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Salmonella Typhimurium invasion induces apoptosis in chicken embryo fibroblast

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***Salmonella* Typhimurium induces apoptosis in macrophages and intestinal epithelial cells. In the present study we report that it induces apoptosis in chicken embryo fibroblast (CEF), by a scatter experiment using flow cytometry. This finding makes CEF the best model for molecular analysis of apoptosis induced by *Salmonella*, since culture and handling of CEF is more convenient than any other specialized cells.**

Keywords: Apoptosis, chicken embryo fibroblast, *Salmonella* Typhimurium.

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APOPTOSIS is a genetically determined form of cell death that plays a central role during development and homeostasis of multicellular organisms[1,2]. Necrotic cell death is usually the consequence of physical injury and does not involve active participation of the cell. Apoptosis can be distinguished from necrosis on the basis of several morphological as well as biochemical parameters, such as nuclear condensation, loss of cell volume, cell shrinkage, DNA fragmentation[2], and phosphatidylserine exposure to the outer face of the plasma membrane[3]. This kind of cell death avoids spillage of intracellular contents in contrast to necrotic cell death, typified by cell and organelle swelling and membrane disruption, resulting in an inflammatory response[3]. The central executioners of apoptosis are a set of cysteine proteases that are part of a large protein family known as caspases[4]. Apoptosis is critically important to development, tissue homeostasis, and in the pathogenesis of various viral and bacterial diseases[5].

The ability of *Salmonella* to promote apoptosis may be important for the initiation of infection, bacterial survival and escape of the host immune response[5].

Flow cytometry is a simple and reproducible method useful for assessing apoptosis of specific cell populations. There are several methods that can be used to quantitate apoptosis by flow cytometry. The simplest method based on biochemical changes includes use of propidium iodide (PI) to stain the DNA and look for the sub-diploid, or Ao population of cells from a cell cycle profile. Staining of isolated nuclei with DNA-binding fluorescent dye PI showed that intensity of fluorescence is correlated with the extent of DNA degradation[6]. Flow cytometric analysis of the apoptotic cell population can be carried out using either single dye or a mixture of dyes.

Salmonella induces apoptosis in macrophages and dendrocytes through the caspase-1 pathway. In the present study we have made an attempt to find whether *Salmonella* induces apoptosis in chicken embryo fibroblast (CEF). The isolates selected for the study were ML-4, ML-7 and ML-5.

Primary CEF culture was prepared. After 45 min of bacterial infection to the fibroblast, the infected cells were washed three times with PBS (7.4) and incubated in fresh tissue culture medium containing 100 µg of gentamicin/ml for 30 min. Then the cells were washed with PBS twice, followed by trypsinization.

For trypsinization, trypsin (0.1 ml/cm^2 of 0.25% trypsin in PBS) was added to the side of the flask opposite the cells. Then the flask was turned over and left stationary for 15–30 s and all but a few drops of the trypsin was withdrawn, making sure that the monolayer was not detached. The cells were further incubated for 5–15 min with flask lying flat, until the cells rounded up when the bottle was tilted and the monolayer was able to slide down the surface. At this stage of trypsinization, culture medium (0.1–0.2 ml/cm^2) was added and the cells were dispensed by repeated pipetting over the surface bearing

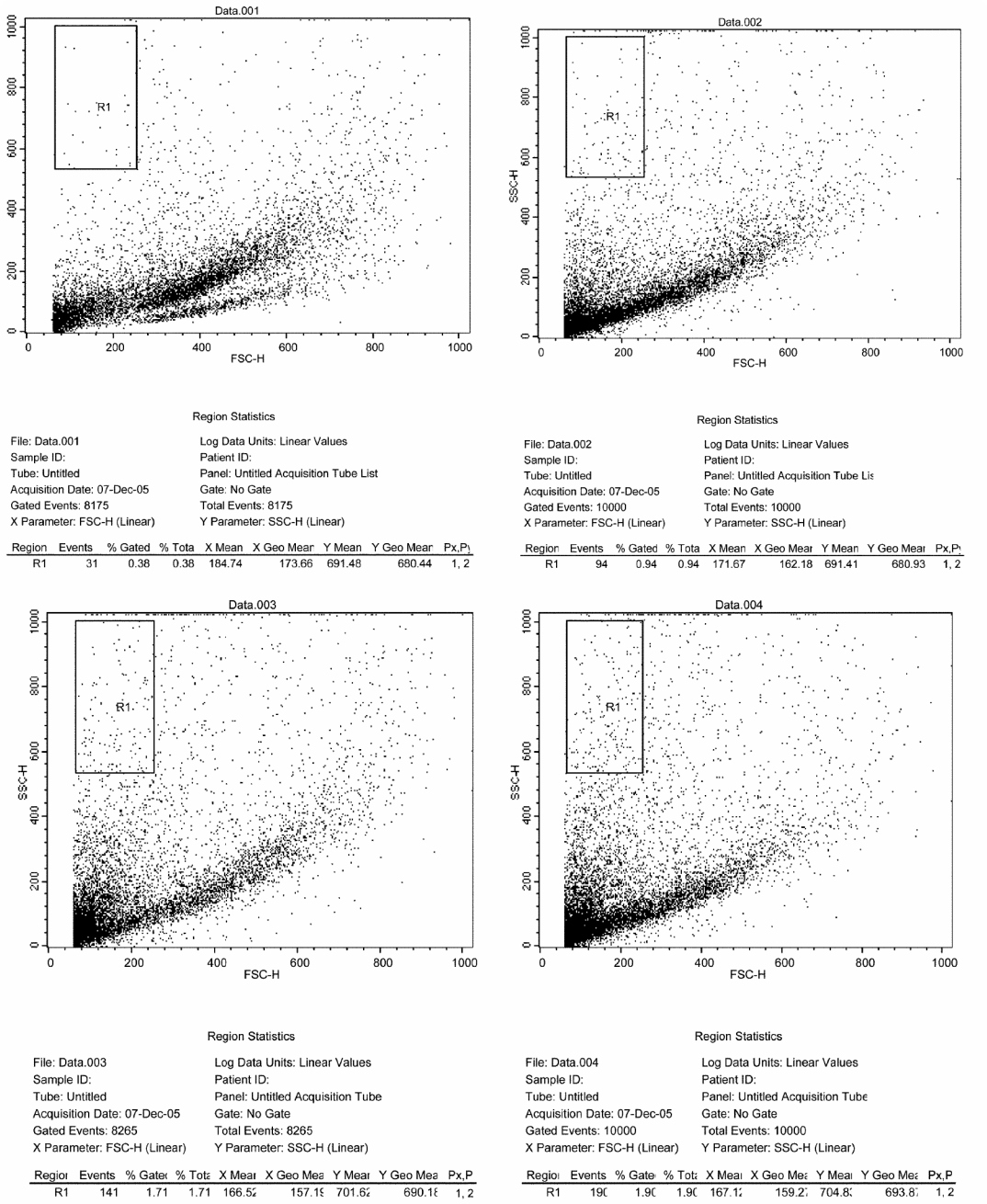

Figure 1. FACS analysis of chicken embryo fibroblast infected with *Salmonella* Typhimurium.

the monolayer. Finally the cell suspension was pipetted up and down few times with the tip of a pipette resting on the bottom corner of the bottle, taking care not to create foam. Pipetting was done sufficiently to dispense cells into a single cell suspension.

Cells were counted using a hemocytometer and the suspension was diluted to approximately 2 million cells/ml. A control cell suspension was also prepared which was free from infection and had been subjected to all the treatments that the test sample has undergone. The cell suspension prepared was centrifuged at 2000 rpm for 10 min, the supernatant removed and thoroughly resuspended in 4 ml PBS. This was centrifuged again for 10 min at 2000 rpm. Then the palette was resuspended in 100 µl PBS and 2 ml of ice cold 70% ethanol added slowly, while vortexing. This was stored at –20°C for at least 2 h, washed twice with 4 ml PBS and finally 25 µg of RNAse per ml of resuspended cells in PBS was added and incubated for 15 min at 37°C in a water bath.

The forward scatter (FCS) and side scatter (SSC) were simultaneously measured using a flow cytometer (FACScalibar, Becton and Dickenson, USA) at the Division of

Animal Biochemistry, IVRI, Izatnagar. The scattered light was recorded as dot plots[7–10] as in Data.001 (Control), Data.002 (ML5), Data.003 (ML4) and Data.004 (ML7; Figure 1). Flow cytometric scatter diagram of infected cells when compared with the control showed obvious morphological changes as represented by a decrease in forward scatter and increase in side scatter, indicating cellular changes that reflected smaller sized cells and fragmentation of the nucleus in the cytoplasm, which is the typical appearance of apoptotic cells. The dot plots recorded with side scatter against forward scatter give the cell size and DNA content.

The region R1 was selected in the plot for comparing the control with infected samples. Statistical data recorded revealed that the control had 0.34% of apoptotic cells, whereas infected cells showed a marginal increase in apoptotic cell number with 0.98% in ML5, 1.71% in ML4 and 1.90% in ML7. On comparison with the control it was clear that *Salmonella* isolates selected for the study induced apoptosis at different degrees in CEF.

An increasing number of bacterial species has been shown to induce apoptosis in phagocytes, including *Salmonella* Typhimurium[5,11], *Shigella flexneri*[12] and *Yersinia pseudotuberculosis*[13]. Programmed cell death is part of normal development and homeostasis. Apoptosis induced by bacteria appears to contribute to infectious diseases. Invasive *Salmonella typhimurium* induces apoptosis in macrophages[5]. To see whether this pathogen induces apoptosis in CEF by flow cytometric analysis, we took three isolates for the study, viz. ML4, ML5 and ML7, and infected CEF for 45 min. A control with no infection was also analysed for ability to induce programmed cell death. Results clearly showed that *Salmonella* induces apoptosis in CEF. The apoptotic cells found in the control were 0.38% of 10,000 events counted, but the treated samples showed significant increase in apoptotic cells with 1.90% in ML7, 1.71% in ML4 and 0.98% in ML5 for 10,000 events. Similar report showed that invasive *Salmonella typhimurium* induces apoptosis in macrophages. But mutants failed to induce the same. The mechanism responsible for this process is unknown. It has already been reported[5] that the Inv-Spa type III secretion apparatus target invasin SipB is necessary and sufficient for the induction of apoptosis. Purified SipB microinjected into macrophages led to cell death. Binding studies show that SipB associates with the proapoptotic protease caspase-1. This interaction results in the activation of caspase-1, as seen in its proteolytic maturation and the processing of its substrate interleukin-1beta. It has been shown that apoptosis occurs *in vivo* during infection not only with *Salmonella* Typhimurium, but also with *Y. pseudotuberculosis* and *S. flexneri*[13,14]. The ability to induce apoptosis is mainly because of the invasive nature of *Salmonella*. Virtually any gene that interrupt the entry *Salmonella* into cells (for example, *hil*A, *org*A and *sip*D) nullifies the apoptotic response to the entry.

Bordetella pertussis, the causative agent of whooping cough, has been shown recently to enter and survive in epithelial cells and macrophages *in vitro*. Cell cytotoxicity mediated by *B. pertussis* occurred through apoptosis, as shown by changes in nuclear morphology and by host cell DNA fragmentation[15]. *Shigella*, the etiological agent of dysentery, kills macrophages by inducing apoptosis. Deletion mutants in the invasion plasmid antigen B (ipaB) of *S. flexneri* are not cytotoxic[10].

In conclusion, invasive *Salmonella* Typhimurium strains induce apoptosis in macrophages and intestinal epithelial cells. In the present study we have demonstrated the ability of *Salmonella typhimurium* to induce apoptosis in CEF using flow cytometric analysis. This suggests that CEF can be used for molecular analysis of apoptosis induced by *Salmonella* Typhimurium.

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