed that stimulated peritoneal macrophages infected with wild-type *S. flexneri,* but not with a noninvasive derivative, release large amounts of IL-1, but not IL-6 or TNFα. Both IL-1α and IL-1β are liberated, but only IL-1β is released in its mature form. We also demonstrated that wild-type shigella cannot induce the de novo production of cytokines and can only release presynthesized IL-1. Our results indicate that macrophage apoptosis is likely to play an essential physiological role in the course of shigellosis, namely signaling the onset of inflammation.

Methods

Bacteria. The wild-type serotype 5 *S. flexneri* strain M90T and its plasmid-cured, noninvasive, noncytotoxic derivative BS176 were previously described (3). Bacteria were grown at 37°C overnight in trypto soy broth, subcultured, washed in macrophage culture medium, and adjusted to the appropriate concentration prior to infection.

Peritoneal macrophages and infection. Male Swiss mice (6–8 wk old) (Elevage Janvier, Le Genest St. Isle, France) were injected with 1 ml i.p. of thioglycollate medium (BioMérieux, Marcy l'Etoile, France) 4 d before they were killed. Cells were recovered by peritoneal lavage with cold PBS, washed three times in cold RPMI-1640 medium (Flow Laboratories, Irvine, Ayrshire, UK), resuspended to 10^6 cells/ml, and allowed to adhere to plastic plates in culture medium (RPMI-1640 containing 10% FCS [Gibco-BRL, Eragny, France], 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM L-glutamine). After 90 min incubation, the adherent cells were washed three times with culture medium and, when indicated, stimulated with 1 μg/ml of purified *Escherichia coli* 0111:B4 LPS (Sigma Chemical Co., St. Louis MO). Macrophages were next washed once with culture medium, infected with *S. flexneri*, or incubated in the indicated conditions, and the plates were centrifuged at 700 *g* for 10 min. The supernatant of the infected macrophages was collected and fresh culture medium replenished to the cell culture every 15 min for the first hour and every 30 min thereafter. For IL-6 and TNFα assays, the culture medium was left in contact with the cells for periods of 60 min after the first hour. After the appropriate incubation time, the supernatant was recovered, filtered through a 0.2 μm membrane (Sartorius, Göttingen, Germany), aliquoted, and frozen immediately. The multiplicity of infection was 100 bacteria per macro-

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phage. Macrophages were incubated at 37°C and 5% CO_2 at all times. All experiments were repeated at least four times.

IL-1 bioassay. IL-1 activity was measured as described (9). Essentially, thymocytes from C3H/HeJ mice (CSEAL, Ardenay, France) were cultured for 72 h at 37°C in RPMI-1640 containing 4% FCS, 2-mercaptoethanol (5×10^{-5}M), 0.1 μg of concanavalin A (ICN, Lisle, IL), and, when appropriate, macrophage culture supernatant. Cell proliferation was monitored by pulsing the cells with [^{3}H]thymidine for the last 7 h of culture. The results are expressed in cpm and represent the means of triplicate cultures. In antibody inhibition experiments, macrophage culture supernatants were incubated with the anti-murine IL-1α and IL-1β antibodies (British Biotechnology, Abingdon, UK) for 1 h before addition to the thymocyte culture at the indicated concentrations.

IL-6 bioassay. IL-6 activity was determined using the specific 7TD1 IL-6-dependent cell line as described (10). In brief, cells were cultured in RPMI-1640 medium supplemented with antibiotics, 2-mercaptoethanol (5×10^{-5}M), and 10% FCS in the presence of serial dilutions of culture supernatants. After 4 d of culture, the cell proliferation was monitored using the 3(4,5-dimethyl-thiazoyl-2-yl)2,5 diphenyltetrazolium salt (MTT) dye method salts and measured with a microELISA automated reader at 540 nm. 1 U of IL-6 corresponds to the half-maximum growth of 7TD1 cells.

TNFα bioassay. Essentially as described in (11), 3×10^4 L929 fibroblasts were cultured overnight in RPMI-1640 10% FCS in 96-well plates. Serial dilutions of culture supernatants were added to the fibroblasts in the presence of actinomycin D (2 μg/ml), and after 18 h incubation, the cells were stained with crystal violet. The microtiter plates were rinsed and the cells were solubilized in 1% SDS. Dye uptake was measured at 540 nm with a microELISA automated reader. 1 U of TNFα activity was defined as the amount required to lyse 50% of L929 target cells.

Cytotoxicity assay. Lactate dehydrogenase (LDH)[1] activity was measured in culture supernatants using the colorimetric Cytotox96 kit (Promega Corp., Madison, WI). Cytotoxicity was calculated as 100× (experimental release − spontaneous release)/(total release − spontaneous release). Spontaneous release was defined as the LDH activity in supernatants of macrophages incubated in medium alone, while LDH activity from macrophage lysates was defined as total release. *S. flexneri* strains have no colorimetric activity by themselves in this assay.

Antibodies, immunoblot analysis and ELISA. Macrophage cell lysates or culture supernatant equivalent to 2×10^4 cells were resolved in SDS-PAGE, transferred to nitrocellulose and incubated with anti-murine IL-1α or IL-1β polyclonal antibodies (British Biotechnology, UK) at a 1:100 dilution. After washing, the blots were further incubated with alkaline phosphatase-labeled secondary antibodies (Nordic Immunology, Tilburg, The Netherlands), revealed with the Western blotting detection kit ECL (Amersham Life Sciences, Amersham, UK), and exposed to X-Omat x-ray films.

IL-1α was quantified with the Intertest-1αx mouse ELISA Kit (Genzyme Corp., Cambridge MA). IL-6 and TNFα were assayed with the appropriate murine ELISA kit (Endogen Inc., Boston MA).

Results

IL-1 release was assayed during *S. flexneri*-induced apoptosis. Peritoneal macrophages were stimulated for 18 h with purified *E. coli* LPS, washed, and infected with either the cytotoxic *S. flexneri* strain M90T or its noncytotoxic, plasmidless derivative BS176. The culture supernatants were assayed for IL-1 bioactivity, as described in Methods.

As early as 15 min after infection of LPS-stimulated macrophages with *S. flexneri* M90T, there was strong IL-1 activity in culture supernatants (Fig. 1). The release of IL-1 peaked in the period between 30 and 45 min postinfection, during which macrophages infected with the wild type strain released eightfold more IL-1 activity than those infected with the nonpathogenic *S. flexneri* derivative. The liberated IL-1 decreased thereafter, reaching control levels about 150 min postinfection (data not shown). The release of IL-1 by BS176 was very similar to background at all points throughout the experiment.

As controls, peritoneal macrophages were either restimulated with LPS or incubated with medium alone. Under neither of these conditions was there substantial IL-1 activity released into the supernatants throughout the experiment (data not shown).

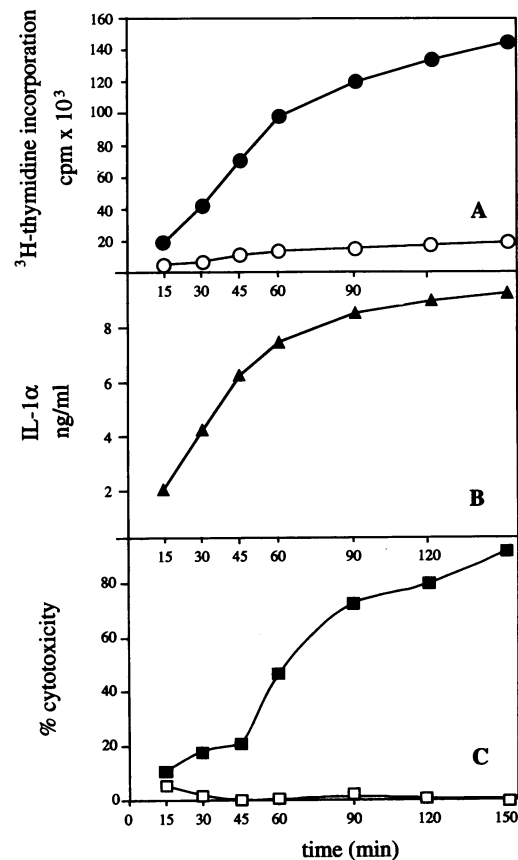

Figure 1. (*A*) IL-1 biological activity in culture supernatants of peritoneal macrophages that were infected with the *S. flexneri* wild-type strain M90T (●) or its noninvasive derivative BS176 (○) 18 h after LPS stimulation. IL-1 biological activity was measured as described in Methods from a 1:10 dilution of culture supernatants. IL-1 is presented as the cumulative activity in supernatants collected every 15 min for the first hour and every 30 min thereafter. Results represent an average of triplicates. The background level of the bioassay was 2537 cpm. (*B*) Cumulative IL-1α present in culture supernatants of peritoneal macrophages infected with the *S. flexneri* wild-type strain M90T 18 h after LPS stimulation (▲). IL-1α was measured using an ELISA as described in Methods. IL-1 is presented as the cumulative activity in supernatants collected every 15 min for the first hour and every 30 min thereafter. (*C*) Cytotoxicity of M90T and BS176 on 18-h LPS-stimulated peritoneal macrophages. Time course of LDH release of the wild-type strain M90T (■) and the noninvasive derivative BS176 (□). These data are representative of a minimum of three experiments.

1. *Abbreviations used in this paper:* ICE, IL-1β converting enzyme activity; LDH, lactate dehydrogenase.

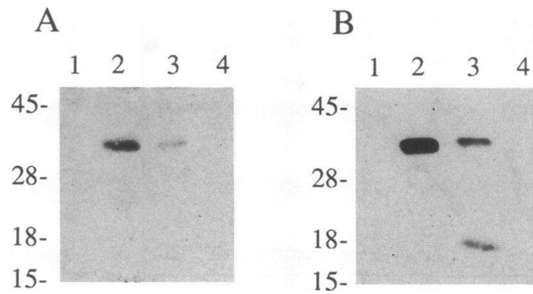

Figure 2. Immunoblot of macrophage cell lysates or culture supernatants with either anti–murine IL-1α or IL-1β antibodies. (*A*) anti-IL-1α antibody, (*B*) anti IL-1β antibody. For both panels: (lane *1*) nonstimulated peritoneal macrophage lysate, (lane *2*) LPS-stimulated peritoneal macrophage lysate, (lane *3*) culture supernatant of M90T–infected peritoneal macrophages, and (lane *4*) culture supernatant of BS176–infected peritoneal macrophages.

IL-6 (12) and TNFα (13) have been reported to be able to promote thymocyte proliferation under the conditions described for the IL-1 bioassay. In order to determine whether the activity detected was in fact due to IL-1, the supernatant of M90T-infected macrophages was preincubated with both anti–murine IL-1α (3 μg/ml final concentration) and IL-1β (30 μg/ml final concentration) antibodies. The antibodies inhibited thymocyte proliferation by more than 85%. These same antibodies did not significantly inhibit the biological activity of a mixture of human recombinant IL-1α and IL-1β, demonstrating the specificity of the antibodies used.

The release of IL-1 activity by macrophages infected with M90T wild-type strain of *S. flexneri,* reached its half-maximal value around 45 min postinfection (Fig. 1 *A*). The total amount of IL-1α in the pooled supernatants of cells infected with wild-type shigella in the experiment reported above was 8.1 ng/ml measured in an ELISA. There were 9.3 ng/ml of IL-1α in the cells before the infection, as assayed in freezed and thawed cultures. Thus, 87% of the IL-1α present in the cells was released after infection with wild-type shigella. Macrophages infected with the plasmid-cured derivative strain BS176 did not release significant amounts of IL-1 activity (Fig. 1 *A*).

When IL-1α was measured antigenically (Fig. 1 *B*), the half-maximal release was attained between 30 and 45 min postinfection, earlier than the release of IL-1 activity. The delay in the release of IL-1 activity, relative to the relapse of IL-1 antigen, might reflect either a difference in the sensitivity of the methodology or a time-dependent conversion of IL-1β from its biologically inactive to its mature form. The cytotoxic strain M90T killed around 50% of peritoneal macrophages after 60 min of infection (Fig. 1 *C*). Neither the plasmidless strain BS176 (Fig. 1 *C*), nor purified *E. coli* LPS (data not shown), were cytotoxic for peritoneal macrophages.

The slopes of curves fitted to the measurements of IL-1 during the first 60 min postinfection with M90T (Fig. 1) are: 1.3 for the antigenically detected IL-1α, 1.1 for the IL-1 biological activity, and 0.7 for cytotoxicity measured by LDH release. The relative rates of IL-1α and LDH release were analyzed with a paired t test and they show a significant difference at $P < .025$, indicating that IL-1 release is slightly faster than that of LDH. These results suggest that apoptotic macrophages do not release IL-1 via a passive leakage of the cytokine.

When Western immunoblots were incubated with either anti–IL-1α (Fig. 2 *A*) or anti–IL-1β antibodies (Fig. 2 *B*), the precursor form of both IL-1α and IL-1β were present in cell lysates of LPS-stimulated peritoneal macrophage (Fig. 2 *A,* lane *2* and Fig. 2 *B,* lane *2*) but not in nonstimulated macrophages (Fig. 2 *A,* lane *1* and Fig. 2 *B,* lane *1*). Only the precursor form of IL-1α (Fig. 2 *A,* lane *3*), and both the precursor and the mature form of IL-1β (Fig. 2 *B,* lane *3*) were detected in the culture supernatant of peritoneal macrophages infected with the cytotoxic strain M90T. Neither IL-1α nor IL-1β were detected in culture supernatants of macrophages infected with the noncytotoxic strain BS176 (Fig. 2 *A,* lane *4* and Fig. 2 *B,* lane *4*). Thus, *S. flexneri* infection provokes the release of the biologically active precursor form of IL-1α and cleaved IL-1β.

*Table I. IL-6 and TNFα Present in Peritoneal Macrophages Supernatants**

First stimulus	Second stimulus	IL-6[‡]	TNFα[‡]
		pg/ml	
LPS	M90T	155	156
LPS	BS176	374	132
LPS	LPS	361	163
LPS	Medium	159	<15
Medium	M90T	ND[§]	591
Medium	BS176	ND	1,161
Medium	LPS	ND	1,096
Medium	Medium	ND	<15

* Peritoneal macrophages were stimulated with *E. coli* LPS 18 h before infection or treatment as indicated. IL-6 and TNFα was assayed with an ELISA kit as described in Methods and reported in pg/ml. [‡] In supernatants collected between 60 and 120 min post-infection. [§] ND, not done.

In order to establish whether apoptosis induced by *S. flexneri* can promote the secretion of IL-6 and TNFα, we tested the culture supernatants of LPS-stimulated and shigella-infected macrophages for the presence of these cytokines. The supernatants of cells 60 to 120 min after infection were measured in an ELISA, and the results are shown in Table I. The release of IL-6 by M90T-infected cells did not exceed that of cells incubated in medium alone, where the remaining IL-6 is consecutive to the LPS pretreatment. As expected, macrophages either infected with the noncytotoxic strain BS176, or restimulated with purified *E. coli* LPS, secreted IL-6. The amount of released TNFα was low (< 200 pg/ml) under all conditions wherein macrophages were preincubated with LPS. When macrophages were not prestimulated with LPS, infection with M90T resulted in very modest (< 600 pg/ml) TNFα release. In contrast, cells infected with BS176 or stimulated with LPS released larger amounts of TNFα (> 1000 pg/ml) (Table I). Taken together, these results indicate that LPS-stimulated macrophages are incapable of de novo cytokine synthesis, and reflect the results described for IL-6. Comparable results were obtained with respect to IL-6 and TNFα activities throughout the time course of infection (data not shown).

We confirmed that infection with shigella induces the release of presynthesized IL-1 and not the de novo expression of the cytokine by comparing the release of IL-1 in macrophages that were incubated for 18 h in the presence or in the absence of

*Table II. IL-1 Biological Activity in Supernatants of Stimulated and Nonstimulated Peritoneal Macrophages**

Second stimulus	IL-1‡	
	LPS-stimulated	Unstimulated
	cpm	
M90T	11,285	1,712
BS176	2,996	1,267
LPS	3,353	1,550
Medium	1,263	1,248

* Peritoneal macrophages were stimulated with *E. coli* LPS or incubated with culture medium alone 18 h prior to infection or treatment as indicated. IL-1 biological activity was assayed as described in Methods and reported in cpm. ‡ In supernatants collected between 0 and 15 min postinfection.

LPS. As described above, LPS pretreated macrophages infected with M90T release large amounts of IL-1. In contrast, wild-type *S. flexneri* does not induce IL-1 release from macrophages preincubated in culture medium; i.e., nonstimulated, either early up to 15 min (Table II) or late (data not shown) after infection.

Discussion

S. flexneri pathogenicity genes are carried on a 220-kb plasmid. Curing this bacterium of the plasmid results in a nonpathogenic derivative (1) incapable of inducing macrophage apoptosis (3). The plasmidless strain is therefore an ideal nonpathogenic control for shigella infections.

We have recently shown that cytotoxic strains of *S. flexneri* induce apoptosis in both peritoneal macrophages and in the macrophage-like cell line J774 (3). We decided to investigate the relationship between the induction of macrophage apoptosis by shigella and the initiation of inflammation during shigella infections. Here, we describe that the wild-type strain of *S. flexneri* induces a rapid and significant release of IL-1 as measured antigenically as well as biologically (Fig. 1). The noncytotoxic strain of shigella or purified LPS are incapable of stimulating this release.

IL-1 is released with slightly but significantly faster kinetics than LDH after a *S. flexneri* infection, suggesting that IL-1 release during *S. flexneri*–induced apoptosis is not a secondary effect of cell injury despite the cytoplasmic localization of both LDH and the precursor forms of IL-1.

Both forms of IL-1 are synthesized as polypeptides of 31 kD and processed through a specific proteolytic cleavage into molecules of 17.5 kD. Both the precursor and the mature IL-1α, but only the mature IL-β are biologically active (4). We tested which forms of IL-1α and IL-1β are released during *S. flexneri*–induced apoptosis. Only the precursor form of IL-1α and both forms of IL-1β are present in infected macrophage supernatants. This suggests that IL-1β converting enzyme activity (ICE)[1] (14, 15) was available in this context, while pro–IL-1α processing enzymes were not (16). Taking together the comparison in the kinetics of IL-1 and LDH release, as well as the cleavage of IL-1β, it appears that the cytokine release is specific and not due to passive leakage.

Macrophages undergoing apoptosis after wild-type *S. flexneri* infection release relatively low amounts of IL-6 or TNFα (Table I), probably because release of these two cytokines requires de novo synthesis and the biosynthetic machinery of an apoptotic cell is seriously compromised. Furthermore, the difference in the TNFα response in macrophages that were preincubated for 18 h in the presence or absence of LPS and then restimulated with LPS (Table I) indicate that after LPS-stimulation macrophages were in a state of anergy (17, 18). This implies that in the paradigm described here infected macrophages cannot effectively synthesize cytokines.

The experiments in Table II show that nonstimulated, naive macrophages, in contrast to stimulated macrophages, do not release IL-1 after shigella infections. These data indicate again that *S. flexneri* cannot induce the production of the cytokine; only its release. Taken together, the experiments described above suggest that wild-type shigella cannot induce the biosynthesis of cytokines in macrophages.

Hogquist et al. (5) reported that ATP- or cytotoxic T cell (CTL)–induced apoptosis of peritoneal macrophages is accompanied by release of mature IL-1α and IL-1β. In their study, both cell death and IL-1 release was detected around 6 h after the induction of apoptosis. The discrepancy between this report and that of Hogquist et al., regarding the maturation of IL-1α, might reflect the difference in the kinetics or in the availability of IL-1α converting enzymes between different pathways of induction of apoptosis. The mechanisms by which apoptotic macrophages process and release IL-1 awaits characterization and may illustrate a pathway for the release of other cytokines that do not contain a signal sequence.

It is interesting to speculate whether apoptosis and IL-1 release are two dissociable events in *S. flexneri* infection. This seems unlikely since apoptosis induced by bacteria or by other effectors (5, 19) are indistinguishable by either their DNA fragmentation patterns or their morphological criteria. Furthermore, a bacterial deletion mutant in *ipaB,* a gene known to be involved in induction of apoptosis, induces neither apoptosis (20) nor IL-1 release (unpublished observation).

Recently Miura et al. (21) demonstrated that ICE can induce apoptosis when expressed in Rat-1, neuroblastoma, and glioma cell lines. These results might support the hypothesis that the release and maturation of cytoplasmic IL-1 are an intrinsic process of macrophage apoptosis. Nevertheless, macrophages can express ICE without necessarily entering apoptosis. The direct role of this enzyme in macrophage-programmed cell death remains to be elucidated.

Taking into consideration the results presented here, the probable sequence of events in a shigella infection would be: (*a*) transcytosis of the bacteria through M cells from the lumen of the colon into the submucosa, (*b*) infection of resident tissue macrophages, especially those contained in lymphoid follicles, and onset of apoptosis, (*c*) release of IL-1 before the host cell is destroyed, (*d*) dissemination of *S. flexneri* through the epithelium and the lamina propria, and (*e*) initiation of inflammation mediated by IL-1. This model supposes that intestinal macrophages have accumulated IL-1α and IL-1β promolecules. Indeed, it is now well established that colonic macrophages are activated (22, 23) and express IL-1 (6, 7), specifically IL-1β (8), probably as a result of exposure to LPS from enteric bacteria.

The importance of apoptosis in the pathogenicity of different infectious diseases is being increasingly recognized (24). The induction of macrophage apoptosis by bacterial pathogens like

S. flexneri and *Bordetella pertussis* (25) are now well established. Our results may have important biological implications since the bacterial induction of apoptosis and the subsequent release of presynthesized IL-1 might serve a pivotal role in the onset of inflammation. Thus, IL-1 would be a distress signal released by macrophages just before cell death by apoptosis.

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