doi: 10.1111/j.1365-3083.2005.01626.x

Expression of the Virulence Factor, BfpA, by Enteropathogenic *Escherichia coli* is Essential for Apoptosis Signalling but not for NF-κB Activation in Host Cells

A. R. Melo*, E. B. Lasunskaia*, C. M. C. de Almeida*, A. Schriefer†‡, T. L. Kipnis* & W. Dias da Silva*

*Laboratório de Biologia do Reconhecer, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Campos dos Goytacazes, RJ; †Serviço de Imunologia – Hospital Universitário Professor Edgard Santos; and ‡Departamento de Ciências da Biointeração – Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, BA, Brazil

Received 21 January 2005; Accepted in revised form 12 April 2005

Correspondence to: Dr W. Dias da Silva, Laboratório de Biologia do Reconhecer, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, Avenue Alberto Lamego, 2000, Campos dos Goytacazes – RJ, CEP:28013-600, Brazil. E-mail: wds@uenf.br

Abstract

Localized adherence (LA) of enteropathogenic *Escherichia coli* (EPEC) to epithelial cells results in attaching and effacing of the surface of these cells. LA depends on the gene *bfpA*, which codes for the BfpA protein. We found that EPEC-E. coli adherence factor (EAF)$^{(+)}$, expressing BfpA, significantly reduced HeLa cell viability in comparison with EPEC-EAF$^{(-)}$, as evaluated by the mitochondrial-dependent succinate dehydrogenase conversion of 3′-[4,5,-dimethylthiazol-2yl]2,5-diphenyltetrazolium bromide (MTT) to its formazan. Apoptosis accounts for a substantial loss of the cell viability, because the cells incubated with EPEC-EAF$^{(+)}$ or with cloned BfpA (data not shown), but not with EPEC-EAF$^{(-)}$, were positive for annexin-V binding, demonstrated chromatin condensation and nuclei fragmentation and exhibited a high level of caspase-3 activity. Because the blockade of bacterial cell-surface-associated BfpA by anti-BfpA immunoglobulin (Ig)Y antibody suppressed apoptotic death induced by EPEC-EAF$^{(+)}$, BfpA may be the trigger for apoptosis. Both EPEC-EAF$^{(+)}$ and EPEC-EAF$^{(-)}$, as well as recombinant BfpA (data not shown), activated nuclear factor (NF)-κB in a similar manner as analysed by the electrophoretic mobility shift assay (EMSA). EMSA supershift analysis demonstrated the presence of p65/RelA in a DNA-binding complex. In contrast to DNA binding, NF-κB-dependent reporter gene transactivation was stimulated more strongly by EPEC B171/EAF$^{(+)}$, suggesting a role for this virulence factor in the regulation of transcriptional activity of NF-κB. Because suppression of NF-κB activation by BAY11-7085, a NF-κB inhibitor, neither induced apoptosis by itself nor blocked apoptosis induction by EPEC-EAF$^{(+)}$, it may be suggested that apoptosis is not regulated by the NF-κB pathway in HeLa cells.

Introduction

Enteropathogenic *Escherichia coli* (EPEC) adheres to epithelial cells in circumscribed clusters designated as localized adherence (LA) phenotype [1]. Primary adhesion is mediated by the bundle-forming pilus (BFP) expressed by EPEC [2]. BFP, an important virulence factor of EPEC [3], is encoded by the gene *BfpA*, localized in 50–70 MDa plasmids [4–6]. LA [7] is followed by the 'attaching and effacing' (AE) lesion, which is characterized by microvilli destruction, intimate adherence of bacteria to the intestinal epithelium, pedestal formation and aggregation of polarized actin and other elements of the cytoskeleton at sites of bacterial attachment [8]. Within adherent microcolonies of EPEC, the BFP organize a meshwork attaching bacteria to themselves and tethering individual bacteria to the host cell surface [9].

Adhesion of EPEC to the epithelial cells was demonstrated to be essential for the activation of nuclear factor (NF)-κB and proinflammatory interleukin (IL)-8 transcription [10], as well as to induce cell death by apoptosis or necrosis [11–14]. Bacterial components responsible for these effects have not been completely elucidated. *E. coli* intimin, the protein responsible for AE, was found not to be important for apoptosis induction, nor for NF-κB activation, because the EPEC *eaeA* deletion mutant CVD206, lacking this protein, continued to be a potent apoptosis [14] and NF-κB inductor [10]. Recently, we demonstrated that immunoglobulin (Ig)Y anti-recombinant

BfpA protein antibody identifies *E. coli*-bearing BfpA, blocks colonization of HeLa cells by EPEC-EAF$^{(+)}$ *in vitro* and inhibits *in vitro* growth of EPEC-EAF$^{(+)}$, but not of EPEC-EAF$^{(-)}$ [15].

In this study, we show that EPEC expressing BfpA, EPEC-EAF$^{(+)}$, but not EPEC-EAF$^{(-)}$, the BfpA cured counterpart bacteria, induced apoptosis in HeLa cells; this effect was blocked by previous neutralization of BfpA with an IgY anti-BFP antibody. These data agree with previous observations indicating that induction of epithelial cell death by *E. coli* depends on the BFP expression by the bacterium [14]. In contrast, expression of BfpA by the bacteria appears not to be essential for NF-κB activation by EPEC.

Materials and methods

Cell culture. HeLa cells and murine lineage A2 cells transfected with *hsp*70 [nitric oxide synthase (NOS) cells obtained from the American Type Culture Collection (Mannasas, VA, USA)] were cultivated in plastic flasks containing Dulbecco's modified Eagle's medium (DMEM) F-12 (Gibco BRL, Grand Island, NY, USA) at 37 °C, under CO_2. For use, desired amounts of cells were harvested at the growth logarithmic phase and transferred to 24-well plates (Corning Glass, Corning, NY, USA).

Escherichia coli growth. B171 is a EAF$^{(+)}$ (O111:NM) EPEC strain (EPEC-EAF$^{(+)}$); B171-4 is a B171 EAF$^{(-)}$ cured derivative (EPEC-EAF$^{(-)}$). The bacterial strains were maintained at −70 °C or in stabs and slants of Luria-Bertrani (LB) agar, for long-term and short-term storage, respectively, and propagated in liquid LB broth supplemented with appropriate antibiotics when required [16].

Treatment of EPEC with IgY antibody anti-recombinant BfpA protein. To block BfpA exposed on the EPEC cell surface, samples of EPEC-EAF$^{(+)}$ were incubated, for 3 h at 37 °C, with 800 μg of IgY anti-BfpA obtained as described [15]. As a control, EPEC samples were similarly treated with control IgY prepared from egg yolks obtained before immunization of the hens with BfpA recombinant protein.

Effect of EPEC on HeLa cell viability. HeLa cells (2.5×10^6) in 100 μl of DMEM were cultured overnight at 37 °C under 5% CO_2 in 24-well plates provided or not provided with glass coverslips. The cells were washed twice with DMEM and incubated with 100 μl of samples of EPEC-EAF$^{(+)}$ or EPEC-EAF$^{(-)}$ containing 5.0×10^4 bacteria for 4 h under the same conditions. Similar cell cultures incubated without bacteria were always included as negative controls. When indicated, similar HeLa cell cultures were incubated or treated (i) with *Salmonella typhimurium* lipopolysaccharide (LPS) (10 ng/ml) (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37 °C; (ii) EPEC-EAF$^{(+)}$ pretreated with IgY anti-BfpA or with control IgY and (iii) with 10 μM of the NF-κB inhibitor BAY11-7085 (Calbiochem, San Diego, CA, USA) for 1 h at 37 °C before EPEC addition. Murine lineage A2 cells transfected with *hsp*70 (NOS cells) incubated with 3 μM adenosine 5′-triphosphate (ATP) (Sigma-Aldrich) for 16 h at 37 °C, when indicated, were used as a positive control for apoptosis.

Representative cell aliquots were removed from each cell culture at different postinfection time points (0, 1, 2 and 4 h) and the cell viability analysed by the 3′-[4,5,-dimethylthiazol-2yl]2,5-diphenyltetrazolium bromide (MTT) test [17]. MTT dye (0.5 mg/ml) was added to the cell samples and incubated for 1 h at 37 °C. In living cells, but not in dead cells, mitochondrial succinate dehydrogenase converts MTT to its formazan, which can be extracted and dissolved in 0.04 M HCl–isopropanol. The absorbance of the resulting solutions was quantified spectrophotometrically at 570 nm on a multiwell plate reader (Model no. 3550, Bio-Rad, Mississauga, Canada). Results are expressed as the mean optical density ± SD obtained from four independent experiments. Significance was analysed by the Student's t-test (discussed later).

Analysis of apoptosis. To determine the cell death type, HeLa cells, incubated with EPEC-EAF$^{(+)}$ or EPEC-EAF$^{(-)}$ strains, were either labelled with fluorescein isothiocyanate-conjugated annexin-V (Boehringer Mannheim, Oberkochen, Germany), which binds to phosphatidylserine exposed on the outer leaflet of cells undergoing apoptosis [18, 19], or stained with a mixture of DNA fluorochromes and acridine orange/ethidium bromide and then analysed for the presence of typical nuclear morphological aspects of apoptotic cells, allowing discrimination between apoptotic and necrotic cells [20]. The apoptotic fluorescent cells, identified by annexin-V binding, were visualized by fluorescence microscopy (×20) (Axoplan, Carl Zeiss, Oberkochen, Germany) and the resulting images photographed. Cells exhibiting extensive condensation of chromatin and internucleosomal DNA fragmentation were determined by visual scoring of a minimum of 200 cells in a fluorescent microscope. Cells with morphologically normal nuclei (living cells) or with condensed and fragmented chromatin (primary apoptosis) stained with acridine orange or with ethidium bromide (secondary apoptosis) were scored. The percentage of primary and secondary apoptotic cells in each sample was calculated as a function of the number of counted cells. Uninfected HeLa cells were used as a negative control. Results are expressed as mean percentage ± SD of apoptotic cells versus the total number of cells from several independent experiments.

Total and nuclear cell extracts. HeLa cell cultures in DMEM after desired times of incubation were collected and centrifuged at 1000 × ***g*** at 4 °C and the pellets washed three times in cold phosphate-buffered saline (PBS). To prepare total cell extracts, pellets corresponding to 2.5×10^7 cells were suspended in 500 μl of low radioimmunoprecipitation (RIPA) buffer [20 mM Tris-buffer, pH 7.5, containing 150 mM ethylenediaminetetraacetic

acid (EDTA), 0.5% TRITON X-100, 1 mM sodium orthovanadate and phenylmethylsulfonyl fluoride (PMSF)] and submitted to three cycles of freezing-thawing in liquid nitrogen. Lysed cells were centrifuged at 1000 × *g* for 10 min at 4 °C. The supernatants were recovered and the protein contents were determined by the Bradford method [21], before adjusting to 60 μg of protein per sample. The proteins were precipitated with six volumes (v/v) of cold acetone and incubated for 30 min at 20 °C. After centrifugation for 10 min at 1000 × *g* at room temperature, the supernatants were discarded and the precipitates dried also at room temperature. To prepare cytosolic and nuclear cell extracts, pellets corresponding to 5×10^6 cells were suspended in 400 μl of cell membrane lysis buffer (10 mM N-[2-Hydroxyethyes piperazine-N^1-[2-ethanesulfonic acid] (HEPES), pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 1.0 mM dithiothreitol (DTT) and 0.5 mM PMSF) and incubated for 20 min on ice, as described [22]. After addition of Nonidet P40 to a final concentration of 0.6%, the samples were allowed to stand for 10 s, stirred and centrifuged for 30 s at 2500 × *g*. The supernatants from each sample were withdrawn and corresponding pellets were used to prepare the nuclear extracts, as described [22]. Briefly, the pellets were complemented with 50 μl of nuclear cell membrane lysis buffer (10 mM HEPES, pH 7.9, containing 0.4 mM NaCl, 1.0 mM EDTA, 1 mM Ethylene glycol bis (B-aminoethylether) N, N, N^1, N^1-tetraacetate (EGTA), 1.0 mM DTT and 1 mM PMSF) and stirred for 15 min at 4 °C. After centrifugation for 5 min at 6000 × *g* at 4 °C, the protein contents were determined and adjusted to 60 μg per sample. Before electrophoresis, 10 μl of sample buffer [62.5 mM Tris, pH 6.8, containing 10% glycerine and 2% sodium dodecyl sulfate (SDS)] was added to each cell extract.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed essentially as described [10]. Nuclear extracts (5 μg), obtained at the indicated time points after HeLa cell infection with EPEC-EAF(+) or EPEC-EAF(–), were subjected to EMSA-binding reaction in buffer containing 10 mM Tris (pH 7.9), 1 mM EDTA, 1 mM DTT, 1 μg of poly (dI-dC), 100 mM NaCl and 10% glycerol in a final volume of 20 μl. Complementary oligonucleotides representing the NF-κB consensus site (upper strand, 5′-AGT TGA GGG GAC TTT CCC AGGC-3′; lower strand, 3′-TCA ACT CCC CTG AAA GGG TCCG-5′) (Promega, Madison, WI, USA) were 3′-end labelled with γ-^{32}P using standard procedures γ-^{32}P ATP (>3000 Ci/mmol) (Amersham Life Sciences, USA), and 10,000 cpm of the probe were incubated with nuclei proteins for 15 min (employed for DNA-binding analysis). Samples were loaded directly onto nondenaturing 4% polyacrylamide gels and the electrophoresis performed in ×0.5 Tris-borate buffer, pH 8.0 containing EDTA (TBE) buffer for 2–3 h at room temperature to separate bound and unbound DNA probe by standard procedures. The specific NF-κB competitive assay was performed with a 50-fold molar excess of unlabelled probe. The gels were dried and exposed to Kodak MS film with appropriate intensifying screens. The band intensity was quantified using a gel documentation system.

Supershift EMSA analysis of DNA-binding NF-κB proteins. Supershift modification of EMSA [10] was used to determine whether p65/RelA, a transactivating NF-κB family member, is activated upon HeLa cell infection with EPEC-EAF(+) or EPEC-EAF(–). The EMSA experiments were performed essentially as described above, with the exception that the anti-p65 antibody was added (Santa Cruz Biotechnology, CA, USA) to the binding reaction and the samples were incubated for an additional period of 30 min at 37 °C.

Transient cellular transfection. Confluent (70–80%) monolayers of HeLa cells cultured in six-well plates were washed with DMEM and transfected with NF-κB/luciferase reporter plasmid, according to the EFFECTENE Qiagen protocol. DNA (0.5 μg) was diluted into 4 μl of Tris-EDTA-buffer, pH 8.0 (TE) buffer (pH 7.4) and the volume completed to 100 μl with EC buffer (pH 7.4). *Enhancer* (3.2 μl) was then added and the mixture stirred strongly for 1 min and incubated at room temperature for 5 min. After addition of 10 μl of the transfecting reagent, EFFECTENE, to the DNA-*enhancer* mixture, the complex was allowed to form upon incubation for 10 min at room temperature. DMEM without fetal calf serum (600 μl) was then added. After careful homogenization, the transfecting complex was added, drop-by-drop, onto the HeLa cells. Following incubation for 18 h at 37 °C in a 5% CO_2 atmosphere, the cells were washed with cold PBS, removed with the aid of trypsin/EDTA solution and reincubated in fresh DMEM. The transfected HeLa cells were incubated with EPEC-EAF(+) or EPEC-EAF(–) and incubated for an additional 6 h under the same conditions. After incubation, transfected cells were washed with PBS, lysed with a luciferase lysis buffer and assayed for luciferase activity, according to the manufacturer's instructions (Promega). Luciferase activity was measured with an Electronic Scintilator-Top Count-3.01 (Promega). Cell transfections were performed in four different experiments with similar results.

Western blot analysis. The presence of particular proteins or phosphorylated forms of proteins was analysed on whole cell proteins extracted by low RIPA buffer from HeLa cells experiments, as indicated above. Each protein sample (60 μg) was mixed 1/1 with ×2 sample buffer [20% glycerol, 4% SDS, 10% 2-B-mencoftoethanol (ME), 0.1% bromophenol blue and 0.125 M Tris-Cl (pH 6.8)], boiled at 95 °C for 5 min, loaded onto 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel and run at 30 mA for 3 h. Cell proteins were electrotransferred to nitrocellulose membranes overnight at 30 V. Equal loading of the proteins (groups) on the blots was

evaluated using Ponceaus S (Bio-Rad Laboratories, Hercules, CA, USA). The resulting nitrocellulose membranes were then blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20. Blotted membranes were treated with primary mouse antibody, anti-p-IκB-α or anti-caspase-3 (1/100, Santa Cruz Biotechnology), for 1 h, and then with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (1/5000 to 1/20,000) (Southern Biotechnology Associates Inc., Birmingham, AL, USA). Immunoreactive bands were developed using a chemiluminescent substrate, ECL Plus (Amersham Pharmacia Biotech, Piscataway, NJ, USA). An autoradiograph was obtained with exposure times of 10 s to 2 min. When indicated, the relative amounts of the proteins in Western blotting bands were quantified by densitometrical analysis of a TIFF image obtained with a commercial scanner at 400 dpi, using a computational program [23].

Data analysis. The number of experiments analysed is indicated in each figure. Statistical analysis was performed using the Student's *t*-test, and the differences were determined to be statistically significant at $P < 0.05$.

Results

Both EPEC-EAF$^{(+)}$ and EPEC-EAF$^{(-)}$ induce apoptosis in HeLa cells

As demonstrated in Fig. 1, HeLa cells, upon incubation with either EPEC-EAF$^{(+)}$ or EPEC-EAF$^{(-)}$, partially lost their ability to convert MTT to its formazan by the action of their mitochondrial-dependent succinate dehydrogenase enzyme, as compared with HeLa cells incubated without bacteria. This effect was significantly higher in the presence of EPEC-EAF$^{(+)}$ (67.35% reduction, $P < 0.001$) rather than EPEC-EAF$^{(-)}$ in the assay (30.65% reduction, $P < 0.05$). Figure 2 shows that the number of cells infected with EPEC-EAF$^{(+)}$ exhibiting typical fluorescence upon labelling with annexin-V was significantly higher (Fig. 2D), as compared either with cells infected with EPEC-EAF$^{(-)}$ (Fig. 2B) or with noninfected cells (Fig. 2A). A significant proportion of ATP-treated NOS cells was also annexin-V-positive (Fig. 2C). Induction of apoptosis HeLa cells by EPEC-EAF$^{(+)}$ was confirmed by quantifying the number of cells exhibiting chromatin condensation and nuclei fragmentation upon staining with the DNA fluorochromes. Approximately 40% and 15% of the cells incubated with EPEC-EAF$^{(+)}$ or EPEC-EAF$^{(-)}$, respectively, underwent the typical apoptotic nuclear alterations (Fig. 3).

EPEC induces procaspase-3 activation

HeLa cells were incubated with EPEC-EAF$^{(+)}$ or EPEC-EAF$^{(-)}$, or only in DMEM for 2 and 4 h at 37 °C. The resulting cell proteins were analysed by Western blot, employing specific polyclonal antibodies to caspase-3. Figure 4 shows that cell extracts obtained from HeLa cells incubated with DMEM or EPEC-EAF$^{(-)}$ exhibited protein bands with a molecular mass of 32 kDa corresponding to procaspase-3. An active caspase-3 band of 17 kDa, resulting from the procaspase-3 cleavage upon apoptosis development, was only detected in extracts

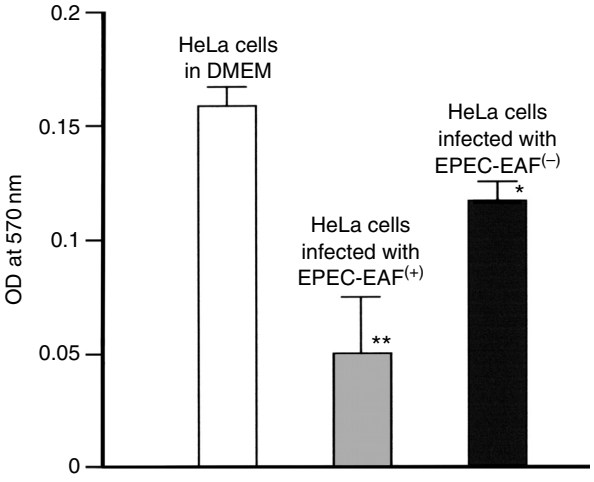

Figure 1 Enteropathogenic *Escherichia coli* (EPEC) reduces HeLa cell viability. HeLa cells (1×10^5) were incubated for 4 h at 37 °C with EPEC-EAF$^{(+)}$ or EPEC-EAF$^{(+)}$ (1×10^4) and evaluated by measuring their capacity to convert MTT dye to its formazan by the action of their mitochondrial-dependent succinate dehydrogenase enzyme. The dark blue crystals of formazan were dissolved and quantified spectrophotometrically. Absorbance (optical density, OD) at 570 nm is presented as the mean ± SE. Data from four independent experiments are shown. Infected cells were compared with uninfected cells (**$P < 0.001$, *$P < 0.05$).

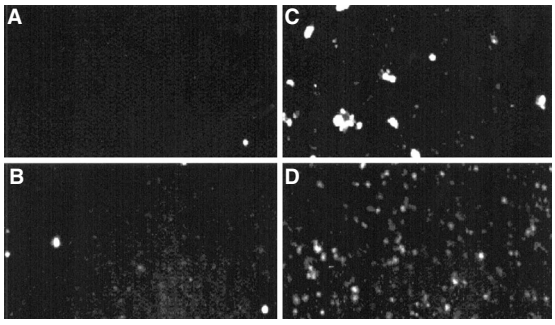

Figure 2 Annexin-V binding in HeLa cells incubated with enteropathogenic *Escherichia coli* (EPEC), demonstrating apoptosis. HeLa cells (1×10^5) were incubated with EPEC-EAF$^{(+)}$ or EPEC-EAF$^{(-)}$ (1×10^4) for 4 h at 37 °C and then incubated with fluorescein isothiocyanate-conjugated annexin-V and examined for annexin-V binding using fluorescence microscopy (×20). Noninfected HeLa cells and NOS cells, induced to apoptosis by adenosine 5′-triphosphate, were included as negative and positive controls, respectively. Noninfected cells (A); EPEC-EAF$^{(-)}$-infected cells (B); NS0 cells (C); EPEC-EAF$^{(+)}$-infected cells (D). Images represent one of five independent experiments.

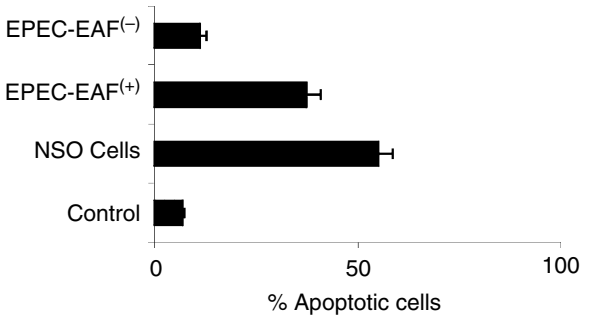

Figure 3 Enteropathogenic *Escherichia coli* (EPEC)-EAF$^{(+)}$ induces alterations typical of apoptosis in epithelial cell nuclei. HeLa cells (1×10^5) were incubated with EPEC-EAF$^{(+)}$ or EPEC-EAF$^{(-)}$ (1×10^4) for 4 h at 37 °C. After incubation, the monolayers were washed and detached cells were collected by centrifugation. Attached and detached HeLa cells were separately stained with a DNA fluorochrome mixture and examined by fluorescence microscopy. The number of cells exhibiting typical apoptotic morphology was counted and the percentage of apoptotic cells was calculated. NOS cells induced to apoptosis by 3 μM adenosine 5′-triphosphate were used as a positive control. Uninfected HeLa cells were used as a negative control. The rate of cells exhibiting apoptotic condensation of chromatin and/or nuclei fragmentation was determined by visual scoring of a minimum of 200 cells per sample in a fluorescent microscope. Results are expressed as a mean percentage ± SD of apoptotic cells versus the total number of cells from several independent experiments.

from the HeLa cells incubated with EPEC-EAF$^{(+)}$. After 4 h of incubation, although a protein band corresponding to procaspase-3 was still detected in cell extracts from cells incubated with EPEC-EAF$^{(-)}$, it disappeared from cells incubated with EPEC-EAF$^{(+)}$.

EPEC induces NF-κB activation in epithelial cells

EMSA was employed to determine whether interaction of EPEC with HeLa cells could induce NF-κB translocation to the nucleus and DNA binding. A double-stranded oligonucleotide NF-κB consensus motif labelled with γ-^{32}P ATP was used to probe nuclear extracts derived from HeLa cells incubated with EPEC-EAF$^{(+)}$ or EPEC-EAF$^{(-)}$. Figure 5(A) demonstrated that both bacterial strains induced a nucleoprotein complex in a similar

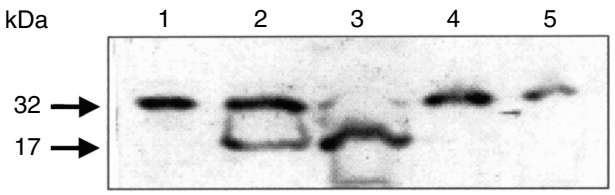

Figure 4 Procaspase-3 is activated in HeLa cells infected with enteropathogenic *Escherichia coli* (EPEC)-EAF$^{(+)}$, but not with EPEC-EAF$^{(-)}$. HeLa cells (2.5×10^6) were incubated with EPEC-EAF$^{(+)}$ or EPEC-EAF$^{(-)}$ (5×10^4) for 2 or 4 h at 37 °C. Uninfected HeLa cells were used as negative controls. After the indicated time of incubation, cells were removed and lysed and the extracts were submitted to Western blotting (10% SDS-PAGE). The protein bands were electrotransferred to nitrocellulose membranes, and the blots were examined with anti-caspase-3 antibody. (1) negative control; (2) EPEC-EAF$^{(+)}$, 2 h; (3) EPEC-EAF$^{(+)}$, 4 h; (4) EPEC-EAF$^{(-)}$, 2 h; (5) EPEC-EAF$^{(-)}$,4 h. Data represent one of five independent experiments.

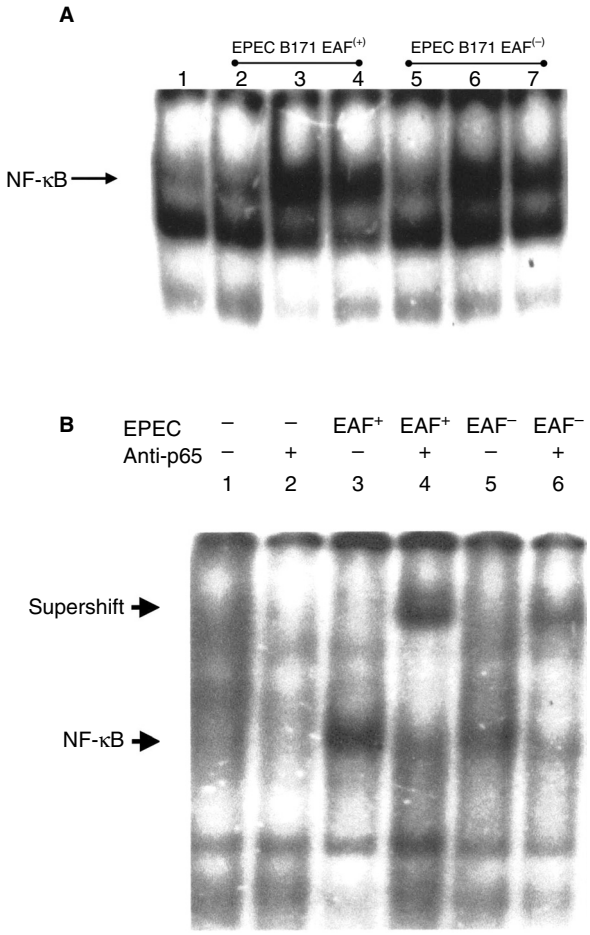

Figure 5 Both enteropathogenic *Escherichia coli* (EPEC)-EAF$^{(+)}$ and EPEC-EAF$^{(-)}$ activate nuclear factor (NF)-κB in HeLa cells. HeLa cells (2.5×10^6) and EPEC-EAF$^{(+)}$ or EPEC-EAF$^{(-)}$ (5×10^4) were incubated for 1–4 h at 37 °C. Uninfected HeLa cell cultures in Dulbecco's modified Eagle's medium were used as negative controls. Cells were lysed and nuclear extracts were submitted to an electrophoretic mobility shift assay (EMSA). The presence of NF-κB-activated proteins in the cell nuclei was demonstrated by binding to oligonucleotide probes containing a single copy of the NF-κB-motif 5′-AGTTGAGGGGACTTTCCCAGGC −3′ (Promega), end labelled with γ-^{32}P ATP. (A) Binding reactions were electrophoresed on native 4% polyacrylamide gels to separate bound and unbound DNA probe: (1) noninfected HeLa cells; (2, 3 and 4) HeLa cells infected for 1–4 h with EPEC-EAF$^{(+)}$; (5, 6, and 7) HeLa cells infected for 1–4 h with EPEC-EAF$^{(-)}$. Data represent one of five independent experiments. (B) Identification of the p65 NF-κB protein in the nuclei of HeLa cells infected with EPEC for 2 h at 37 °C. DNA-binding reaction was performed in the presence or in the absence of anti-p65/Rel A NF-κB antibody and then submitted to EMSA: (1) noninfected cells; (2) noninfected cells + antibody; (3) EPEC-EAF$^{(+)}$ (2 h); (4) EPEC-EAF$^{(+)}$ + antibody; (5) EPEC-EAF$^{(-)}$ (2 h); (6) EPEC-EAF$^{(-)}$ (2 h) + antibody. Data represent one of three independent experiments.

manner, whereas noninfected HeLa cells exhibited no NF-κB activity. NF-κB–DNA binding was detectable within 1 h of incubation and maintained up to 4 h with maximal binding at 2 h postinfection. To identify the presence of p65/RelA, a transcriptionally active NF-κB family member in the nucleoprotein complex was observed using the supershift EMSA. The reaction mixture was incubated in the presence of the anti-p65 antibody and submitted to electrophoresis. Figure 5(B) shows the supershifted nucleoprotein complex in samples from cells infected with the EPEC, confirming the similar character of NF-κB activation by both strains.

EPEC induces phosphorylation of IκB-α

IκB-α phosphorylation precedes degradation of this inhibitory molecule and subsequent nuclear translocation of NF-κB. A representative PAGE (Fig. 6A) shows that in HeLa cells incubated with EPEC-EAF$^{(+)}$, accumulation of phosphorylated IκB-α was observed to be significantly more pronounced than in HeLa cells incubated with EPEC-EAF$^{(-)}$ or with LPS. Densitometry of the protein bands separated by Western blot confirmed the visual inspection (Fig. 6B).

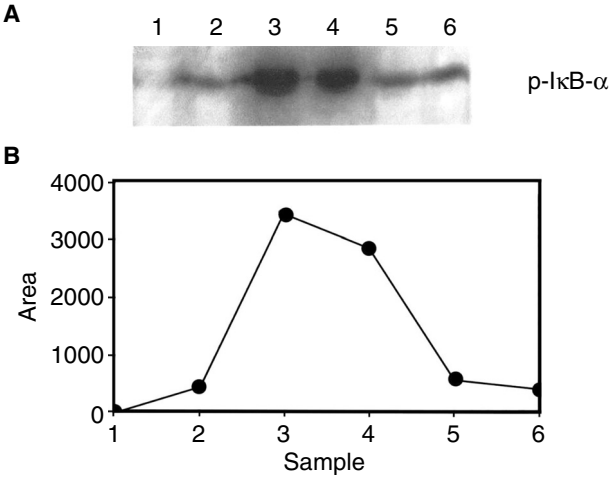

Figure 6 The IκB-α phosphorylation is induced differently by enteropathogenic *Escherichia coli* (EPEC) expressing or not BfpA. HeLa cells (2.5×10^6) were incubated either with EPEC-EAF$^{(+)}$ or EPEC-EAF$^{(-)}$ (5×10^4). Untreated HeLa cells or cells incubated in the presence of lipopolysaccharide (LPS) for 2 h were used as negative and positive controls, respectively. After incubation, the cells were lysed and whole cell proteins were examined by Western blotting, employing an antibody against the phosphorylated form of IκB-α. (A) Western blot of the electrophoretic mobility shift assay analysis: (1) negative control; (2) LPS (10 ng); (3) EPEC-EAF$^{(+)}$, 2 h; (4) EPEC-EAF$^{(+)}$, 4 h; (5) EPEC-EAF$^{(-)}$, 2 h; (6) EPEC B171/EAF$^{(-)}$, 4 h. (B) Densitometric quantification of proteins in Western blotting bands. Data represent one of three independent experiments.

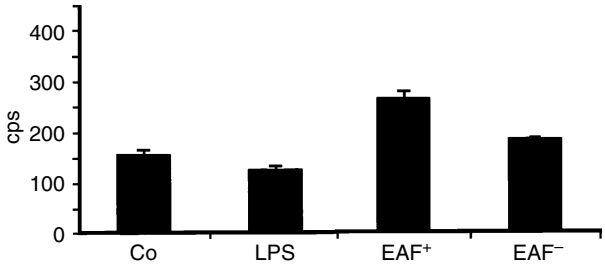

Figure 7 Nuclear factor (NF)-κB-dependent gene transcription is more strongly stimulated with BfpA-expressing EPEC. HeLa cells, transfected with NF-κB/luc reporter plasmid, were incubated with EPEC-EAF$^{(+)}$ or EPEC-EAF$^{(-)}$. Similar transfected cells were incubated in Dulbecco's modified Eagle's medium (negative control) or treated with lipopolysaccharide (10 ng). After washing, the cells were lysed and the luciferase activity was quantified. Data represent the chemiluminescence ratio relative to stimulated and unstimulated transfected cells. The data presented represent one of four independent experiments.

EPEC induces transcriptional activity of NF-κB in HeLa cells

In order to evaluate NF-κB transactivation capacity, HeLa cell monolayers were transfected with the NF-κB/luc reporter plasmid. Transfected cells were then incubated with EPEC and the luciferase activity was measured. Figure 7 shows that EPEC-EAF$^{(+)}$ induces higher reporter gene activity than EPEC-EAF$^{(-)}$. LPS, used as control, failed to activate this gene.

BfpA blockade with anti-BfpA IgY, but not NF-κB inhibition, prevents apoptosis induced by EPEC-EAF$^{(+)}$

To verify the importance of BfpA expression by EPEC-EAF$^{(+)}$ for apoptosis induction, HeLa cell cultures were incubated in parallel with EPEC-EAF$^{(+)}$ previously treated with anti-BfpA IgY antibody or with control IgY. As shown in Fig. 8, blockade of BfpA in EPEC-EAF$^{(+)}$ with IgY anti-BfpA IgY, but not with control IgY, inhibits its ability to induce apoptosis in HeLa cells. Previous incubation of HeLa cells with BAY11-7085, a chemical compound known to decrease NF-κB activation by inhibiting IκB-α phosphorylation [24], neither inhibited apoptosis induced by EPEC-EAF$^{(+)}$ (43% of apoptotic cells) nor facilitated induction of apoptosis by EPEC-EAF$^{(-)}$ (14.3% of apoptotic cells); furthermore, it did not induce apoptosis by itself.

Discussion

Induction of intestinal epithelium cell death is one pathogenetic factor favouring enterobacterial growth in infected organisms. A variety of enterobacteria such as *Salmonella*, *Shigella* and *Yersinia* induce apoptosis through intimate contact or invasion of epithelial cells and liberation of cytotoxic factors, *Avr A* [25, 26], *IpaB* [27] or *Yop* [28]. EPEC has also been demonstrated to induce apoptosis in epithelial cells [11–13]. The BFP protein has been suggested to be responsible for induction of cell death [14],

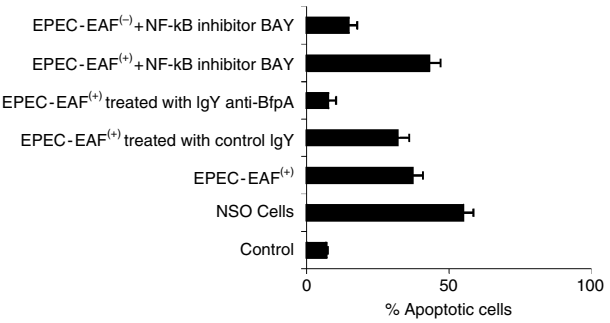

Figure 8 Enteropathogenic *Escherichia coli* (EPEC)-induced apoptosis in HeLa cells is suppressed through BfpA blockade with IgY-specific antibody, but not through inhibition of nuclear factor (NF)-κB activation. HeLa cells (1×10^5) were incubated with EPEC B171/EAF$^{(+)}$ (1×10^4) pretreated, or not, with IgY anti-BfpA or with control IgY for 4 h at 37 °C. Similar HeLa cells cultures were incubated for 1 h at 37 °C with the NF-κB inhibitor, BAY11-70, and then infected with untreated EPEC-EAF$^{(+)}$ with IgY anti-BfpA. Noninfected HeLa cells incubated in Dulbecco's modified Eagle's medium (DMEM) or in DMEM plus dimethylsulfoxide (negative controls) or NOS cells treated with 3 μM ATP (positive control) were included. After 4-h incubation with bacteria, the cells were stained with DNA fluorochromes and examined for apoptotic morphology, as indicated in the legend to Fig. 3. Results are expressed as mean percentage ± SD of apoptotic cells versus the total number of cells from four independent experiments.

although the pathway and mechanism of death were not determined. In the current investigation, we addressed the role of BfpA in the induction of epithelial cell apoptosis, employing bacterial strains that were positive or negative for BfpA expression.

The data herein described provide evidence that EPEC-EAF$^{(+)}$, which is equipped with the gene *bfpA* coding for BfpA, but not its counterpart EPEC-EAF$^{(-)}$ that lacks this gene, activates the apoptotic death programme in HeLa cells.

The apoptotic nature of death was confirmed by annexin-V binding to phosphatidylserine on the outer surface of the cellular membrane (Figs 1 and 2) and by the typical apoptotic alterations, such as fragmentation and condensation of chromatin, observed in the nucleus of the cells infected with EPEC-EAF$^{(+)}$, but not of cells infected with EPEC-EAF$^{(-)}$ (Fig. 3). One family of mediators implicated in the apoptosis pathway is the aspartate-specific cysteinyl proteases or caspases. A member of this family, caspase-3 (CPP32, apopain, YAMA), has been identified as being a key mediator of apoptosis in mammalian cells [29]. As apoptosis induction and caspase-3 activation occurred in parallel in EPEC-EAF$^{(+)}$, but not in EPEC-EAF$^{(-)}$ (Fig. 4), both phenomena are thought to be intimately implicated.

E. coli-induced apoptosis was characterized by rapid permeabilization of cell membrane and secondary necrosis, providing morphological markers of both apoptosis and necrosis observed in the majority of dead cells after infection. Nonetheless, typical features in the cells' nuclei suggest an apoptotic nature of the death induced by EPEC-EAF$^{(+)}$. EPEC-EAF$^{(-)}$ also provoked a slight reduction in epithelial cell viability, indicated by the MTT assay (Fig. 1). This basal cell death can be explained as possibly resulting from a toxic effect of the bacteria on the epithelial cell metabolism and succinate dehydrogenase enzyme activity, but no significant apoptosis was observed.

The possible involvement of BfpA in apoptosis induced by EPEC-EAF$^{(+)}$ is supported by the observation that previous treatment of these bacteria with monospecific IgY anti-BfpA reduces the number of apoptotic cells infected with EPEC-EAF$^{(+)}$ to control levels. This finding is in agreement with recent data describing cell death induction in human epithelial cell lines infected with BfpA-expressing EPEC [14] and allows us to conclude that the BfpA is a critical virulence factor signalling for programmed death in the intestinal epithelium colonized by EPEC.

The NF-κB pathway is an essential component of signal transduction leading not only to proinflammatory gene transcription, but also to transactivation of antiapoptotic regulators (for review, see Kucharczak [30]). Inhibition of NF-κB activation by cytotoxic enterobacterial factors has been proven to contribute to apoptosis induction by *Salmonella* [25, 26], *Shigella* [27] and *Yersinia* [28], while negative regulation of host cell apoptosis by NF-κB activation has been well documented in a variety of infections provoked by intracellular parasites [30, 31]. However, NF-κB activation has been shown not to be important for regulation of apoptosis in other instances such as infections with *Helicobacter pylori* and herpes simplex virus [32, 33], although it mediates apoptosis induction in virus-infected cells [34, 35].

EPEC induces NF-κB activation in intestinal epithelial cells [10]. To find out whether the EPEC virulent factor BfpA also participates in the NF-κB activation, we analysed some events known to occur in this cell-signalling system in cells infected with EPEC-EAF$^{(+)}$ or EPEC-EAF$^{(-)}$. Our results clearly show that EPEC-EAF$^{(+)}$ and EPEC-EAF$^{(-)}$ are able to activate NF-κB in a similar manner, as demonstrated by the DNA band shift assay (Fig. 5). This indicates that factors other than BfpA are sufficient for the activation of this signalling cascade pathway in target cells. Analysis of DNA-binding proteins revealed the presence of a RelA-/p65-transactivating NF-κB member in the nuclei of cells incubated with EPEC-EAF$^{(+)}$ and EPEC-EAF$^{(-)}$, suggesting that both bacteria are able to induce transcription of NF-κB-dependent genes. Unexpectedly, the ability of NF-κB to induce transactivation of the NF-κB/luc reporter gene was different in HeLa cells treated with BfpA-positive and BfpA-negative bacteria (Figs 5 and 7). The phosphorylation of IκB-α was also significantly higher in HeLa cells infected with EPEC-EAF$^{(+)}$, as compared with cells infected with EPEC-EAF$^{(-)}$, despite similar levels of NF-κB binding to the target cells DNA (Fig. 6). This fact could be explained by diverse transcriptional activities induced by EPEC-EAF$^{(+)}$ and

EPEC-EAF$^{(-)}$, which could lead to different levels of NF-κB-dependent IκB-α gene expression, thus affecting IκB-α resynthesis and phosphorylation. Additional studies are necessary to clarify which synergistic signalling pathways are triggered by BfpA-enhancing basal transcriptional activity of NF-κB induced by EPEC. Our results with LPS indicate that this gram-negative cell wall component apparently is not involved in the apoptosis induction by EPEC-EAF$^{(+)}$.

In order to verify the possible role of NF-κB activation in the regulation of BfpA-mediated apoptosis, we analysed the effect of NF-κB suppression by a specific NF-κB inhibitor on the apoptosis induced by EPEC. The inhibitor of IκB-α phosphorylation, BAY11-7085, has been previously shown to block NF-κB activation and translocation to the nucleus in HeLa cells [24], because apoptosis was observed only in HeLa cells pretreated with BAY11-7085 and subsequently infected with EPEC-EAF$^{(+)}$, but not with EPEC-EAF$^{(-)}$, or in controls without bacteria; these results suggest that NF-κB activation apparently is not involved in the apoptosis induced by EPEC bearing BfpA (Fig. 8).

In conclusion, the data obtained in this study indicate that (i) the BfpA virulence factor plays a significant role in eliciting mechanisms by which EPEC induces apoptosis and caspase-3 activation in susceptible host cells. Preliminary data using cloned BfpA proteins to stimulate HeLa cells as a substitute for EPEC-EAF$^{(+)}$ reinforce the presumptive involvement of this EPEC virulent factor in apoptosis induction and in the activation of NF-κB (data not shown); (ii) the expression of BfpA is not essential for NF-κB activation by EPEC; (iii) EPEC bacterial virulence factors distinct from BfpA, expressed by both EPEC-EAF$^{(+)}$ and EPEC-EAF$^{(-)}$, are triggers for NF-κB activation.

Acknowledgments

The authors thank Dr Nicola Conran for English review of the manuscript and Dr N. Rice, NCI-Frederic Cancer Research and Development Center, USA, for providing us with antibodies: anti-NF-κB proteins and rabbit anti-p65 (1226, 1207).

This work was supported by CNPq, Proc. 4727452003-4 and Proc. 521941/95-4 (NV); FAPERJ, Proc. E-26/151/501/99 and E-26152-497/2002, and FENORTE Proc E-5/022024/03. CMCA and ARM were the recipients of scholarships from FAPERJ, Proc. E-26/150.830./97 and FENORTE, respectively.

References

1 Donnenberg MS, Kaper JB. Enteropathogenic *Escherichia coli*. Infect Immun 1992;60:3953–61.

2 Giron JA, Ho AS, Schoolnik GK. An inducible bundle-forming pilus of enteropathogenic *Escherichia coli*. Science 1991;254:710–3.

3 Bieber DS, Ramer W, Wu CY *et al.* Type IV poli, transient bacterial aggregates, and virulence of enteropathogenic *Escherichia coli*. Science 1998;280:2114–8.

4 Baldini MM, Kaper JB, Candy DC, Moon HW. Plasmid-mediated adhesion in enteropathogenic *Escherichia coli*. J Pediatr Gastroenterol Nutr 1983;2:534–8.

5 Sohel IJ, Puente L, Ramer SW, Bieber D, Wu C-Y, Scoolnik GK. Enteropathogenic *Escherichia coli*: identification of a gene cluster coding for bundle-forming pilus morphogenesis. J Bacteriol 1996;178:2613–28.

6 Stone KD, Zhang HZ, Carlson LK, Donnenberg MS. A cluster of fourteen genes from enteropathogenic *Escherichia coli* is sufficient for biogenesis of a type IV pilus. Mol Microbiol 1996;20:325–8.

7 Donnenberg MS, Girón JA, Nataro JP, Kaper JB. A plasmid-encoded type IV fimbrial gene of enteropathogenic *Escherichia coli* associated with localized adherence. Mol Microbiol 1992;6:3427–37.

8 Frankel G, Phillips AD, Rosenshune I, Dougan G, Kapper JB, Knutton S. Enteropathogenic and enterohemorrhagic *Escherichia coli* more subversive elements. Mol Microbiol 1998;30:911–21.

9 Knutton S, Shaw RK, Anantha RP, Donnenberg MS, Zorgani AA. The type IV bundle-forming pilus of enteropathogenic *Escherichia coli* undergoes dramatic alterations in structure, associated with bacterial adherence, aggregation, and dispersal. Mol Microbiol 1999;33:499–509.

10 Savkovic SD, Koutsouris A, Heccht AG. Activation of NF-κB in intestinal epithelial cells by enteropathogenic *Escherichia coli*. Am J Physiol 1997;273:C1160–7.

11 Baldwin TJ, Lee-Delaunay MB, Knutton S, Williams PH. Calcium calmodulin dependence of actin accretion and lethality in cultured HEp-2 cells infected with enteropathogenic *Escherichia coli*. Infect Immun 1993;61:760–3.

12 Barnett-Foster D, Abul-Milh M, Huesca M, Lingwood CA. Enterohemorrhagic *Escherichia coli* induces apoptosis which augments bacterial binding and phosphatidylethanolamine exposure on the plasma membrane outer leaflet. Infect Immun 2000;68:3108–15.

13 Crane JK, Majumdar S, Pickhardt DF III. Host cell death due to enteropathogenic *Escherichia coli* has features of apoptosis. Infect Immun 1999;67:2575–84.

14 Abul-Milh M, Wu Y, Lau B, Lingwood CA, Foster DB. Induction of epithelial cell death including apoptosis by enteropathogenic *Escherichia coli* expressing bundle-forming pili. Infect Immun 2001;69:7356–64.

15 Almeida CMC, Quintana-Flores VM, Medina-Acosta E, Schriefer A, Barral-Netto M, Dias da Silva W. Egg yolk anti-BfpA antibodies as a tool for recognizing and identify enteropathogenic *Escherichia coli*. Scand J Immunol 2003;57:573–82.

16 Schriefer A, Maltez JR, Silva N, Stoeckle MY, Barral-Netto M, Riley LW. Expression of a pilin subunity BfpA of the bundle-forming pilus of enteropathogenic *Escherichia coli* in a aroA live *Salmonella* vaccine strain. Vaccine 1999;17:770–8.

17 Berridge MV, Tan AS. Characterization of the cellular reduction of 3-(4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, subcellular dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys 1993;303:474–82.

18 Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometry detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin. J Immunol Methods 1995;184:39–51.

19 Van Engeland MF, Ramackers C, Schutte B, Reutelingsperger CP. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry 1996;24:131–9.

20 Martin D, Leonardo M. Morphological and biochemical assays of apoptosis. In: Coligan JE, Kruisbeck AM, Margulis DH *et al.*, eds. Current Protocols in Immunology. New York, N.Y., (USA): John Wiley and Sons, 1998:3.17.1–4.

21 Bradford M. A rapid and sensitive method quantification of microgram quanties of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
22 Dignam J, Lebovitz A, Roeder R. Accurate transcription initiation by RNA-polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 1983;11:1475–89.
23 Retamal CAP, Thiebaut P, Alves EW. Protein purification from polyacrilamide gels by sonification extraction. Anal Biochem 1999;268:15–20.
24 Darieva Z, Lasunskaia EB, Campos MNN, Kipnis TL, Dias da Silva W. Activation of phosphatidylinositol 3-kinase and c-Jun – N terminal kinase cascades enhances NF-κB-dependent gene transcription in BCG-stimulated macrophages through promotion of p65/p300 binding. J Leukoc Biol 2004;75:689–97.
25 Collier-Hyams LS, Zeng J, Sun H *et al. Salmonella* AvrA effector inhibits the key proinflammatory, anti-apoptotic NF-κB pathway. J Immunol 2002;69:2846–50.
26 Haraga A, Miller SI. A *Salmonella* enterica serovar *Typhimurium* translocated leucine-rich repeat effector protein inhibits NF-κB-dependent gene expression. Infect Immun 2003;71:4052–8.
27 Hilbi H, Moss JE, Hersh D *et al. Shigella*-induced apoptosis is dependent on caspase-1 which binds to IpaB. J Biol Chem 1998;273:32895–900.
28 Zhang Y, Bliska JB. Role of Toll-like receptor signaling in the apoptotic response of macrophages to *Yersinia* infection. Infect Immun 2003;71:1513–9.
29 Kothakota S, Azuma A, Reinhard C *et al.* Caspase 3-generated fragments of gelsolin: effector of morphological change in apoptosis. Science 1997;278:294–8.
30 Kucharczak JM, Simmons J, Fan Y, Gelinas C. To be, or not to be: NF-κB is the answer-role of Rel/NF-κB in the regulation of apoptosis. Oncogene 2003;22:896–82.
31 Heussler VT, Kuenzi P, Rottenberg S. Inhibition of apoptosis by intracellular protozoan parasites. Int J Parasitol 2001;31:1166–76.
32 Santoro MG, Rossi A, Amici C. NF-κB and virus infection: who controls whom. EMBO J 2003;22:2552–60.
33 Chu SH, Kim JW, Kim H. Nf-κB and Bcl-2 in *Helicobacter pilori*-induced apoptosis in gastric epithelial cells. Ann N Y Acad Sci 2003;1010:568–72.
34 Taddeo B, Luo TR, Zhang W, Roizman B. Activation of NF-κB in cells productively infected with HSV-1 depends on activated protein kinase R and plays no apparent role in blocking apoptosis. Proc Natl Acad Sci USA 2003;100:12408–13.
35 O'Donnell SM, Hansberger MW, Dermody TS. Viral and cellular determinants of apoptosis induced by mammalian reovirus. Int Rev Immunol 2003;22:477–503.