CIENCIA 15(3), 444 - 451, 2007
Maracaibo, Venezuela

Use of FACS to determine cell death process in *Chlamydia trachomatis* Infected cells

Beatriz E. Millán-Mendoza[1,2] y Adrián Eley[2]*

[1]*Universidad de los Andes, Facultad de Odontología. Grupo de Investigaciones Biopatológicas de la Facultad de Odontología (GIBFO), Apartado Postal 133. Mérida, Venezuela.*
[2]*Division of Genomic Medicine. The University of Sheffield, Sheffield, S10 2RX, United Kingdom.*

Recibido: 15-02-06 Aceptado: 08-06-07

Abstract

Cytotoxicity due to infection of obligate intracellular pathogen *Chlamydia trachomatis,* has been reported for many years, but the mechanisms involved in the cell death process remain controversial. *C. trachomatis* is responsible for several important ocular and genital tract infections worldwide and few techniques have been used to assess the cell death process of the mammalian host cells. In this report, simultaneous cell staining with Annexin V-FITC, a phospholipid binding protein, and the non-vital dye propidium iodide (PI), followed by fluorescence-activated cell sorting (FACS) analysis were used to characterize the cell death process in infected HeLa cells. Apoptosis found not to be activated in the infected cells until some point between 24 and 48 h in a dose-dependant relation; after 48 h, necrosis took over the cell death process. Thus, we demonstrate that the use of annexin V-FITC/PI staining followed by FACS analysis offers an alternative for assessing the cell death process.

Key words: Apoptosis; annexin V; *Chlamydia trachomatis*; flow cytometry; necrosis.

Uso de la Citometría de flujo para determinar el tipo de muerte en células infectadas con *Chlamydia trachomatis*

Resumen

La citotoxicidad debido a la infección con *Chlamydia trachomatis*, el cual es un patógeno intracelular obligatorio, responsable de infecciones oculares y del tracto genital en todo el mundo, ha sido reportado por años. El mecanismo involucrado en este proceso continua siendo un punto controversial y numerosas técnicas han sido utilizadas para caracterizar el tipo de muerte celular producido en la células hospederas. En este reporte se describe una tinción simultanea con annexin V-FITC y yoduro propídico (PI) un colorante no vital, los cuales fueron utilizados para analizar el proceso de muerte celular en células HeLa infectadas con *C. trachomatis* estudiado a través de citometría de flujo (FACS). Apoptosis fue observada entre 24 y 48 h post-infección estando directamente relacionada con la dosis utilizada, y después de 48 h el proceso de necrosis resultó predominante en la muerte celular. Con esto, se ha demostrado que el uso de la doble tinción celular con annexin V-FITC / PI seguida de análisis por Citometría de flujo es una alternativa para caracterizar la muerte celular en células infectadas con *C. trachomatis*.

Palabras clave: Annexin V; apoptosis; *Chlamydia trachomatis*; necrosis; Citometría de flujo.

* Autor para la correspondencia. E-mail: bmillanm@ula.ve

Introduction

*Chlamydia*e are obligate intracellular Gram-negative bacteria which grow and develop inside an inclusion body within host cells. They are a common cause of ocular and genital tract infections in humans such as cervicitis, salpingitis, female urethral syndrome, pelvic inflammatory disease, postpartum endometritis, male urethritis, and epididymitis (1).

The development of chlamydial disease is mainly caused by persistent intracellular infection by the organism. A typical chlamydial infection cycle is initiated by the entry of an infectious extracellular elementary body (EB) into epithelial cells where they are internalized into vacuoles that avoid fusion with host cell lysosomes. After incubation of 12 h the EBs differentiate into noninfectious but metabolically active reticulate bodies, which multiply and differentiate back to EBs and around 48-72 h postinfection cells begin to lyse and release infectious EBs to start a new cycle (2).

Apoptosis or programmed cell death is a process whereby cells play an active role in their own death and is an innate mechanism of eukaryotic cell suicide, which plays a major role in many physiological and pathological processes. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis different from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems (3, 4).

After receiving specific signals instructing the cells to undergo apoptosis, a number of distinctive biochemical and morphological changes occur in the cell. A family of proteins known as Caspases is typically activated in the early stages of apoptosis. These proteins breakdown or cleave key cellular substrates that are required for normal cellular function, including structural proteins in the cytoskeleton and nuclear proteins, such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNAses, which begin to cleave the DNA in the nucleus. Thus, the result of these biochemical changes are reflected in morphological changes in the cell and is marked by a series of reorganization characteristics such as chromatin condensation, loss of cell volume and membrane blebbing which are some of the most evident morphological changes of apoptotic cells. However, when the apoptotic mechanisms are triggered and, in order to promote their phagocytosis by macrophages, rapid alterations in the organization of phospholipids in most cell types occur and translocation of phosphatidylserine (PS) from the inner leaflet of the cell to the outer surface is noticeable. This leads to its exposure on the cell surface which is now seriously considered as a general hallmark of apoptosis (5).

Intracellular pathogens have developed strategies to survive for extended periods inside their host cells. These include avoidance of host microbicidal effectors often by sequestration in a protected subcompartment of the host cells or in other cases the cell invader exerts an antiapoptotic effect that prolongs the life of the infected host cell (6). Both strategies can be applied to *Chlamydia* but the mechanisms involved remain largely unknown and is subject of current studies.

In a study of apoptotic processes, in the interaction between *C. trachomatis* and mammalian host cells, antiapoptotic activity was found (7), which seems to be very important for the first stages of the infection. However, a proapoptotic activity has also been reported in epithelial cells and macrophages infected with *C. psittaci* (8,9) and also during genital tract infections in mice with *C. trachomatis* (10), but all of these studies have used different methods to assess cell death.

There are several methods that can be used to assess apoptosis such as micro-

scopic examination, detection of DNA laddering by gel electrophoresis (3,11), analysis by flow cytometry (5), ADP/ATP ratio assay (12) and more recently caspase activity measurement (13,14). One of the most simple flow cytometric methods require the use of propidium iodide to stain the DNA and evidence the sub-diploid, or Ao, population of cells from a cell cycle profile. Other flow cytometric based methods include the TUNEL assay, which measures DNA strand breaks and Annexin V binding, detecting relocation of membrane phosphatidyl serine from the intracellular surface to the extracellular surface (15).

Reports of cell death studies of cells infected with *Chlamydia trachomatis* using cytometric analysis of double labelling technique with annexin V-FITC/PI are limited. However, reports describe the use of this technique for HeLa cells infected with *C. psittaci* (8) and, murine embryonic fibroblasts infected with *Chlamydia muridarum* (the mouse pneumonitis strain of *Chlamydia trachomatis*) (16).

Annexin V is a member of a family of proteins which exhibit Ca^{2+}-dependent phospholipid-binding properties (17) and when conjugated with a fluorochrome isothiocyanate (FITC) is widely employed in cytometry and microscopy as an early marker for apoptosis, because of its binding affinity for phosphatidylserine (PS), which is exposed at the cell surface early in the process (18, 19). Its simultaneous use with a DNA-binding dye such as Propidium Iodide (PI) allow to differentiate between apoptosis and necrosis, provided necrosis is accompanied by the loss of cell membrane integrity and leakage of cellular constituents into the environment.

Despite the fact that flow cytometry provides a way to accurately analyze a large number of cells for evidence of *Chlamydial* infection, its use has been limited. In the present report, a cytometric analysis of HeLa cells performed for detection and differentiation of cells undergoing programmed cell death (known as apoptosis), cells dying from necrosis, or cells remaining viable, after an infection with *C. trachomatis* serovar (LGV1) at time intervals, using a fluorochrome-conjugated annexin V and DNA-binding dye Propidium Iodide is described.

Material and Methods

Cell Lines: McCoy cells (Mouse fibroblast cell line) and HeLa 229 (Human Cervical Carcinoma cell line) obtained from the American Type Culture Collection (ATCC) (Manassas,Va.) and maintained according to the supplier's instructions.

Cell Culture: McCoy and HeLa cells cultured in 25-cm^2 flasks in Eagle minimal essential medium (EMEM) with L-glutamine (Bio-Whittaker 12-611F) supplemented with 10% v/v heat inactivated Foetal Calf Serum (FCS) (Life Technologies) and maintained at 37°C in an atmosphere of 5%CO_2.

C. trachomatis serovar LGV1 was propagated in a semiconfluent McCoy cell sheet for 48-72 h in EMEM medium with 10% v/v FCS and cycloheximide [2ug/mL]. EB's were harvested as described previously (20).

The EB's were quantified and the number of inclusion forming units (IFU) per milliliter was calculated. McCoy cells cultured on sterile coverslips in 24-well tissue culture plates with a serial dilution of purified EB's (10^{-2}-10^{-6}). The plates centrifuged for 1 h. at 2,000g prior incubation for 48 h. at 37°C in 5%CO_2. After incubation, coverslips were fixed and stained with fluorescein isothiocyanate-labeled antichlamydial monoclonal antibody (Imagen-DAKO) following the manufacturers instructions and the number of inclusion bodies for each dilution counted using a fluorescence microscope at X40 magnification.

To determine the number of inclusion forming units (IFU) per milliliter the total number of inclusion bodies per coverslip for each dilution tested was calculated and the multiplicity of infection (MOI) was determined.

Infection Step: An 80% confluent HeLa cells layer was infected using MOI 0.5, 1.0, 1.5 of purified LGV1. After inoculation, cells centrifuged at 3000g for 1h and then incubated for 2h at 37°C in 5%CO2, then the medium replaced and cells incubated for different periods of time (24, 48, 72 h) under the same incubation conditions described earlier.

Cell Harvesting: After the incubation period, HeLa cells evaluated microscopically to determine the degree of infection and then harvested by trypsinization, avoiding damage to the cells. The medium collected and both adherent cells and cells in suspension collected for subsequent analysis. The cells were centrifuged in a conical tube at 500 g for 5 min and then washed with PBS. A suspension of approximately 2 x 10^6 cells was made 500 μL of the cells were centrifuged at 500g for 2 min and resuspended in the same volume of Annexin V- binding buffer (0.1M Hepes/NaOH, pH 7.4, 1.4 M NaCl, 25 mM $CaCl_2$)(PharMingen) and used for the FACS analysis. HeLa cells treated with an apoptosis inducer, staurosporine (Sigma) 1μM x 4 h used as a positive control.

Flow Cytometry: For Fluorescence-activated cell sorter (FACS) analysis, cells were stained with Annexin V-FITC (PharMingen) and PI (Sigma) following the manufacturer's staining protocol. Briefly, 100 μL of cell suspension (1X Binding Buffer) [1x10^5] stained with 2.5 μL of annexin V-FITC and 5 μL. of PI [50 μg/mL], gently mixed and incubated for 15 min at room temperature in the dark, and then 200 μL. of 1X Annexin binding buffer added and cells immediately analyzed by flow cytometry.

Flow cytometry analysis performed on a FACSCalibur (Becton Dickinson, Oxford, United Kingdom) equipped with a single argon ion laser emitting an excitation light at 488nm. Data on 10000 cells collected at a low flow rate and analyzed using CellQuest software. The annexin V-FITC signal detected by FL1 and PI detected by FL2.

The discrimination between living, necrotic and apoptotic cells was based on changes in PS asymmetry of cell membrane, detected by annexin V binding. Analysis of the cell death process using simultaneous staining with FITC-Annexin V (green fluorescence) and the non-vital dye propidium iodide (red fluorescence) allowed discrimination of intact cells (FITC-PI-), early apoptotic (FITC+PI-) and, late apoptotic or necrotic cells (FITC+PI+).

Statistical analysis: All experiments were performed at least twice. The data shown are means ± standard deviation of triplicate cultures. The statistical significance of the differences between each dose and its negative control was examined by a one-way analysis of variance (ANOVA) on a transformed data.

Results

Infectivity experiments: The percentage of HeLa cells infected with *C. trachomatis* serovar LGV1 was proportional to the infectious inoculum used and inclusion bodies were easily distinguished by fluorescence microscopy using the FITC-monoclonal antibody (Figure 1). The number of detached cells was also proportional to the inoculum used and time of incubation (data not shown). However, all the cells were collected during harvesting.

Figure 2 represent death and behavior of (summarizes the kind of cell death present in) HeLa cells using a flow cytometric assessment of annexin V-FITC/ PI stain. After 24 h of incubation, death cell level was low and proportional to the concentration of the inoculum used. Microscopically, also the size of the inclusion bodies in the infected cells was small. However, at 48 h post-infection the number of detached cells increased and the size of the inclusion bodies were considerable larger, some cells already burst and a new generation of EB's were ready to continue infecting new target cells. At this point, flow cytometry results showed that the level of apoptosis and necrosis increased consid-

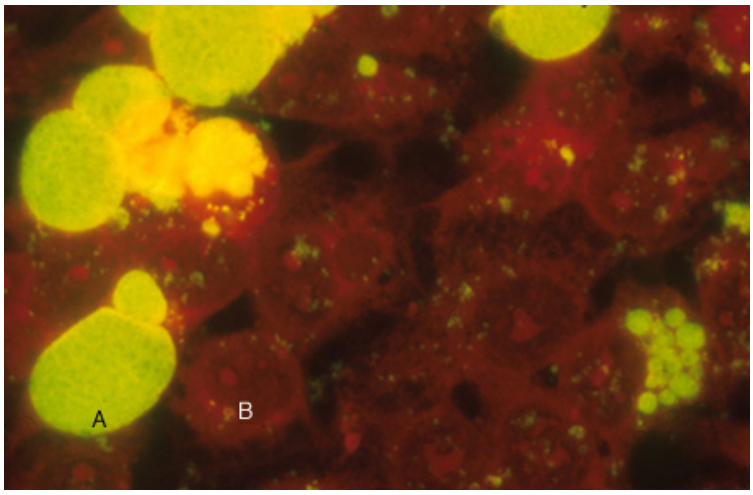

Figure 1. Fluorescence micrographs of HeLa cells labeled with FITC-conjugated anti-chlamydial antibody (100X). A: indicates a *C. trachomatis* inclusion. B: Uninfected HeLa cell.

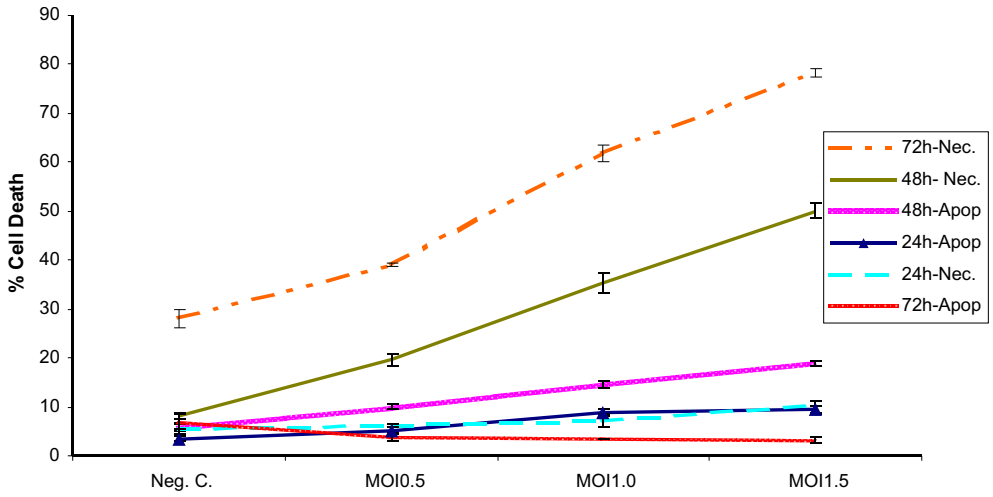

Figure 2. Cell death characterization of HeLa cells infected with *Chlamydia trachomatis* serovar LGV1 at three different MOI concentration (0.5, 1.0, 1.5) for periods of 24,48, and 72 h measured by flow cytometry using annexin V-FITC/PI staining. Values represent means ± standard deviation from 3 experiments. (Nec=Necrosis, Apop= Apoptosis).

erably and was shown to be dose dependant where almost half of the cells had died when infected at a MOI of 1.0, predominantly through a necrosis process (35%) ($P<0.001$).

At 72h post infection the majority of the cells had detached from the flask and died through necrosis. This process was directly proportional to the amount of inoculum used and as the necrosis took over the cell death process, the rate of apoptotic cells decreased.

The non-infected cells treated with the apoptotic inducer staurosporine for 4 h used as a positive control, showed an apoptosis ratio of 10.5% and 14.5% at 24h and 48h incubation periods and the percentage of ne-

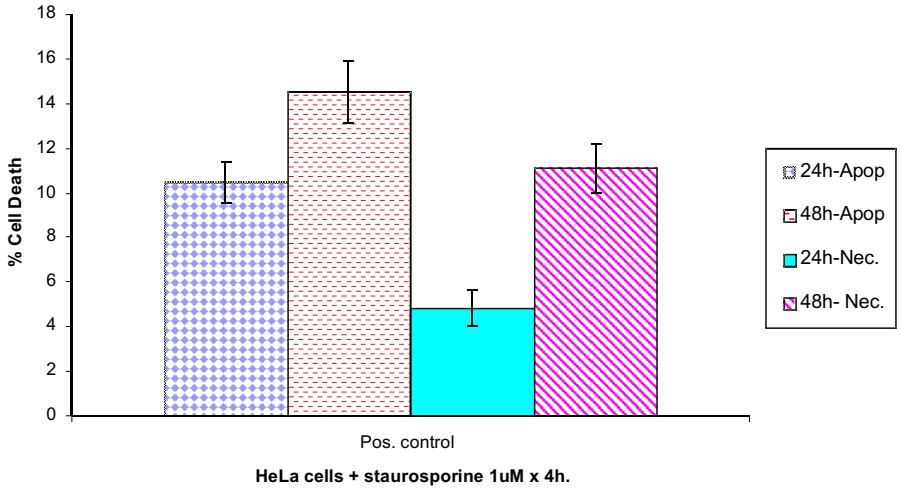

Figure 3. Cell death characterization of HeLa cells exposed to an apoptotic inducer, staurosporine. (Apop= apotosis at 24 h and 48h, Nec.= Necrosis at 24h and 48h.).

crotic cells for cases, 4.8% and 11.1% respectively (Figure 3).

Discussion

In the present study a flow cytometric approach was used to analyze infections of cell lines with the obligate intracellular pathogen, *C. trachomatis* using annexin V-FITC/PI staining. This study showed that the overall percentage of apoptotic HeLa cells after infection with three different concentrations of C*. trachomatis* serovar LGV1 is less than 20%. This percent value was low at 24 h post-infection and increased in a dose dependant response after 48 h, at this time the percentage of necrotic cells also increased, taking over the cell death process at 72 h post infection. The low percentage of apoptotic cells found in our experiments is similar to previous findings where cells infected with *Chlamydia* had an antiapoptotic effect (7) allowing maturation of the host cells. However, after 48 h of infection, cells started to become necrotic relative to the dose applied. This also could be explained due to the nature of the *Chlamydia* life cycle, where the host cells are lysed and at some point a new generation of infective EB is released into the medium. Perfettini et al. (2003) (16), studied the effect of Bax on host cell death in vitro using a primary cell line infected with *C. muridarum* and using double labelling technique with annexin V-FITC/PI. They found, that at 48h Bax-/- murine embryonic fibroblasts cells die through necrosis more often than Bax+/+ cells (7% apoptosis - 34% necrosis and 30% apoptosis - 10% necrosis respectively). In our investigation, the same double labelling technique were used to asses the cell death process but in a carcinoma cell line infected with *C. trachomatis*, and some similarity in the percentage of necrosis at 48h post infection using MOI 1.0 (35%) was found. Results analysis show that the double staining technique could be used successfully to assess cell death process independently the cell line and the infecting microorganism.

The use of annexin V staining allowed the quantification of cells in the early stages of apoptosis or when apoptosis occurred in the absence of DNA fragmentation due to loss of plasma membrane asymmetry, which seems to be a hallmark event of apoptosis. Therefore, measurement of annexin V binding, performed simultaneously with propidium iodide (PI) is a satisfactory assay to detect apoptotic cells and to discriminate between apoptosis and necrosis (21). This

assay when compared with traditional methods like microscopy, DNA electrophoresis, and TUNEL, reported methodology appeared to be sensitive and correlated with other reports, and additionally has the advantage of being less time consuming, easy, and reliable. Its use in determination of adherent cells has been limited due to concerns over any damage of the plasma membrane during the process of detaching cells from the flask where a great deal of care needs to be taken. Despite this limitation, reports of the use of this technique using detached cells have been made (22) and the results obtained in our experiments could be related to previous findings in the area showing that the limitation of the use of the flow cytometry technique using annexinV binding could be overcome (8-16).

Schöier et al. (23) studied the effect of *C.trachomatis* serovar E in infected and uninfected McCoy cells using FACS only with PI and TUNEL techniques. The apoptotic index found with both techniques was similar to our results. Additionally, they found a similar correlation of cell death to be dose-dependant. Greene et al. (24), found that Chlamydia-infected cells are resistant to apoptosis induction, although the extent of the antiapoptotic ability varied between serovars, demostrating that an anti- but not proapoptotic activity is the prevailing event in chlamydia-infected cultures, however, none of them discriminate between necrosis and apoptosis.

In summary, the present work demonstrated that the use of annexin V-FITC /PI staining procedure for a flow cytometric analysis of chlamydia infected cells and differentiation between necrotic and apoptotic cell death is feasible. Results analysis indicate that *C. trachomatis* serovar LGV1 are resistant to apoptosis and only a low level of apoptosis in HeLa cells could be detected in a dose dependant manner at 48 h post-inoculation, but it is at this point, where necrosis takes over the cell death process, probably due to tissue damage which, when exacerbated by reinfection, can lead to the release of cellular mediators to activate the cascade for a necrotic process, or as it is suggested by Prozialeck et al. (25), that the ability of *Chlamydia trachomatis* to accumulate substances which serves as an important structural component of adherent junctions in epithelial cells like â-catenin in the intracellular inclusions, could disrupt one of the pathways that lead to apoptotic cell death, or the degradation of BiK, Puma, and Bim, three upstream proapoptotic BH3-only proteins of the Bcl-2 family that can transmit death signals to mitochondria by inhibiting the Bcl-2 antiapoptotic proteins and/or activating the Bcl-2 proapoptotic members, such as Bax and Bak (26). However, all these unclear mechanisms are an interesting area which is currently under research by different groups (26-30).

References

1. MORSE S.A., MORELAND A A., THOMPSON S.E. ***Sexually Transmitted Diseases***. J.B. Lippincott. Philadelphia, Capitulo 6, 1990.
2. GILLESPIE S.H., BAMFORD K.B. ***Medical Microbiology and Infection at a glance***. Blackwell Science Ltd. Malden U.S.A. p. 48-4, 2000.
3. KERR J.F., WYLLIE A.H., CURRIE A.R. ***Br J Cancer*** 26: 239-257,1972.
4. COHEN J.J. ***Chest*** 103: 99S-101S, 1993.
5. DARZYNKIEWICZ Z., BRUNO S., Del BINO G., GORCZYCA W., HOTZ M.A., LASSOTA P., TRAGANOS F. ***Cytometry*** 13: 795-708, 1992.
6. TSE S.M., MASON D., BOTELHO R.J., CHIU B., REYLAND M., HANADA K., INMAN R.D., GRINSTEIN S. ***J Biol Chem*** 280: 25210-25215, 2005.
7. FAN T., LU H., HU H., SHI L., McCLARTY G.A., NANCE D.M., GREENBERG A.H., ZHONG G. ***J Exp Med*** 187: 487-496, 1998.
8.
9. OJCIUS D.M., SOUQUE P., PERFETTINI J.L., DAUTRY-VARSAT A**.** ***J Immunol*** 161: 4220-4226, 1998.

10. GIBELLINI D., PANAYA R., RUMPIANESI F. ***Zent bl Bakteriol*** 288: 35-43, 1998.

11. PERFETTINI J.L., DARVILLE T., GACHELIN G., SOUQUE P., HUERRE M., DAUTRY-VARSAT A., OJCIUS D.M. ***Infection and Immunity*** 68: 2237-2244, 2000.

12. COMPTON M.M. ***Cancer Metastasis Rev*** 11: 105-119, 1992.

13. BRADBURY D.A., SIMMONST D., SLATER K.J., CROUCH S.P. ***J Immunol Methods*** 240: 79-92, 2000.

14. GORMAN A.M., HIRT U.A., ZHIVOTOVSKY B., ORRENIUS S., CECCATELLI S. ***J Immunol Methods*** 226: 43-48, 1999.

15. AMSTAD P.A., YU G., JOHNSON G.L., LEE B.W., DHAWAN S., PHELPS D.J. ***Biotechniques*** 31(3): 608-614, 2001.

16. O'BRIEN I.E., REUTELINGSPERGER C.P., HOLDAWAY K.M. ***Cytometry*** 29: 28-33, 1997.

17. PERFETTINI J.L., OJCIUS D.M., ANDREWS C.W. Jr., KORSMEYER S.J., RANK R.G., DARVILLE T. ***J Biol Chem*** 278: 9496-9502, 2003.

18. ANDREE H.A., REUTELINGSPERGER C.P., HAUPTMANN R., HEMKER H.C., HERMENS W.T., WILLEMS G.M. ***J Biol Chem*** 265: 4923-4928, 1990.

19. MARTIN S.J., REUTELINGSPERGER C.P., McGAHON A.J., RADER J.A., VAN SCHIE R.C., LaFACE D.M., GREEN D.R. ***J Exp Med*** 182:1545-1556, 1995.

20. REUTELINGSPERGER C.P., VAN HEERDE W.L. ***Cell Mol Life Sci*** 53: 527-532, 1997.

21. TARAKTCHOGLOU M., PACEY A.A., TURNBULL J.E., ELEY A. ***Infection and Immunity*** 69: 968-976, 2001.

22. VERMES I., HAANEN C., STEFFENS-NAKKEN H., REUTELINGSPERGER C. ***J Immunol Methods*** 184: 39-51, 1995.

23. VAN ENGELAND M., RAMAEKERS F.C., SCHUTTE B., REUTELINGSPERGER C.P. ***Cytometry*** 24:131-139, 1996.

24. SCHÖIER J., OLLINGER K., KVARNSTROM M., SODERLUND G., KIHLSTROM E. ***Microbial Pathogenesis*** 31: 173-174, 2001.

25. GREENE W., XIAO Y., HUANG Y., McCLARTY G., ZHONG G. ***Infection and Immunity*** 72: 451-60, 2004.

26. PROZIALECK W.C., FAY M.J., LAMAR P.C., PEARSON C.A., SIGAR I., RAMSEY K.H. ***Infection and Immunity*** 70: 2605-2613, 2002.

27. DONG F., PIRBHAI M., XIAO Y., ZHONG Y., WU Y., ZHONG G. ***Infection and Immunity*** 73:1861-1864, 2005.

28. PERFETTINI J.L., REED J., ISRAEL N., MARTINOU J.C., DAUTRY-VARSAT A., OJCIUS D.M. ***Infection and Immunity*** 70: 55-61, 2002.

29. FISCHER S.F., HARLANDER T., VIER J., HACKER G. ***Infection and Immunity*** 72: 1107-15, 2004.

30. JENDRO M.C., FINGERLE F., DEUTSCH T., LIESE A., KOHLER L., KUIPERS J.G., RAUM E., MARTIN M., ZEIDLER H. ***Medical Microbiology Immunology*** 193: 45-52, 2004.

31. YING S., SEIFFERT B.M., HACKER G., FISCHER S.F. ***Infection and Immunity***. 73: 1399-403, 2005.