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APOPTOSIS vs. NECROSIS

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GENERAL INTRODUCTION

It is generally accepted that cell death can either be the consequence of a passive, degenerative process, or the consequence of an active process. The former type of cell death is termed necrosis, the latter apoptosis. Apoptosis was originally identified morphologically. The explosion of studies on apoptosis in recent years has clarified that it represents the mode of death that is actively driven by the cell, a complex process that is indicated as programmed cell death. On the opposite, necrosis represents a passive consequence of gross injury to the cell. It is morphologically different from apoptosis, and its physiological consequences are also very different from those of apoptosis. Being apoptosis a very common phenomenon during embryogenesis as well as adult life, and a frequent response of cells to drugs in the course of therapy, its quantitative evaluation represents an issue of considerable relevance. Flow cytometry is the choice technique for the quantitation of apoptosis. A multitude of methods have been described to identify apoptotic cells by flow cytometric analysis (see for review, Darzynkiewicz Z. et al., *Cytometry* 27: 1-20, 1997), each of them differently suitable to different experimental conditions. One general problem that arises virtually always with the flow cytometric analysis of apoptosis is the distinction between necrotic and apoptotic cells. Moreover, although necrotic cells are usually felt as contaminating objects during the quantification of apoptosis, one might be interested in the quantification of both apoptosis and necrosis in a given cell population. There is no clear cut parameter that allows the separation by flow cytometry of necrotic from apoptotic cells, particularly at their late stages. On the contrary, such a distinction is immediate by morphological techniques. Even the mere presence of apoptotic cells in a given population should be always validated by morphological observations. Therefore, flow cytometry and optical and electron microscopy all contribute, from different perspectives, to the identification of apoptosis and to its distinction from necrosis. Therefore, here will be described some common flow cytometric and morphological procedures for the identification and measurement of apoptotic cells with a particular emphasis on the discrimination of apoptotic from necrotic cells.

APOPTOSIS vs. NECROSIS BY FLOW CYTOMETRY

1. Introduction

It may be useful to make some general comments on the flow cytometric distinction of apoptotic from necrotic cells before describing the methods.

- 1) A univocal method to distinguish apoptotic from necrotic cells by flow cytometry does not exist. Rather, it is necessary to discriminate necrotic cells from apoptotic cells in each of the many flow cytometric methodologies available to measure apoptosis. Only the scatter method can apply in combination with most of the other methods to analyze apoptosis by FACS, which makes this method, that has some limitations *per se*, very useful.
- 2) Given the fact that most methodologies concentrate on the detection of apoptotic cells, often the problem is to discriminate healthy from necrotic cells. This can generally be achieved by the scatter method. The combination of a specific methodology designed to reveal apoptosis and the scatter analysis of the cell population generally allows a satisfactory distinction between healthy, necrotic and apoptotic cells.
- 3) A bigger problem arises with debris. It is of the utmost importance that everyone using flow cytometry to quantify apoptosis realizes that the fate of a apoptotic cell in vitro is to give origin to many small apoptotic bodies, which generally end up as debris at the flow cytometric analysis, exactly as the final products of cell necrosis. Even if it was possible to count the apoptotic bodies, this would be useless, since it would be impossible to know how many apoptotic cells they derive from. Therefore, all the techniques that we use to enumerate apoptosis, work until the apoptotic cell is still a cell. Being apoptosis a asynchronous process, it is evident that we can analyze only a window of the whole process.
- 4) Morphological analysis is essential to confirm the flow cytometric data. This is why after the flow cytometric methodologies, a section will follow in this chapter, in which the distinction between apoptosis and necrosis will be described by morphological techniques.

2A. First protocol: light scatter

2A.1. Materials

- PBS, pH 7.0
- Flow cytometer with forward (FSC) and side (90°) scatter (SSC) detection, laser tuned at 488nm wavelength

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- PBS, pH 7.0
- Flow cytometer with forward (FSC) and side (90°) scatter (SSC) detection, laser tuned at 488nm wavelength

2A.2. Methodology

1. Put in a tube 1×10^5 cells in which you want to test the presence of apoptosis, and resuspend them in 300-500 ul of PBS.
2. If possible, put in a tube 2×10^5 healthy cells of the same type, and resuspend them in 600-1000 ul of PBS. Divide this sample in two, by putting vol/2 in a new tube. Take one of the tubes and freeze and thaw it 3 times, to generate a population of necrotic cells. The intact cells of the other tube will serve as control for healthy cells.
3. If allowed by the type of instrumentation, set the flow cytometer paying particular attention to the light scatter detection system. Use a 488 nm wavelength laser emission. It can be useful to put a 488 nm band pass filter before the SSC photomultiplier (PMT).
4. Analyze the cells by flow cytometry. Start the analysis with the sample that contains healthy cells on the cytogram (FSC vs SSC). Place the population at mid-high FSC values and mid SSC values working on signal gains and PMT voltage. This will allow to have some space on the cytogram at lower FSC values and slightly higher SSC values.
5. Analyze the necrotic cell sample. It is likely that the necrotic population will appear at lower FSC and SSC values, frequently merging with debris.
6. Analyze the sample which should contain apoptotic cells. Depending on the cell type and on the nuclear/cytoplasmic (N/C) ratio of the intact cell, a third population will appear, generally at lower FSC and slightly higher SSC values with respect to healthy cells.

This scheme mainly refers to thymocytes, and does not have a general value, although other cell types may give similar results. Identification of apoptotic and necrotic cells as distinct populations by scatter parameters may not be possible in some cell types, and therefore each cell type must be tested in pilot experiments. It is also advisable to sort (if possible) each population (few hundreds of cells directly on a slide will be sufficient), and look at them under the microscope after Wright or Giemsa staining.

3A. Commentary**3A.1. Background information**

When a cell passes through a laser beam in a flow cytometer, generates light scatter. FSC provides information about cell size, SSC about the cell's morphological complexity. When a cell dies, its morphology changes, and changes in light scatter may reflect these phenomena.

Necrosis: when a cell dies by necrosis, both FSC and SSC tend to increase, likely as a consequence of cell swelling. However, as a consequence of plasma membrane damage and leakage of cell constituents, both FSC and SSC rapidly decrease.

Apoptosis: when a cell dies by apoptosis, its major morphological changes take place in the nucleus. Only in the late apoptotic stages the cytoplasm and the plasma membrane are seriously damaged. It has in fact been demonstrated that the higher is the N/C ratio in a given cell, the better is the distinction between apoptotic, necrotic and healthy cells by light scatter.

3A.2. Time considerations

The flow cytometric procedure will not take more than 30 min.

3A.3. Key references

1. Zamai, L., Falcieri, F., Zauli, G., Cataldi, A., and Vitale, M. 1993. Optimal detection of apoptosis by flow cytometry depends on cell morphology. *Cytometry* 14: 891.
2. Ornerod, M. G., Cheetham, F. P. M., and Sun, X. M. 1995. Discrimination of apoptotic thymocytes by forward light scatter. *Cytometry*, 21: 300.

2B. Second protocol: membrane permeability by propidium iodide**2B.1. Materials**

- PBS, pH 7.0
- propidium iodide (PI) 40 ug/ml in PBS (always wear gloves when using PI)

2B.2. Methodology

1. Put in a tube 1×10^5 cells in which you want to test the presence of apoptosis, and resuspend them in 300-500 ul of PBS.
2. If possible, put in a tube 2×10^5 healthy cells of the same type, and resuspend them in 600-1000 ul of PBS. Divide this sample in two, by putting vol/2 in a new tube. Take one of the tubes and freeze and thaw it 3 times, to generate a population of necrotic cells. The intact cells

2. If possible, put in a tube 2×10^5 healthy cells of the same type, and resuspend them in 600-1000 μ l of PBS. Divide this sample in two, by putting vol/2 in a new tube. Take one of the tubes and freeze and thaw it 3 times, to generate a population of necrotic cells. The intact cells of the other tube will serve as control for healthy cells.
3. Centrifuge and resuspend the pellets with the PI solution. Leave the samples at RT for an interval between 30 min and 1.5 hr. This time has to be adjusted on the cell type.
4. Analyze by flow cytometry the healthy cell sample s red fluorescence on a log scale. Place the peak at low fluorescence values (between 10^0 and 10^1) working on signal gain and PMT voltage.
5. Analyze the necrotic cell sample. These cells will be all brightly stained by PI, and will appear as a peak at very high fluorescence values.
6. Analyze the sample which may contain healthy, apoptotic and necrotic cells. Apoptotic cells will appear as a dimly fluorescent population.

3B. Commentary

3B.1. Background information

The intact membrane of living cells excludes cationic dyes, such as PI or trypan blue. Due to their extensive membrane damage, necrotic cells are quickly stained by short incubations with PI. Apoptotic cells (with the exception of late apoptoses, which, from this standpoint, behave as necrotic cells) show an uptake of PI that is much lower than that of necrotic cells. It is therefore possible to distinguish healthy (PI negative), apoptotic (PI dim) and necrotic (PI bright) cells from each other. This method can be usefully combined with the scatter method.

During the method optimization, it is useful to sort (if possible) a few hundreds cells on a slide and directly look at them under the fluorescence microscope.

3B.2. Troubleshooting

Usually the distinction between apoptotic and necrotic cells is quite evident, while the discrimination between healthy and apoptotic cells may be difficult. If these two populations tend to merge, increase the time of incubation with PI. If distinction is good but the peak of healthy cells is too high, decrease the time of incubation with PI.

3B.3. Time considerations

The whole procedure will take 3 hr or less, depending on the length of the incubation with PI.

3B.4. Key references

1. Lyons, A. B., Samuel, K., Sanderson, A., and Maddy, A. B. 1992. Simultaneous analysis of immunophenotype and apoptosis of murine thymocytes by single laser flow cytometry. *Cytometry* 13: 809.
2. Vitale, M., Zamai, L., Mazzotti, G., Cataldi, A., and Falcieri, E. 1993. Differential kinetics of propidium iodide uptake in apoptotic and necrotic thymocytes. *Histochemistry* 100: 223.
3. Zamai, L., Falcieri, E., Marhefka, G., and Vitale, M. 1996. Supravital exposure to propidium iodide identifies apoptotic cells in the absence of nucleosomal DNA fragmentation. *Cytometry* 23: 303.

2C. Third protocol: membrane permeability by Hoechst/PI

2C.1. Materials

- PBS, pH 7.0
- Hoechst (HO) 33342 1.5 μ g/ml in PBS
- propidium iodide (PI) 5 μ g/ml in PBS (always wear gloves when using PI)

2C.2. Methodology

1. Incubate 1×10^6 cells with HO for 15 min at RT, then stop the reaction on ice for 3 min.
2. Centrifuge the cells, discard the supernatant and resuspend the pellet in 300-500 μ l of PBS containing PI.
3. Analyze the sample by flow cytometry. Use a UV excitation (356 nm). Collect the blue (430 nm) fluorescence of HO and the red (630 nm) fluorescence of PI.

3C. Commentary

3C.1. Background information

Apoptotic cells show a high HO staining and a low PI staining, since they initially tend to exclude PI. Necrotic cells are brightly stained with PI. while healthy cells are dimly stained by HO and not stained by PI.

Apoptotic cells show a high HO staining and a low PI staining, since they initially tend to exclude PI. Necrotic cells are brightly stained with PI, while healthy cells are dimly stained by HO and not stained by PI.

3C.2. Time considerations

The whole procedure will take about 30 min.

3C.3. Key references

1. Belloc, F., Dumain, P., Boisseau, M. R., Jalloustre, C., Reiffers, J., Bernard, P., and Lacombe, F. 1994. A flow cytometric method using Hoechst 33342 and propidium iodide for simultaneous cell cycle analysis and apoptosis determination in unfixed cells. *Cytometry* 17: 59.
2. Ormerod, M. G., Sun, X. M., Snowden, R. T., Davies, R., Fearhead, H., and Cohen, G. M. 1993. Increased membrane permeability of apoptotic thymocytes: a flow cytometric study. *Cytometry* 14: 595.

2D. Fourth Protocol: DNA content

2D.1. Materials

- PBS pH 7.0
- Staining solution: a freshly prepared solution of (20 ug/ml propidium iodide + 2 mg/ml DNase-free RNase in PBS) (always wear gloves when using PI)
- 70% ethanol

2D.2. Methodology

1. Fix the cells in suspension by adding 1 ml of cold (4°C) 70% ethanol to the resuspended pellet. Incubate 30 min on ice. Cells can be stored in ethanol at -20°C up to 1 or 2 weeks.
2. Centrifuge the cells, and discard ethanol.
3. Resuspend the pellet in 300-500 ul of the staining solution, and incubate for 15 min at RT.
4. Analyze by flow cytometry, at an excitation wavelength of 488 nm. Collect PI fluorescence at > 600nm.

3D. Commentary

3D.1. Background information

Fixation of cells with precipitating fixatives (such as ethanol or acetone) causes the leakage of the cleaved low MW DNA fragments that are produced during apoptosis. As a consequence, apoptotic cells can be identified as a hypodiploid peak, while healthy cells generate a typical cell cycle histogram. Necrotic cells are generally found among the healthy ones. The scatter methodology described before can help in the distinction between healthy and necrotic cells.

3D.2. Time considerations

The whole procedure will take about 1.5-2 hr.

3D.3. Key references

1. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. 1991. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J. Immunol. Meth.* 139: 271.
2. Zauli, G., Vitale, M., Re, M.C., Furlini, G., Falcieri, E., Gibellini, D., Visani, G., Davis, B.R., Capitani, S., La Placa, M. 1994. In vitro exposure to human immunodeficiency virus type 1 induces apoptotic cell death of the factor-dependent TF-1 hematopoietic cell line. *Blood* 83: 167.

APOPTOSIS vs. NECROSIS BY ELECTRON MICROSCOPY

1. Introduction

When analysed by light microscopy or fluorescence microscopy, (i.e. after Giemsa, Hoechst, DAPI, PI stainings and others) necrotic cells appear deeply different from apoptotic ones. The differences mainly concern cell shape and cell structural features. In numerous cell types frequently observable in culture and animal models, as well as human tissues, surface blebbing is considered a pattern specific of apoptosis. It is due to a deep cytoskeleton rearrangement, causing progressive changes in cell shape and organelle distribution. The final stage of this process is the cell splitting in numerous cellular portions, termed apoptotic bodies, whose most common final fate in vivo is to be engulfed by phagocytes. Blebs can be occasionally also described on the surface of cells undergoing necrosis, but, in this condition, they are followed by the rapid appearance of membrane discontinuities, causing water influx and strong ion distribution. No cell splitting appears in the course of necrosis, but a general cell hydration occurs, followed by cell swelling and disruption. Electron microscopy

provides a detailed characterization of both phenomena, allowing the analysis of different cell compartments and, particularly, of the nucleus and its components.

Apoptotic and necrotic cells can be studied and distinguished by TEM (transmission electron microscopy), SEM (scanning electron microscopy) and FF (freeze-fracture), as well as by means of some more specific and complex ultrastructural approaches (immuno-localization of particular proteins, cytochemical localization of substrates, etc.) (1).

1. TEM allows the analysis of sectioned specimens and provides a qualitative bidimensional image of the inner cell, after fixation, embedding, and staining.

2. SEM describes the cell surface and gives information about shape modifications and membrane specializations, with a relatively low detail resolution, but giving an overall information.

3. The FF technique utilizes frozen cells and the possibility to cleave them under high vacuum, exposing novel surfaces of the fractured cells, which are immediately platinum-carbon coated. These replicas, chemically purified from organic underlying tissue, are analyzed by the transmission electron microscope. New information on cell membrane and organelles can thus be obtained.

a) **Necrosis**: when a cell dies by necrosis the early changes can be identified on plasma membrane. It shows progressive discontinuities (Figure 1), which cause general cell hydration and swelling, as well as organelle disruption. This condition is clearly evidenced by the early rearrangement of the freeze-fractured plasma membrane (2), as well as after SEM procedure of later necrotic stages (Figure 3).

The cytoplasm and plasma membrane are therefore the first target of the necrotic process, while the nucleus appears relatively well preserved, at least concerning its inner constituents (Figure 1), for some time.

b) **Apoptosis**: in the case of apoptotic death, the cell nucleus is early and specifically involved. TEM shows, indeed, a particular chromatin margination, followed by its compaction towards the nuclear periphery, to form one or several frequently cup-shaped masses. In the residual diffuse chromatin, remnants of deeply modified nucleoli can be still revealed (Figure 2). The nucleus appears therefore strongly rearranged, if compared to the normal one, which shows a perinuclear and a perinucleolar dense heterochromatin, clearly distinguishable from the diffuse interchromatin. (Figure 2). Surprisingly, plasma membrane and organelles are preserved for long, except for cytosol condensation and blebbing phenomenon, which characterize some apoptotic models (Figure 4). Subsequently, the nucleus generates numerous compact electron dense micronuclei, frequently released in the extracellular space. Cell splitting in a number of apoptotic bodies represents generally the final stage of apoptosis. The most common mechanism of apoptotic cell deletion in vivo is the engulfment by phagocytes. Differently, in vitro apoptotic cells undergo a late process of secondary necrosis.

FF reveals very peculiar nuclear aspects in apoptotic cells. Cryoprotected frozen cells or tissues can be indeed fractured in different possible ways. The nucleus can be cleaved, thus showing chromatin arrangement and allowing the morpho-metrical study of its fibres (3). More frequently, membrane leaflets are exposed, and details of membrane architecture and specializations can be described.

In particular, the nuclear envelope of apoptotic cells displays a characteristic clustering of nuclear pores (Figure 6), which are regularly located - as TEM and FF comparison confirms - in close relationship of the diffuse chromatin, being the dense chromatin masses pore-free. This arrangement, never present in normal cells (Figure 5), has a specific functional meaning and is typical of apoptotic cells (4).

2. Protocol

2.1. Materials

2.5% glutaraldehyde in phosphate buffer 0.1 M pH 7.3
 1% osmium tetroxide in phosphate buffer 0.1 M pH 7.3
 0.2 M phosphate buffer solution (Sorensen)
 0.15 M phosphate buffer solution (Sorensen)
 alcohol-distilled water solutions 50%, 70%, 95%
 alcohol 100%
 propylene oxide
 araldite resin: 50% component A, 50% component B, added with 1.5% component C
 1% toluidine blue in distilled water, added with 0.5% sodium carbonate
 3% uranyl acetate in water: absolute alcohol, 1:1
 lead citrate: 1.33 g lead nitrate + 1.76 g sodium citrate in 50 ml distilled water, added with 8 ml 1N NaOH
 poly-L-lysine 1 mg/ml in distilled water
 30% glycerol in 0.1 M phosphate buffer pH 7.3
 freon 22
 liquid nitrogen
 commercial bleach
 chloroform:methanol, 2:1
 Reichert Ultracut FC4 Ultramicrotome
 nickel or copper 100/200 mesh grids, coated with 4% formvar in chloroform
 Balzers MED10 Critical Point Drying device
 Balzers Gold Sputter device
 Balzers BAF 400D Freeze-Fracture device
 Philips CM10 Transmission Electron Microscope
 Cambridge Stereoscan 200 Scanning Electron Microscope

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2.2. Methodology

TEM

1. At least 500.000 cells or a 1 mm x 1 mm tissue fragment are necessary to have a specimen proper for ultrastructural analysis.
2. The glutaraldehyde fixation must be performed immediately, possibly preceded by a PBS washing, to remove culture or biological medium. During fixation, cells must not be touched, to avoid resuspension, which could cause cell loss.
3. After 1 h at RT, a washing is required, with 0.15 M phosphate buffer, which keeps the physiological osmolarity, to prevent shape changes or distortions. The permanence in buffer can last until 8-10 days, at 4°C, eventually changing the buffer with a new fresh one.
4. Postfixation is carried out in 1% OsO₄ in the same buffer, for 1 h RT, followed by a rapid wash in the same buffer.
5. CAUTION: Both glutaraldehyde and OsO₄ fixation cause vapour exhalations and manipulations must be carried out with care and under chemical hood.
6. Specimen dehydration follows by means alcohol-water solutions of increasing concentrations (50%, 70%, 95%, 100%, 15-20 min each) and final treatment with 100% propylene oxide for 30 min. Araldite embedding starts with a 1:1 mixture of propylene oxide:araldite for 1 h, followed by 1:3 mixture overnight, at RT.
7. After an additional treatment in undiluted resin for 1 h, polymerization is performed at 60°C for 3-4 days.
8. Sectioning is generally preceded by the analysis of semithin sections, stained at 40°C with 1% toluidine blue, which gives an overall information about the specimen, thus facilitating the choice of thin section areas.
9. Staining of thin sections, collected on nickel or copper grids, is performed by means of uranyl acetate and lead citrate for 10 and 15 min, respectively. Careful washing is necessary between the two treatments, as well as at the end of the procedure.

Observations with conventional transmission electron microscope are generally performed at 80 KV.

SEM

1. Smaller cell amounts can be usefully processed for this ultrastructural approach. In the case of isolated cells, they must be fixed in suspension (not as a pellet, as for TEM) with the same glutaraldehyde as above, after having carefully removed the medium, with a gentle centrifugation before fixation.
2. For SEM, cell adhesion to a substrate is required and it is obtained by cell suspension deposition on poly-L-lysine coated slides and storing at 4°C, in a moist chamber, overnight.
3. Slides are therefore postfixed and dehydrated as above. A crucial step for SEM procedure is solvent removal in the absence of specimen distortions or drying artifacts. This is now performed by a critical point drying device, which utilizes the progressive substitution of the solvent with liquid CO₂, successively evaporated by high pressure-heating.
4. Finally, specimens are mounted on silver paint-coated conventional stubs, and sputtered with gold to stabilize their surface and prevent electric charge effects. Observations with conventional scanning electron microscope are generally performed at 1-10 KV, depending on specimen characteristics and coating quality.

FF

1. Specimens, fixed with 2.5% glutaraldehyde as above, and washed, are mounted on gold supports and cryoprotected with 30% glycerol in 0.1 M phosphate buffer pH 7.3, for 1-3 h, to prevent ice crystal formation, which could disrupt membranes and organelles.
2. Subsequently, they are quickly frozen in Freon 22 and rapidly transferred into liquid nitrogen. The fracture is performed at -115°C and platinum-carbon/carbon replicas of the exposed surfaces are obtained, checking the thickness with a quartz film monitor. Organic tissue deletion is obtained by cleaning with commercial bleach and degreasing with a chloroform-methanol mixture.
3. Purified replicas are gently washed with distilled water and collected on nickel grids, previously coated with formvar and carbon.

3. Commentary

3.1. Background information

TEM procedures provide detailed information about necrotic and apoptotic cells, particularly in the case of very early and minimal changes, which are very difficult to detect with the other experimental approaches. Nevertheless, they are qualitative and do not generally provide quantitative information. Particularly, both necrosis and apoptosis, are generally focal phenomena and quantitation must be performed, before TEM, by light/ fluorescence microscopy or flow cytometry.

SEM can utilize very low magnification observations, so providing an overall picture of the specimen, but has some limits. Firstly SEM analysis is performed at low power resolution and, consequently, with a minor investigative potential. Secondly, it analyses the cell surface and no information about the inner cell are available. Finally, FF provides very detailed information. Pt-C thin replicas are indeed analyzed with a transmission electron microscope. This allows high magnifications and some possible morpho-functional correlations. In addition, the shadowing technique provides a 3-D image, which represents a complementary approach to conventional TEM. Unfortunately, and in contrast with TEM sections, the fracture plane is casual and its direction (over or inside necrotic, apoptotic or normal cells) is unpredictable.

3.2. Critical Parameters

The most critical parameters for TEM, SEM and FF are the total number of cells necessary to process the samples and the percentage of necrotic or apoptotic cells present. This can be previously checked, in similar experimental conditions, by flow cytometry.

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3.3. Troubleshooting

A good and immediate fixation, to which a particular care must be devoted, is crucial.

3.4. Time considerations

Complete TEM procedure takes 4-5 days, SEM takes 2 days and FF takes 1 day. The time spent for the electron microscope observation is variable and strongly dependent on the operator's experience.

3.5. Key References

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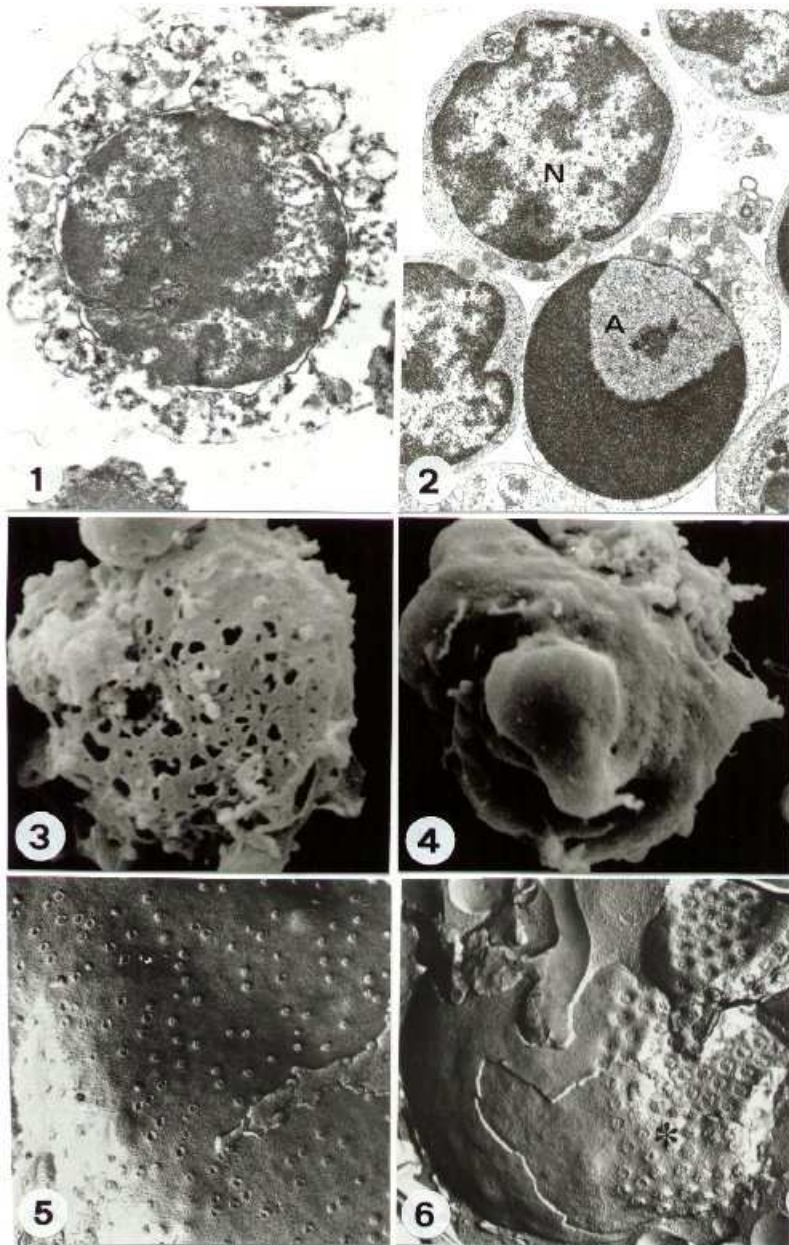


FIGURE 1

TEM of a necrotic cell: the disruption of plasma membrane and organelles is observable. A relative preservation of nuclear

morphology appears.
(original magnification: x 10,000)

FIGURE 2

TEM of an apoptotic (A) and a normal (N) cell. The characteristic chromatin rearrangement appears in A, strongly different from its normal organization (N). The good preservation of membrane and organelles is also evident. (original magnification: x 8,000)

FIGURE 3

SEM of a necrotic cell. Numerous lesions appear on the cell surface. (original magnification: x 5,000)

FIGURE 4

SEM of an apoptotic cell. Surface blebbing is evident. (original magnification: x 5,000)

FIGURE 5

FF of normal cell, nuclear envelope. The regular distribution of nuclear pores is visible. (original magnification: x 30,000)

FIGURE 6

FF of apoptotic cell. The nuclear envelope shows a characteristic clustering (asterisc) of nuclear pores. (original magnification: x 35,000)