Apoptosis of Human Intestinal Epithelial Cells after Bacterial Invasion

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Abstract

Epithelial cells that line the human intestinal mucosa are the initial site of host invasion by bacterial pathogens. The studies herein define apoptosis as a new category of intestinal epithelial cell response to bacterial infection. Human colon epithelial cells are shown to undergo apoptosis following infection with invasive enteric pathogens, such as *Salmonella* or enteroinvasive *Escherichia coli*. In contrast to the rapid onset of apoptosis seen after bacterial infection of mouse monocyte-macrophage cell lines, the commitment of human intestinal epithelial cell lines to undergo apoptosis is delayed for at least 6 h after bacterial infection, requires bacterial entry and replication, and the ensuing phenotypic expression of apoptosis is delayed for 12–18 h after bacterial entry. TNF-α and nitric oxide, which are produced as components of the intestinal epithelial cell proinflammatory program in the early period after bacterial invasion, play an important role in the later induction and regulation of the epithelial cell apoptotic program. Apoptosis in response to bacterial infection may function to delete infected and damaged epithelial cells and restore epithelial cell growth regulation and epithelial integrity that are altered during the course of enteric infection. The delay in onset of epithelial cell apoptosis after bacterial infection may be important both to the host and the invading pathogen since it provides sufficient time for epithelial cells to generate signals important for the activation of mucosal inflammation and concurrently allows invading bacteria time to adapt to the intracellular environment before invading deeper mucosal layers. (*J. Clin. Invest.* 1998. 102:1815–1823.) Key words: TNF-α • *Salmonella* • nitric oxide • pathogenesis • infection

Introduction

A single layer of columnar epithelial cells, which are derived from the crypt compartment, lines the intestinal mucosa. These cells, which have a life span of 3–5 d, proliferate in the crypt compartment and differentiate as they migrate to the luminal surface of the colon or up the villous surface in the small intestine (1). To maintain stable numbers of enterocytes, proliferation is counterbalanced by programmed cell death (apoptosis). Apoptosis occurs spontaneously in epithelial cells in the crypt compartment, where it maintains the critical balance in cell number between newly generated and surviving cells, and at the luminal surface of the colon and villous tips in the small intestine, where differentiated epithelial cells are lost (2–4).

Apoptosis results in the deletion of epithelial cells from the intestinal mucosa with little, if any, accompanying inflammatory response since cells undergoing apoptosis are ingested by phagocytic cells or shed into the intestinal lumen. Moreover, morphologic studies suggest that cell death by apoptosis results in little disruption of the intestinal epithelial barrier integrity (5). In contrast, necrosis in response to cell injury is associated with the rupture of the cell membrane and the release of cellular contents, which often is accompanied by an inflammatory response.

Apoptosis is a regulated multistep intracellular process that can be signaled from the external environment or from within the cell. For example, apoptosis of cultured human colon epithelial cells can be induced by stimulation of cells with cytokines (e.g., IFN-γ in combination with TNF-α) (6, 7) or by ligation of Fas receptor (CD95/Apo-1) on the cell surface (6, 8). Apoptosis of human colon crypt and surface epithelial cells also has been observed after mucosal infection with HIV-1 (9) and during acute *Shigella* colitis (10), but it is not known whether this epithelial response is a direct consequence of the infection or is due to mediators released during the accompanying inflammation.

The intestinal mucosa is an important route of entry for microbial pathogens. Furthermore, intestinal epithelial cells are the initial sites of interaction of the host with invasive enteric pathogens. Following bacterial entry, intestinal epithelial cells rapidly (i.e., within 60–90 min) upregulate the expression of an inflammatory gene program that includes the cytokine TNF-α and an array of proinflammatory chemokines that chemoattract neutrophils and mononuclear phagocytes (11–13). The early epithelial response to bacterial infection also includes the upregulated expression and production of inducible nitric oxide synthase (iNOS)[1] and nitric oxide (NO) (14). Thus, in the first few hours after bacterial invasion, human colon epithelial cells produce mediators that have the potential to orchestrate the onset of the mucosal inflammatory response (15). Some of these mediators also have the potential to induce apoptosis of human colon epithelial cells (6, 7, 16). In other systems, mouse monocyte-macrophage cell lines were shown to rapidly undergo apoptosis within 1–2 h of infection with the enteric pathogens *Shigella*, *Salmonella*, and *Yersinia* (17–19), although

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1. *Abbreviations used in this paper:* AG, aminoguanidine; CM, conditioned media; $DiOC_6(3)$, 3′3-dihexyloxacarbocyanine iodide; GFP, green fluorescent protein; iNOS, inducible nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; SNAP, S-nitroso-*N*-acetyl-D,L-penicillamine.

these pathogens did not activate apoptosis of human macrophages or human cervical epithelial cells when examined over a similar time frame (19–21).

We hypothesized that apoptosis may be a delayed epithelial response to infection with enteroinvasive bacteria and linked to the epithelial cell proinflammatory response, and the present studies tested this notion. It is shown that human colon epithelial cells undergo apoptosis in response to infection with enteroinvasive bacteria. Moreover, apoptosis occurs as a relatively late event, requires bacterial invasion and replication, and is linked to the activation of the early epithelial cell inflammatory program. Thus, TNF-α and NO, which are upregulated as key components of the host's early proinflammatory gene program in response to bacterial infection of epithelial cells, play an important role in regulating the apoptotic process in those cells. These results broaden current concepts regarding the range of epithelial cell responses to bacterial infection and suggest new functions for host cell apoptosis in the pathogenesis of mucosal infection.

Methods

Cell lines

HT-29 human colon epithelial cells (ATCC HTB 38) and Caco-2 human ileocecal epithelial cells (ATCC HTB 37) were grown in RPMI 1640 medium with 10% FCS. T84 human colon epithelial cells were grown in 50% DME, 50% Ham's F12 medium, and 5% newborn calf serum as previously described (22). In some experiments, as indicated, HT-29 cells were grown for 24 h before use in arginine-free DME with 10% dialyzed FCS.

Bacteria

These studies used a *Salmonella dublin* lane strain (11) and two isogenic mutants of *S. dublin* lane. SL5647 is an *aroA* mutant of *S. dublin* lane that was prepared by Dr. B. Stocker, Stanford University (23, 24). SB133 is an *invA* invasion defective mutant of *S. dublin* (11, 25). In addition, these studies used *S. typhimurium* SL1344 (provided by Dr. D. Guiney, UCSD) and an *aroA* mutant of *S. typhimurium* SL1344, enteroinvasive *Escherichia coli* O29:NM (ATCC 43892), and a nonpathogenic *E. coli*, DH5α (GIBCO BRL, Gaithersburg, MD). Bacteria were grown to late log phase at 37°C in tryptic soy broth before infection as described before (11, 26).

Cytokines and other reagents

Recombinant human TNF-α and goat anti–human TNF-α (IgG isotype) were purchased from R&D Systems (Minneapolis, MN). Normal goat IgG was purchased from Sigma Chemical Co. (St. Louis, MO). FITC-conjugated goat anti–*Salmonella* common structural antigen-1 was purchased from Kirkegaard and Perry Laboratories Inc. (Gaithersburg, MD). Chloramphenicol and aminoguanidine were obtained from Sigma Chemical Co. S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) was from Cayman Chemical Co. (Ann Arbor, MI). N^G-nitro-L-arginine methyl ester (L-NAME) was from ICN Biomedical Inc. (Aurora, OH). The DNA dye Hoechst 33258 (2′-(4-hydroxyphenyl)-5-(4-methyl-piperazinyl)-2,5′-bi-1H-benzimidazole) was from CalBiochem (La Jolla, CA). 3′3-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) was from Molecular Probes (Eugene, OR).

Infection protocol

Cells were seeded into 6-well tissue culture plates (Costar, Cambridge, MA) at 10^6 per well and grown to confluence (i.e., ~ 24 h for HT-29 cells, 48 h for Caco-2 cells, > 96 h for T84 cells). Cultures were infected with bacteria for 1 h to allow bacterial entry to occur. Extracellular bacteria were removed by washing, and the cultures were incubated in the presence of gentamicin (50 μg/ml) to kill remaining extracellular bacteria (11). To prepare conditioned media (CM), supernatants from bacteria-infected cultures were removed 18 h postinfection, filtered through a 0.22 μm filter, and kept frozen at −80°C until use. Numbers of intracellular bacteria were determined after lysing epithelial cells in distilled water (11).

Assays for apoptosis in epithelial cell lines

Morphologic assessment. Cells undergoing apoptosis were identified by staining monolayers with the DNA dye Hoechst 33258 (5 μg/ml) or by staining adherent and nonadherent cells with acridine orange and ethidium bromide (27, 28). For the latter, adherent cells were detached using 0.25% trypsin/0.25% EDTA in PBS for 3–5 min, washed, pooled with washed nonadherent cells, and adjusted to 5×10^5 cells/ml. 100 μl of a mixture of 100 μg/ml each of acridine orange and ethidium bromide were added to 5×10^5 cells in a 1-ml volume. Cell preparations were examined by epifluorescence microscopy.

$DiOC_6(3)$ staining. Mitochondrial transmembrane potential ($\Delta\psi_m$) was assessed using $DiOC_6(3)$ staining and flow cytometry (29). Reduced mitochondrial potential is known to occur late in the apoptotic process relative to the release of cytochrome c from mitochondria (30). Briefly, adherent cells were detached, pooled with nonadherent cells, and washed with cold PBS, after which cells (5×10^5/ml) were incubated with $DiOC_6(3)$ (40 nM in PBS) for 15 min at 37°C followed by analysis on a flow cytometer (FACScan®; Becton Dickinson, Sunnyvale, CA).

FITC-labeled annexin V and propidium iodide staining. FITC-conjugated annexin V, which binds to phosphatidylserine (31, 32), and propidium iodide were added to 10^5 cells, after which cells were incubated for 15 min at room temperature in the dark according to the manufacturer's instructions (Apoptosis Detection Kit; R&D Systems), and cells were analyzed by flow cytometry. Early apoptotic cells stained with annexin V alone, whereas necrotic cells and late apoptotic cells stained with both annexin V and propidium iodide.

Cell death detection ELISA. To assess DNA fragmentation, nucleosomes were quantitated in the cytoplasm using a sandwich ELISA (Cell Death Detection ELISA^{plus} kit; Boehringer Mannheim, Indianapolis, IN). Briefly, adherent cells were detached and 5×10^4 cells were lysed, after which cytosolic oligonucleosomes were quantitated using biotin-coupled mouse monoclonal anti-histone antibody as the capturing antibody and peroxidase-conjugated mouse monoclonal anti-DNA antibody as detecting antibody, and ABTS (2,2′-azino-di[3-ethylbenzthiazolin-sulfonat]) as the developing reagent. The relative increase in nucleosomes in the cytoplasm is expressed as an enrichment factor which was calculated as the ratio of specific absorbance in lysates from bacteria-infected compared with uninfected cells as described by the manufacturer.

Bacteria labeling with green fluorescent protein (GFP)

S. dublin bacteria were grown in LB broth to an OD_{600} of 0.5, after which approximately 10^9 bacteria in 100 μl were electroporated with 2 μg of a plasmid, pKEN2-GFPmut2, encoding GFP (33). Transformants were selected on LB agar plates supplemented with 50 μg/ml carbenicillin.

Determination of nitrite and TNF-α production

NO is rapidly converted to the stable end products nitrite and nitrate. The concentration of nitrite in culture supernatants was quantitated using the Griess reaction (34). Briefly, 100 μl of culture supernatant was combined with an equal volume of Griess reagent (0.5% sulfanilamide/0.05% naphthylethylene-diamine dihydrochloride in 2.5% H_3PO_4) and incubated for 10 min at room temperature. Absorbance was measured at 550 nm with an ELISA reader and compared with a standard curve generated with sodium nitrite. Sensitivity of the assay was ~ 1 μM. NO production by cells was inhibited by incubating cells with the NOS inhibitors L-NAME or aminoguanidine or by growing cells in arginine-free medium supplemented with dialyzed FCS. TNF-α levels in culture supernatants were assayed by ELISA. The TNF-α ELISA (Quantikine HS; R&D Systems) was sensitive to 0.2 pg/ml.

Results

Enteroinvasive bacteria induce apoptosis in human colon epithelial cell lines

Monolayers of HT-29 human colon epithelial cells were infected with two *Salmonella* strains, *S. dublin* and *S. typhimurium*, or enteroinvasive *E. coli* (serotype O29:NM), as prototypic invasive bacteria known to activate strongly an epithelial cell inflammatory gene program (11–14, 26), and apoptosis was assessed by several methods. *S. dublin*– or enteroinvasive *E. coli*–infected monolayers of HT-29 cells contained three- to sixfold more apoptotic cells than control uninfected monolayers. Fig. 1 shows, as an example, nuclear fragmentation characteristic for apoptosis in *S. dublin*–infected HT-29 cells stained with the DNA dye Hoechst 33258. Similar nuclear condensation and fragmentation were observed in *S. dublin*–infected HT-29 cells stained with acridine orange and ethidium bromide (data not shown). Morphologic assessments of apoptosis were confirmed by staining cells with FITC-annexin V to detect externalization of phosphatidylserine to the outer leaflet of the cell membrane (31, 32), by $DiOC_6(3)$ staining to detect reduced mitochondrial transmembrane potential (29) (Table I and Fig. 2), and by the release of oligonucleosomes into the cytoplasm using a cell death detection ELISA (Table I). The ratio of apoptotic cells in infected compared with control cultures was similar with each of the above methods, although absolute numbers differed (Table I). Similar increases in apoptotic cell numbers were noted following *S. dublin* or enteroinvasive *E. coli* infection of two additional human colon epithelial cell lines, Caco-2 and T84. The percentage of apoptotic cells in Caco-2 cultures was 17.6±0.2% after *S. dublin* infection and 14.7±1.5% after enteroinvasive *E. coli* infection compared with 6.3±1.1% in uninfected controls. The percentage of apoptotic cells in T84 cultures was 18.8±0.6% after *S. dublin* infection and 18.6±1.3% after *E. coli* infection compared with 8.2±1.0% in uninfected controls ($DiOC_6$ (3) staining 24 h after infection; mean±SE, $n = 3$).

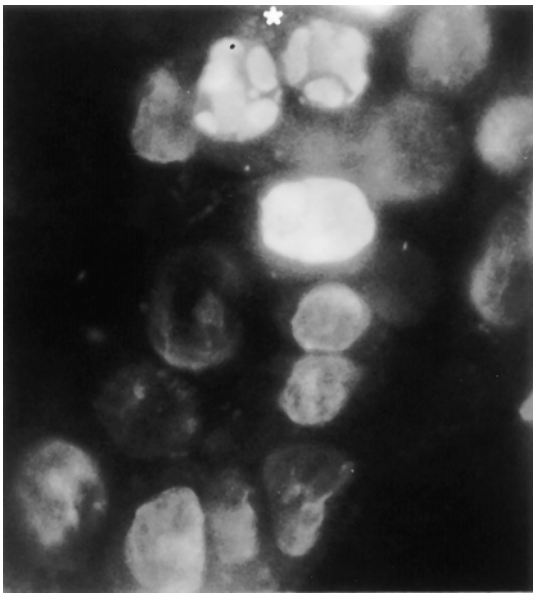

Figure 1. Apoptosis in S. dublin-infected HT-29 monolayers. Monolayers of HT-29 cells were infected with 5×10^7 *S. dublin.* After 48 h, monolayers were fixed with 2% paraformaldehyde, and were stained with Hoechst dye 33258. At the upper margin of the figure (*), two cells are visible with nuclear fragmentation typical for apoptosis.

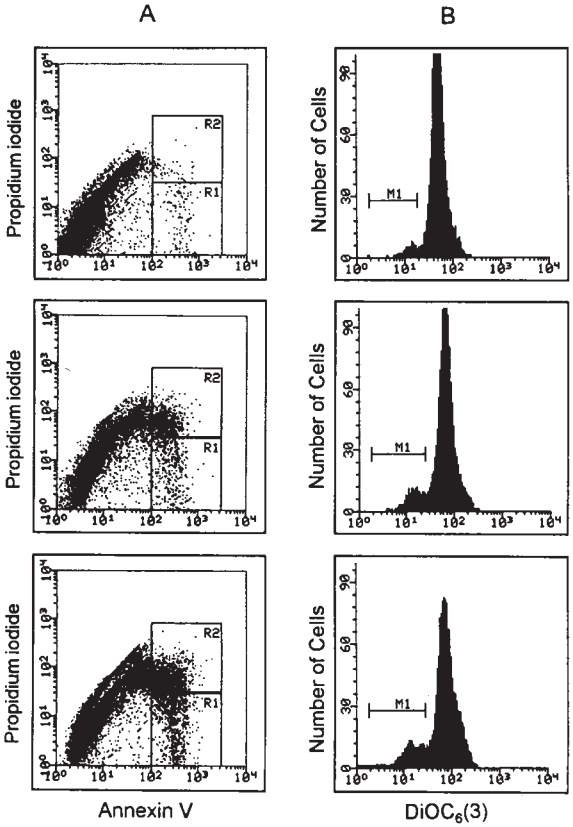

Figure 2. Flow cytometric analysis of apoptosis of colon epithelial cells infected with *S. dublin* or enteroinvasive *E. coli*. (*A*) Confluent monolayers of HT-29 human colon epithelial cells in 6-well plates were left uninfected as controls (*top panel*), or were infected with 5×10^8/well enteroinvasive *E. coli* serotype O29:NM (*middle panel*), or 5×10^7/well *S. dublin* (*bottom panel*) and further incubated for 24 h in the presence of gentamicin (50 μg/ml) to kill remaining extracellular bacteria as described in Methods. Adherent and nonadherent cells were pooled, incubated with FITC-annexin V and propidium iodide, and analyzed by flow cytometry. Data are from a representative experiment. Early apoptotic cells have increased annexin V, but not propidium iodide staining as indicated by the area marked R1 (control 1.4% of cells; *E. coli* O29:NM 4.3% of cells; and *S. dublin* 9.2% of cells). Necrotic and late apoptotic cells stained with both annexin V and propidium iodide as indicated by the area marked R2 (control 0.7% of cells; *E. coli* O29:NM 7.1% of cells; and *S. dublin* 12.9% of cells). Quantitative data for R1 obtained in several independent experiments are presented in Table I. (*B*) Culture and infection conditions are the same as in *A.* Adherent and nonadherent cells were incubated with 40 nM $DiOC_6(3)$ for 15 min at 37°C and analyzed by flow cytometry. The area marked as M1 to the left of the major peak contains the apoptotic cell population (M1 area in control, *E. coli* O29:NM infected, and *S. dublin*–infected cultures contains 4.0%, 10.4%, and 15.3% of the total cells, respectively).

Apoptosis in colon epithelial cell lines is a relatively late response after bacterial infection and requires bacterial invasion, as well as bacterial replication and protein synthesis

Apoptosis was a relatively late epithelial cell response to bacterial infection. As shown in Fig. 3, apoptosis was first appar-

*Table I. Apoptosis of HT-29 Cells in Response to Infection with Invasive Bacteria**

	Assay				
	Annexin V and propidium iodide‡		$DiOC_6(3)$‡		Cell death detection ELISA
	Apoptotic cells (%)	Ratio infected/ control	Apoptotic cells (%)	Ratio infected/ control	Enrichment factor§
Control	1.4±0.3‖	1	4.6±0.3‖	1	1
E. coli O29:NM	4.1±0.3	2.9	11.7±1.0	2.5	4.6±0.6‖
S. dublin	7.9±0.9	5.6	17.8±1.0	3.9	7.4±0.9
S. typhimurium	ND¶		ND		3.1±0.5

*Confluent monolayers of HT-29 cells in 6-well plates were infected with 5×10^7 *S. dublin*, 10^8 *S. typhimurium*, or 5×10^8 enteroinvasive *E. coli* O29:NM as described in Methods. Apoptosis was assessed 24 h later. ‡Assay includes adherent and nonadherent cells. §Enrichment factor is fold increase in bacteria-infected compared with control cells. Assay includes adherent cells only. ‖Values are means±SE of three or more separate experiments. ¶ND, not done.

ent 12–18 h after *S. dublin* or enteroinvasive *E. coli* infection and continued to increase over the ensuing 48 h as assessed by $DiOC_6(3)$ staining. Similar kinetics were obtained using the cell death detection ELISA (data not shown).

Since activation of IL-8 expression and an epithelial cell inflammatory program (11, 12, 26, 35) in human colon epithelial cells is associated with bacterial invasion, we asked whether the ability of bacteria to invade was important for initiating the apoptotic response. This was the case since apoptosis was not increased in HT-29 cells cultured with a noninvasive, nonpathogenic *E. coli* (Fig. 3) or an invasion-defective mutant of *S. dublin*, SB133 (Table II), which is isogenic with *S. dublin* lane, but 50 to 100-fold less invasive (25). Further, intracellular replication of invaded bacteria was important for the maximal induction of apoptosis. Thus, SL5647, an *aroA* mutant of *S. dublin*, which is isogenic with *S. dublin* lane and as invasive for HT-29 cells as the parental strain, but replicates more slowly inside cells, had a decreased ability to induce apoptosis compared with the wild-type parental strain (Table II). In contrast, production of the chemokine IL-8 did not differ significantly in HT-29 cultures infected with the replication-deficient *S. dublin* SL5647 compared with the parental *S. dublin* lane strain (data not shown).

Apoptosis increased with increasing bacterial inocula (Fig. 4). However, induction of apoptosis required a higher bacterial inocula after infection with enteroinvasive *E. coli* compared with *S. dublin.* This could either reflect decreased invasiveness of enteroinvasive *E. coli* compared with *S. dublin*, or a lower efficiency of invaded *E. coli* in activating apoptosis. The latter appeared to be mostly responsible since HT-29 cells infected with identical inocula of *S. dublin* and enteroinvasive *E. coli* differed by only two- to fivefold in the numbers of recovered viable intracellular bacteria as measured 4 h after infection.

To determine if protein synthesis by invaded bacteria was required for the induction of apoptosis, HT-29 monolayers were infected with *S. dublin* or enteroinvasive *E. coli* and further incubated in the presence of the cell permeant antibiotic chloramphenicol to inhibit bacterial protein synthesis. Addition of chloramphenicol immediately after the 1-h infection period decreased the number of apoptotic cells in *S. dublin*–infected cultures by ~ 70% (Table III) and the number of apoptotic cells in enteroinvasive *E. coli*–infected cultures by ~ 40% (data not shown) as assessed 24 h after infection. Chloramphenicol also inhibited apoptosis, albeit to a lesser extent, when added as late as 6 h after *S. dublin* infection (Table III). These data indicate bacterial protein synthesis in the initial several hours after infection is required to activate the later apoptotic response.

To assess further the relationship between bacterial infection and apoptosis, HT-29 monolayers in 6-well plates were infected with 5×10^7 GFP-expressing *S. dublin* per well. 24 and 48 h later, cells were stained with Hoechst dye 33258 and examined by epifluorescence microscopy for the presence of intracellular bacteria and apoptotic nuclei. Approximately 20% of cells contained bacteria. Although a few infected cells contained as many as 5–10 bacteria per cell, which is similar to the numbers seen after infection with the same inocula of unlabeled *S. dublin*, bacterial replication in many of the cells infected with GFP-labeled bacteria was much lower (e.g., 2–3 bacteria per cell). Consistent with this low level of intracellular replication, only 5–7% of cells were apoptotic. Moreover, among the apoptotic cells, some contained bacteria while oth-

*Table II. Apoptosis of HT-29 Human Colon Epithelial Cells in Response to Infection with Wild-type and Mutant S. dublin**

Added bacteria	$DiOC_6(3)$ staining (% apoptotic cells)	Cell death detection ELISA (enrichment factor)
None	5.1±0.6	1
S. dublin	17.1±1.1‡	7.4±0.5‡
S. dublin SB133 (*invA*)	5.1±0.5	1.2±0.3
S. dublin SL5647 (*aroA*)	ND§	2.9±0.3‡

*Monolayers of HT-29 cells in 6-well plates were infected for 1 h with 10^8 *S. dublin*, 10^8 of the *S. dublin invA* mutant, SB133, or 10^8 replication defective *S. dublin aroA* mutant, SL5647 and further incubated for 24 h in the presence of gentamicin (50 μg/ml). Apoptosis was assessed by $DiOC_6(3)$ staining and flow cytometry and by the cell death detection ELISA. Values are means±SE of three or more separate experiments. Invasion of HT-29 cells with wild-type *S. dublin* and replication defective *S. dublin* SL5647 were almost identical (i.e., 1.3% and 1.4% of the infecting dose of those strains, respectively, were recovered from HT-29 cells 4 h postinfection). The doubling time of the isogenic *S. dublin* SL5647 was ~ twofold longer than that of wild-type *S. dublin* in HT-29 cells over the 24 h period postinfection, which resulted in eight-fold greater numbers of intracellular bacteria in cells infected with the parental compared with the mutant replication defective strain. ‡Significantly increased compared to control ($P < 0.05$). §ND, not done.

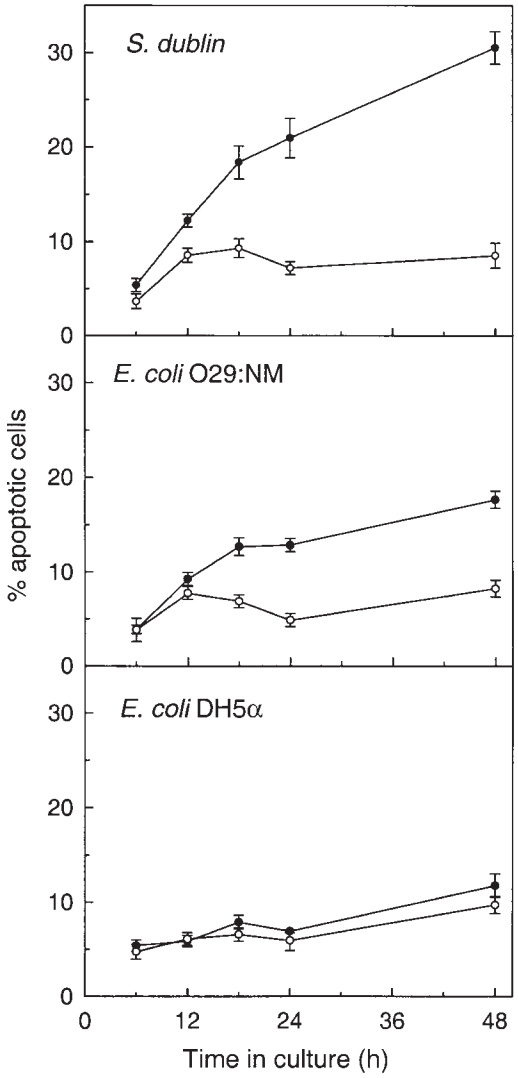

Figure 3. Time course of apoptosis in HT-29 cells after infection with *S. dublin*, enteroinvasive *E. coli* O29:NM, or nonpathogenic *E. coli* DH5α. Confluent monolayers of HT-29 cells in 6-well plates were infected with 5×10^7/well *S. dublin* or 5×10^8/well enteroinvasive *E. coli* or nonpathogenic *E. coli* as described in Fig. 2. Cells were assayed for $DiOC_6(3)$ staining at the indicated times after infection. The percentage of apoptotic cells is shown for bacteria-infected (*filled circles*) and uninfected controls (*open circles*). Values are means±SE of three separate experiments.

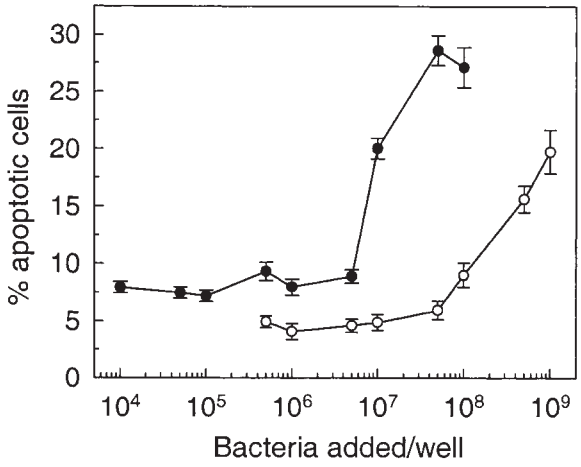

Figure 4. Relationship between bacterial inoculum and apoptosis. Confluent monolayers of HT-29 cells in 6-well plates were infected with increasing inocula of *S. dublin* (*filled circles*) or enteroinvasive *E. coli* O29:NM (*open circles*). Cells were assayed for $DiOC_6(3)$ staining 48 h postinfection to determine the percentage of apoptotic cells. Values are means±SE of three or more separate experiments.

ers did not. These data suggest either that bacteria are killed in cells undergoing apoptosis, possibly by the entry of gentamicin from the culture medium and/or their exit from cells undergoing apoptosis, or that apoptosis occurs in uninfected "bystander" cells. The former appears to be mainly the case since apoptotic cells were mostly present as dispersed single cells scattered throughout the culture and were not surrounded by adjacent apoptotic cells as would be expected if apoptosis occurred mainly in bystander cells.

Role of TNF-α and NO in mediating bacteria-induced apoptosis

Role of TNF-α. TNF-α production is upregulated in *S. dublin*– or enteroinvasive *E. coli*–infected human colon epithelial cell lines (12, 26). In the present studies, maximal levels of TNF-α were noted by 3 h after infection and remained relatively constant over the ensuing 24-h culture period [TNF-α levels in supernatants from HT-29 cultures infected with 10^8/well *S. dublin* or 5×10^8/well enteroinvasive *E. coli* were 112±10.6 pg/ml and 34.3±5.8 pg/ml, respectively, whereas uninfected cultures contained < 0.2 pg/ml TNF-α (values are mean±SE, $n = 4$)]. Since TNF-α had been shown to contribute to apoptosis in HT-29 cells (6, 7), we determined whether TNF-α, produced as part of the epithelial cell response to bacterial infection, played a role in activating apoptosis after bacterial infection. As shown in Fig. 5 for *S. dublin*–infected cells, when endogenous TNF-α was blocked by adding anti–TNF-α antibody, bacteria-activated apoptosis was inhibited reproduc-

*Table III. Bacterial Protein Synthesis Is Required for Apoptosis of S. dublin–infected HT-29 Cells**

Additions to culture	Time after infection of chloramphenicol addition (*h*)	Apoptotic cells (%)‡
None	—	6.1±0.4
S. dublin	—	23.7±0.6
S. dublin + chloramphenicol	0	11.4±1.0§
S. dublin + chloramphenicol	6	18.2±0.4§
S. dublin + chloramphenicol	12	21.4±0.4
S. dublin + chloramphenicol	18	23.9±0.2

*Confluent monolayers of HT-29 cells in 6-well plates were infected with 10^8 *S. dublin* for 1 h, washed, and further incubated in the presence of gentamicin (50 μg/ml) for 24 h. Chloramphenicol (100 μg/ml) was added immediately after the 1 h infection period (time 0 h) or at the other indicated times after infection. Apoptosis was assessed using $DiOC_6(3)$ staining and flow cytometry. Chloramphenicol alone did not alter apoptosis in uninfected control cultures (5.2±2.0% at 24 h). ‡Values are means±SE of three separate experiments. §Values significantly different from those in *S. dublin*–infected cultures without chloramphenicol ($P < 0.05$).

ibly by ~20%. Anti–TNF-α treatment also significantly inhibited apoptosis of enteroinvasive *E. coli*–infected HT-29 cells ($P < 0.01$) (enrichment factor in cell death detection ELISA: *E. coli*–infected, 3.9±0.2; *E. coli*–infected + anti–TNF-α, 2.1±0.1; *E. coli*–infected + control Ig, 3.7±0.6; mean±SE, $n = 3$). Consistent with these findings, the addition of CM from *S. dublin* or enteroinvasive *E. coli*–infected HT-29 cells to uninfected HT-29 cells induced a two- to threefold increase in the number of apoptotic cells after 48 h in culture, and those responses were completely blocked by anti–TNF-α, but not control antibody (data not shown). Moreover, endogenous TNF-α in infected cultures was sufficient to mediate a maximal apoptotic response to that mediator since adding recombinant TNF-α (20 ng/ml) to *S. dublin* or enteroinvasive *E. coli*–infected HT-29 cells did not lead to greater numbers of apoptotic cells than induced by infection alone. Taken together, these data support an autocrine/paracrine role for TNF-α in mediating apoptosis of bacteria-infected human colon epithelial cells. Further, the inability to block completely apoptosis by blocking TNF-α suggests that mediators other than TNF-α are involved in the activation of apoptosis after bacterial infection.

Role of NO. NO production is upregulated in human colon epithelial cell lines in response to infection with *S. dublin* or enteroinvasive *E. coli* (14). We determined whether NO, produced by human colon epithelial cells during bacterial infection, was linked to increased apoptosis of those cells. As shown in Fig. 6 for *S. dublin* infection, at 48 h after infection, apoptosis of HT-29 cells was significantly inhibited by L-NAME or AG at concentrations that inhibited epithelial cell NO production by 75–80%. L-NAME and AG also decreased apoptosis, although to a lesser extent, in *S. dublin*–infected HT-29 cells at 24 h after infection (inhibition by L-NAME 23.8±4.6% [$n = 6$] and by AG 25.4±1.7% [$n = 3$]; values are mean±SE). Furthermore, when NO production was inhibited by 90–95% by culturing cells in arginine-free medium, apoptosis was inhibited by 66.7±4.1% in *S. dublin*–infected and by 62.7±9.2% in enteroinvasive *E. coli*–infected cultures, as assessed 48 h after infection. In contrast, apoptosis of HT-29 cells primed with IFN-γ and stimulated with TNF-α was not affected by culturing cells in arginine-free medium (enrichment factor in cell death detection ELISA was 5.4 in arginine-free medium and 5.3 in control medium). Inhibition of NO production did not alter bacterial entry or replication when assessed 4 and 24 h after infection (data not shown).

Since pathways involving either TNF-α or NO were shown to regulate apoptosis in bacteria-infected HT-29 cells, we assessed whether blocking the production of both mediators, in combination, was more effective at inhibiting apoptosis than inhibiting each individually. Inhibiting TNF-α and NO in *S. dublin*–infected HT-29 cells yielded an additive but not

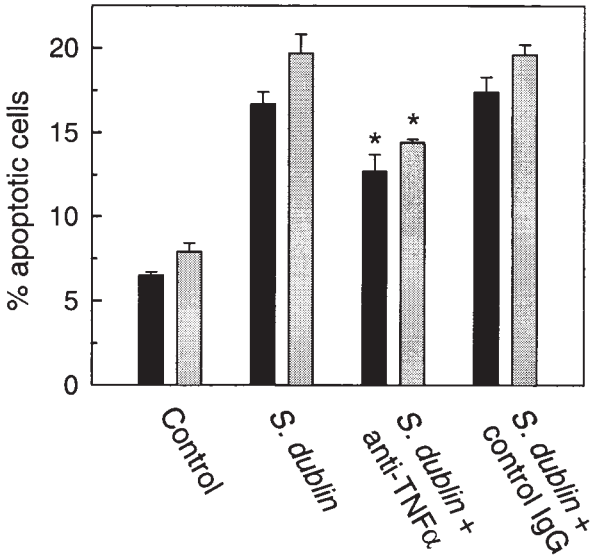

Figure 5. Blocking endogenous TNF-α decreases apoptosis in bacteria-infected colon epithelial cells. Confluent monolayers of HT-29 cells in 6-well plates were left uninfected (*control*) or were infected with 10^8/well *S. dublin* for 1 h and further incubated with 20 μg/ml of anti–TNF-α or normal goat IgG as a control antibody for 18 h (*black bars*) or 24 h (*gray bars*). Cells were assayed for $DiOC_6(3)$ staining to determine the percentage of apoptotic cells. Values are means±SE of three separate experiments. Asterisks indicate values with added antibody that are significantly different from *S. dublin*–infected groups without added antibody ($P < 0.05$). Similar results were obtained using the cell death detection ELISA (enrichment factor at 24 h: *S. dublin* alone 5.2±0.4 and *S. dublin* + anti–TNF-α 3.2±0.1). Anti–TNF-α treatment of HT-29 cells did not alter bacterial invasiveness or intracellular replication. Thus, at 4 h after infection, 2.9±0.6% and 3.0±0.6% of the infecting inoculum was recovered from *S. dublin*–infected compared with *S. dublin*–infected + anti–TNF-α treated cultures, respectively. At 24 h after infection, 9.5±0.3% and 9.2±0.2% of the infecting inoculum was recovered from infected compared with infected + anti–TNF-α treated cultures, respectively.

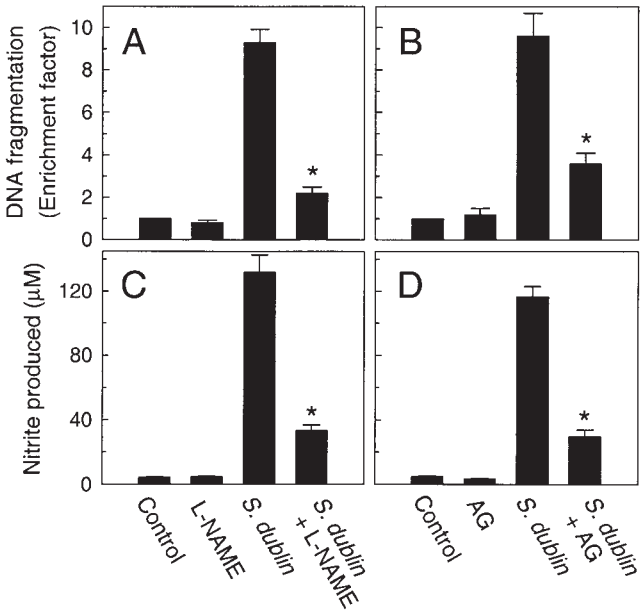

Figure 6. Inhibition of NOS and NO production by bacteria-infected cells inhibits apoptosis. (*A* and *B*) Confluent monolayers of HT-29 cells in 6-well plates were infected with 10^8/well *S. dublin* for 1 h, and further incubated in the presence of NOS inhibitors, L-NAME (1 mM, *A*) or AG (100 μM, *B*) for 48 h. Apoptosis was assessed using a cell death detection ELISA. Numbers refer to DNA fragmentation as measured by the enrichment factor. (*C* and *D*) culture and infection conditions were identical to *A* and *B*. After 48 h, culture supernatants were harvested and NO_2^- concentrations were determined by the Griess reaction. Values in *A*–*D* are means±SE of three or more separate experiments. Asterisk indicates value significantly different from *S. dublin*–infected cells without added NOS inhibitor ($P < 0.05$).

a synergistic effect (% inhibition of apoptosis assayed by cell death detection ELISA 24 h after infection: anti–TNF-α alone 19.2±2.9%, L-NAME alone 13.9±1.3%, anti–TNF-α + L-NAME 31.0±3.3%, $n = 3$). Consistent with this, when both TNF-α (20 ng/ml) and an NO donor, SNAP (0.5 mM), were added to uninfected HT-29 cultures for 24 h, the increase in apoptosis was additive and not synergistic (enrichment factor in cell death detection ELISA: SNAP alone 3.10±0.39; TNF-α alone 2.32±0.14; SNAP + TNF-α 4.93±0.67; mean±SE, $n = 3$). Blocking NO production by L-NAME treatment or culturing cells in arginine-free medium had no effect on *S. dublin*– or enteroinvasive *E. coli*–induced TNF-α release (data not shown). Further, addition of anti–TNF-α to culture did not affect NO production by *S. dublin*–infected cells (e.g., *S. dublin*–infected cells, 45.9±6.4 μM nitrite produced; *S. dublin*–infected cells + anti–TNF-α, 47.7±6.2 μM; values obtained 24 h postinfection, mean±SE, $n = 3$) or enteroinvasive *E. coli*–infected HT-29 cells (data not shown). Moreover, anti–TNF-α did not block apoptosis of HT-29 cells activated by exogenous NO donors (data not shown).

Discussion

These studies define apoptosis as a new category of response of human intestinal epithelial cells to bacterial infection and provide a link between the early activation of the epithelial cell proinflammatory program and the later onset of epithelial apoptosis. The onset of apoptosis in bacteria-infected intestinal epithelial cells is a relatively late event that is first noted 12–18 h after bacterial entry. Moreover, TNF-α and NO, which are produced as part of the epithelial cell proinflammatory response in the initial few hours after bacterial invasion (12, 14, 26), were shown herein to have an important role in regulating the later apoptotic response of bacteria-infected human intestinal epithelial cells.

Apoptosis can be induced in several cell types by TNF-α (6, 7, 36, 37). Following bacterial infection, TNF-α production is upregulated and reaches maximal levels within 3 h (26). As shown herein, blocking TNF-α activity in infected cultures blocked, in part, the activation of apoptosis. The expression of TNF-α receptors and sensitivity of HT-29 cells to TNF-α–induced apoptosis are increased by IFN-γ priming (6, 7, 38), a finding we confirmed (data not shown). However, neither HT-29 cells nor the other human colon epithelial cell lines tested herein (i.e., Caco-2, T84) express IFN-γ (12, 22), and apoptosis of these cells after bacterial infection occurred in the absence of added IFN-γ.

Increased NO production in bacteria-infected HT-29 cultures was linked to increased apoptosis, since inhibition of NO production by NO inhibitors or growing cells in arginine-free media decreased bacteria-induced apoptosis. Although exogenous addition of NO donors, or the potent oxidant peroxynitrite, induced apoptosis in cultured human colon epithelial cells (16, 39), endogenous peroxynitrite did not appear to play a role in bacteria-induced apoptosis of the cells studied herein since no evidence was found for a differential representation of proteins containing nitrotyrosine residues in bacteria-infected and control cells (data not shown). In addition, although apoptosis can be activated in cultured human colon epithelial cells by ligation of Fas receptor (CD95/Apo-1) (6, 8), bacteria-induced apoptosis was not mediated by extracellular signaling through Fas receptor (data not shown).

Our findings with human colon epithelial cells stand in marked contrast to earlier reports that studied apoptosis of mouse monocyte-macrophage cell lines following bacterial infection. Bacteria-induced activation of apoptosis was first apparent at 12–18 h after *Salmonella* infection of cultured human colon epithelial cell lines. Further, detectable activation of caspase-3 (CPP32), which is known to play a central role in the caspase cascade leading to apoptosis (40), was not seen until 6 h after infection (data not shown). In contrast, *Shigella*, *Salmonella*, and *Yersinia* induced apoptosis in mouse monocyte-macrophage cell lines within 1–2 h of infection (17–19), although *Shigella* did not induce apoptosis of human macrophages, suggesting important species differences between these cells in mice and humans (20). Of note, *Shigella* and *Yersinia* did not induce apoptosis of a human cervical epithelial cell line after infection (19, 21). However, apoptosis was assessed only during the first few hours after infection, which is prior to the time apoptosis of human colon epithelial cells was seen in our studies. Apoptosis in mouse macrophage cell lines after *S. flexneri* or *Y. enterocolitica* infection was linked to the production of bacterial type III secretory proteins that are important for bacterial entry (19, 41). The IpaB protein of *S. flexneri*–activated IL-1 converting enzyme (i.e., ICE or caspase-1) and subsequent apoptosis in mouse macrophages, whereas apoptosis in response to *Y. enterocolitica* infection depended on the YopP protein and did not require bacterial internalization (19, 42). After *Salmonella* infection of mouse macrophages, apoptosis was an early event associated with membrane ruffling (18). In contrast to murine monocyte-macrophage cell lines, apoptosis of human colon epithelial cells, which are nonphagocytic, was associated with bacterial entry and replication. The commitment of cells to undergo apoptosis at later time points was delayed for up to 6 h after infection, and morphologic and biochemical changes characteristic of apoptosis were delayed for 12–18 h after infection, suggesting that epithelial cells activate antiapoptotic mechanisms. The delayed onset of apoptosis might be explained, in part, by activation of the transcription factor NF-κB after bacterial entry (43). In this regard, NF-κB activation appears to promote cell survival and suppress signals for cell death since inhibition of NF-κB results in increased apoptosis in human and mouse cell lines stimulated with TNF-α and increased apoptosis of cultured human endothelial cells infected with *Rickettsia rickettsii* (36, 44, 45).

Increased apoptosis was observed in three different human intestinal epithelial cell lines that were infected using two different invasive pathogens, *Salmonella* and enteroinvasive *E. coli.* These bacteria use different strategies for host cell invasion and uptake and, following invasion, have different intracellular locations (i.e., *Salmonella* resides in membrane vesicles, whereas enteroinvasive *E. coli* lyse such vesicles and reside in the cytoplasm) (46). These findings suggest that epithelial cell apoptosis is a conserved response of intestinal epithelial cells to bacterial invasion. Consistent with this, we found that human intestinal epithelial cells in an intestinal xenograft model (47) undergo apoptosis following infection of the xenografts with *Salmonella* (data not shown) and others have noted apoptosis of colon epithelial cells in vivo during the course of *Shigella* enteritis (10), although it is not known if this is a direct or indirect response to bacterial invasion.

Enteroinvasive bacteria can pass through epithelial cells within hours after infection (11). Thus, elimination of cells by apoptosis, which occurs later, is unlikely to prevent systemic

host infection by clearing bacteria-infected epithelial cells, although apoptosis could delete epithelial cells that remain persistently infected. However, we suggest that the delayed onset of epithelial cell apoptosis has other important functions for both the host and the invading pathogen. It provides sufficient time for epithelial cells to generate signals required for the rapid activation of mucosal inflammation which can benefit host survival. However, it provides invading bacteria sufficient time to adapt to the intracellular environment and multiply before invading deeper mucosal layers. We further suggest a major role for apoptosis after bacterial infection may be to delete populations of epithelial cells from the intestinal mucosa to restore normal epithelial cell growth regulation. Thus, mucosal infection can result in increased crypt mitotic activity (48, 49), crypt hyperplasia and branching, and increased epithelial cell proliferation with heaping up of epithelial cells (50, 51). Consistent with altered epithelial cell growth, prostaglandin H synthase-2, which inhibits apoptosis in bacteria-infected intestinal epithelial cells (data not shown) as well as noninfected cells (52), is upregulated for more than 24 h after bacterial infection in both infected and uninfected neighboring epithelial cells (26). From the host perspective, deleting those populations of epithelial cells by apoptosis could restore normal regulation of epithelial growth and differentiation, while preserving the integrity of the epithelial mucosal barrier.

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References

1. Wright, N.A., and M. Irwin. 1982. The kinetics of villus cell populations in the mouse small intestine. I. Normal villi: the steady state requirement. *Cell Tissue Kinet.* 15:595–609.

2. Hockenbery, D. 1995. Defining apoptosis. *Am. J. Pathol.* 146:16–19.

3. Hall, P.A., P.J. Coates, B. Ansari, and D. Hopwood. 1994. Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. *J. Cell Sci.* 107:3569–3577.

4. Potten, C.S. 1992. The significance of spontaneous and induced apoptosis in the gastrointestinal tract of mice. *Cancer Metastasis Rev.* 11:179–195.

5. Iwanaga, T., H. Han, K. Adachi, and T. Fujita. 1993. A novel mechanism for disposing of effete epithelial cells in the small intestine of guinea pigs. *Gastroenterology.* 105:1089–1097.

6. Abreu-Martin, M.T., A. Vidrich, D.H. Lynch, and S.R. Targan. 1995. Divergent induction of apoptosis and IL-8 secretion in HT-29 cells in response to TNFα and ligation of Fas antigen. *J. Immunol.* 155:4147–4154.

7. Browning, J.L., K. Miatkowski, I. Sizing, D. Griffiths, M. Zafari, C.D. Benjamin, W. Meier, and F. Mackay. 1996. Signaling through the lymphotoxin β receptor induces the death of some adenocarcinoma tumor lines. *J. Exp. Med.* 183:867–878.

8. Yonehara, S., A. Ishii, and M. Yonehara. 1989. A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J. Exp. Med.* 169:1747–1756.

9. Clayton, F., S. Reka, W.J. Cronin, E. Torlakovic, S.H. Sigal, and D.P. Kotler. 1992. Rectal mucosal pathology varies with human immunodeficiency virus antigen content and disease stage. *Gastroenterology.* 103:919–933.

10. Islam, D., B. Veress, P.K. Bardhan, A.A. Lindberg, and B. Christensson. 1997. In situ characterization of inflammatory responses in the rectal mucosae of patients with shigellosis. *Infect. Immun.* 65:739–749.

11. Eckmann, L., M.F. Kagnoff, and J. Fierer. 1993. Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry. *Infect. Immun.* 61: 4569–4574.

12. Jung, H.C., L. Eckmann, S.K. Yang, A. Panja, J. Fierer, E. Morzycka-Wroblewska, and M.F. Kagnoff. 1995. A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J. Clin. Invest.* 95:55–65.

13. Yang, S.K., L. Eckmann, A. Panja, and M.F. Kagnoff. 1997. Differential and regulated expression of C-X-C, C-C and C-chemokines by human colon epithelial cells. *Gastroenterology.* 113:1214–1223.

14. Witthöft, T., L. Eckmann, J.M. Kim, and M.F. Kagnoff. 1998. Enteroinvasive bacteria directly activate expression of iNOS and NO production in human colon epithelial cells. *Am. J. Physiol.* 275:G564–G571.

15. Kagnoff, M.F., and L. Eckmann. 1997. Epithelial cells as sensors for microbial infection. *J. Clin. Invest.* 100:6–10.

16. Sandoval, M., X. Liu, P.D. Oliver, X.-J. Zhang, D.A. Clark, and M.J.S. Miller. 1995. Nitric oxide induces apoptosis in a human colonic epithelial cell line, T84. *Mediat. Inflammat.* 4:248–250.

17. Zychlinsky, A., M.C. Prevost, and P.J. Sansonetti. 1992. *Shigella flexneri* induces apoptosis in infected macrophages. *Nature.* 358:167–169.

18. Monack, D.M., B. Raupach, A.E. Hromockyj, and S. Falkow. 1996. *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. *Proc. Natl. Acad. Sci. USA.* 93:9833–9838.

19. Mills, S.D., A. Boland, M.P. Sory, P. van der Smissen, C. Kerbourch, B.B. Finlay, and G.R. Cornelis. 1997. *Yersinia enterocolitica* induces apoptosis in macrophages by a process requiring functional type III secretion and translocation mechanisms and involving YopP, presumably acting as an effector protein. *Proc. Natl. Acad. Sci. USA.* 94:12638–12643.

20. Fernandez-Prada, C.M., D.L. Hoover, B.D. Tall, and M.M. Venkatesan. 1997. Human monocyte-derived macrophages infected with virulent *Shigella flexneri* in vitro undergo a rapid cytolytic event similar to oncosis but not apoptosis. *Infect. Immun.* 65:1486–1496.

21. Mantis, N., M.C. Prevost, and P. Sansonetti. 1996. Analysis of epithelial cell stress response during infection by *Shigella flexneri. Infect. Immun.* 64: 2474–2482.

22. Eckmann, L., H.C. Jung, C. Schurer-Maly, A. Panja, E. Morzycka-Wroblewska, and M.F. Kagnoff. 1993. Differential cytokine expression by human intestinal epithelial cell lines: regulated expression of interleukin 8. *Gastroenterology.* 105:1689–1697.

23. Segall, T., and A.A. Lindberg. 1993. Oral vaccination of calves with an aromatic-dependent *Salmonella dublin* (O9,12) hybrid expressing O4,12 protects against *S. dublin* (O9,12) but not against *Salmonella typhimurium* (O4,5,12). *Infect. Immun.* 61:1222–1231.

24. Hoiseth, S.K., and B.A. Stocker. 1981. Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature.* 291:238–239.

25. Galan, J.E., and R.3. Curtiss. 1991. Distribution of the invA, -B, -C, and -D genes of *Salmonella typhimurium* among other *Salmonella* serovars: invA mutants of *Salmonella typhi* are deficient for entry into mammalian cells. *Infect. Immun.* 59:2901–2908.

26. Eckmann, L., W.F. Stenson, T.C. Savidge, D.C. Lowe, K.M. Barrett, J. Fierer, J.R. Smith, and M.F. Kagnoff. 1997. Role of intestinal epithelial cells in the host secretory response to infection by invasive bacteria: bacterial entry induces epithelial prostaglandin H synthase-2 expression, and prostaglandin E_2 and $F_{2\alpha}$ production. *J. Clin. Invest.* 100:296–309.

27. Duke, R.C., R.Z. Witter, P.B. Nash, J.D. Young, and D.M. Ojcius. 1994. Cytolysis mediated by ionophores and pore-forming agents: role of intracellular calcium in apoptosis. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 8:237–246.

28. Filep, J.G., C. Baron, S. Lachance, C. Perreault, and J.S. Chan. 1996. Involvement of nitric oxide in target-cell lysis and DNA fragmentation induced by murine natural killer cells. *Blood.* 87:5136–5143.

29. Zamzami, N., P. Marchetti, M. Castedo, C. Zanin, J.L. Vayssiere, P.X. Petit, and G. Kroemer. 1995. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death in vivo. *J. Exp. Med.* 181:1661–1672.

30. Kluck, R.M., S.J. Martin, B.M. Hoffman, J.S. Zhou, D.R. Green, and D.D. Newmeyer. 1997. Cytochrome c activation of CPP32-like proteolysis plays a critical role in a *Xenopus* cell-free apoptosis system. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:4639–4649.

31. Martin, S.J., C.P. Reutelingsperger, A.J. McGahon, J.A. Rader, R.C. van Schie, D.M. LaFace, and D.R. Green. 1995. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* 182:1545–1556.

32. Koopman, G., C.P. Reutelingsperger, G.A. Kuijten, R.M. Keehnen, S.T. Pals, and M.H. van Oers. 1994. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood.* 84: 1415–1420.

33. Cormack, B.P., R.H. Valdivia, and S. Falkow. 1996. FACS-optimized mutants of the green fluorescent protein (GFP). *Gene.* 173:33–38.

34. Green, L.C., D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok, and S.R. Tannenbaum. 1982. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal. Biochem.* 126:131–138.

35. Huang, G.T., L. Eckmann, T.C. Savidge, and M.F. Kagnoff. 1996. Infection of human intestinal epithelial cells with invasive bacteria upregulates apical

intercellular adhesion molecule-1 (ICAM-1) expression and neutrophil adhesion. *J. Clin. Invest.* 98:572–583.

36. Van Antwerp, D.J., S.J. Martin, T. Kafri, D.R. Green, and I.M. Verma. 1996. Suppression of TNFα-induced apoptosis by NF-κB. *Science.* 274:787–789.

37. Grell, M., G. Zimmermann, D. Hulser, K. Pfizenmaier, and P. Scheurich. 1994. TNF receptors TR60 and TR80 can mediate apoptosis via induction of distinct signal pathways. *J. Immunol.* 153:1963–1972.

38. Ruggiero, V., J. Tavernier, W. Fiers, and C. Baglioni. 1986. Induction of the synthesis of tumor necrosis factor receptors by interferon-γ. *J. Immunol.* 136:2445–2450.

39. Sandoval, M., X.-J. Zhang, X. Liu, E.E. Mannick, D.A. Clark, and M.J.S. Miller. 1997. Peroxynitrite-induced apoptosis in T84 and RAW 264.7 cells: attenuation by L-ascorbic acid. *Free Radic. Biol. Med.* 22:489–495.

40. Enari, M., H. Sakahira, H. Yokoyama, K. Okawa, A. Iwamatsu, and S. Nagata. 1998. A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature.* 391:43–50.

41. Zychlinsky, A., and P. Sansonetti. 1997. Perspectives series: host/pathogen interactions. Apoptosis in bacterial pathogenesis. *J. Clin. Invest.* 100:493–495.

42. Thirumalai, K., K.S. Kim, and A. Zychlinsky. 1997. IpaB, a *Shigella flexneri* invasin, colocalizes with interleukin-1β-converting enzyme in the cytoplasm of macrophages. *Infect. Immun.* 65:787–793.

43. Dyer, R.B., C.R. Collaco, D.W. Niesel, and N.K. Herzog. 1993. *Shigella flexneri* invasion of HeLa cells induces NF-κB DNA-binding activity. *Infect. Immun.* 61:4427–4433.

44. Beg, A.A., and D. Baltimore. 1996. An essential role for NF-κB in preventing TNFα-induced cell death. *Science.* 274:782–784.

45. Clifton, D.R., R.A. Goss, S.K. Sahni, D. Van Antwerp, R.B. Baggs, V.J. Marder, D.J. Silverman, and L.A. Sporn. 1998. NF-κB dependent inhibition of apoptosis is essential for host cell survival during *Rickettsia rickettsii* infection. *Proc. Natl. Acad. Sci. USA.* 95:4646–4651.

46. Finlay, B.B., and P. Cossart. 1997. Exploitation of mammalian host cell functions by bacterial pathogens. *Science.* 276:718–725.

47. Savidge, T.C., A.L. Morey, D.J. Ferguson, K.A. Fleming, A.N. Shmakov, and A.D. Phillips. 1995. Human intestinal development in a severe-combined immunodeficient xenograft model. *Differentiation.* 58:361–371.

48. Mathan, M.M., and V.I. Mathan. 1991. Morphology of rectal mucosa of patients with shigellosis. *Rev. Infect. Dis.* 13 (Suppl 4):S314–S318.

49. Savidge, T.C., A.N. Shmakov, J.A. Walker-Smith, and A.D. Phillips. 1996. Epithelial cell proliferation in childhood enteropathies. *Gut.* 39:185–193.

50. Islam, M.M., A.K. Azad, P.K. Bardhan, R. Raqib, and D. Islam. 1994. Pathology of shigellosis and its complications. *Histopathology.* 24:65–71.

51. Sachdev, H.P., V. Chadha, V. Malhotra, A. Verghese, and R.K. Puri. 1993. Rectal histopathology in endemic *Shigella* and *Salmonella* diarrhea. *J. Pediatr. Gastroenterol. Nutr.* 16:33–38.

52. Tsujii, M., and R.N. DuBois. 1995. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell.* 83:493–501.