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# Analysis of infected human mononuclear cells by atomic force microscopy (*)

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**Summary.** — The surfaces of the human lymphoid cells of the line H9 chronically infected with the Human Immunodeficiency Virus HIV-1, and of human monocytes acutely infected *in vitro* with *Mycobacterium Tuberculosis* (MTB) were dried, fixed and imaged with atomic force microscopy (AFM). These images were compared with those of non-infected samples. Dried and fixed samples of infected cells can be distinguished from non-infected ones by AFM technology due to their different surface structures and by the presence of pathogenic (viral or mycobacterial) agents on the cell surface.

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## 1. – Introduction

Recently, a new generation of non-optical microscopes, the so-called scanning probe microscopes, is gaining an increasing attention because of the astonishing resolution achievable [1, 2]. The atomic force microscope [3] has become one of the most widely used in several fields of biology [4, 5], as it allows to confirm and enrich the information obtained by optical microscopes. The important aspects of AFM studies are: i) no need for treatments that might alter the molecular and conformational structure of the sample; ii) three-dimensional images that allow detection of new aspects of cell morphology, cell-cell interaction and impact of pathogenic infections [6]; iii) applicability to a great variety of surfaces despite their conductivity, allowing to observe biological material both fixed [7] and in its physiological environment [8, 9]. Another aspect is to investigate the changes of the cell surface during differentiation [10], activation or infection.

(*) In honour of Prof. Gianfranco Chiarotti on the occasion of his 70th birthday.

 

Microbes (viruses and bacteria) enter the eukaryotic cell by endocytosis and phagocytosis. The first step of the microbe-host interaction is of paramount importance in the establishment of infection and disease [11]. Tuberculosis and AIDS represent two of the most serious infections in man [12]. There is no information available on their contact with the membranes of human cells.

In this work we studied at membrane level these two different mechanisms of host cell reaction to contact with HIV and MTB. Morphological characteristics of non-infected lymphoid H9 cells and HIV-infected H9/HTLV-IIIB cells were easily imaged from fixed and dried cell preparations. Some particles, presumably of viral origin (120–130 nm size), were also observed in proximity of the cell surface of H9/HTLV-IIIB cells. Samples of human monocyte-derived macrophages acutely infected *in vitro* with MTB were analyzed for their surface structure. In some cases it was possible to recognize, in the AFM images at the top of the membrane surface, particles of size and typical shape of mycobacteria.

## 2. – Materials and methods

2·1. *Sample preparation.* – The cell line H9 used for these experiments is a clonal derivative of the human T cell line HuT 78 isolated originally from a patient with Sezary syndrome. H9 cells are routinely used for detection, isolation and continuous propagation of HIV-1 and related cytopathic variants of human T cell lymphotropic retroviruses. The infected cell line H9/HTLV-IIIB is characterized as human T cell line (origin H9) that produces Human Immunodeficiency Virus (HIV, HTLV-III). Both cell lines were grown in suspension in tissue culture medium RPMI 1640 containing 2.0 g/l sodium bicarbonate, supplemented with 1% L-Glutamine and 10% FCS (heat inactivated fetal calf serum) and cultivated in tissue culture flasks (Corning) at 37 °C and 5% $CO_2$ humidified atmosphere. Medium has been changed twice per week, adjusting cell numbers to $1–5 \times 10^5$/ml.

Fresh human monocytes were obtained either by elutriation or by adherence to plastic from peripheral blood mononuclear cells (PBMC) isolated by Ficoll Hypaque gradient centrifugation from peripheral blood of healthy donors. Monocytes were infected according to a protocol developed in our laboratory. In brief, $4–6 \times 10^6$ monocytes were pelletted and incubated at 37 °C/5% $CO_2$ humidified atmosphere in a conical polypropylene tube (Falcon) with the same number of Mycobacteria as a titered stock of MTB human H37R strain. After 2 hours, cells were washed 3 times in tissue culture medium, suspended at $0.5–1 \times 10^6$/ml and incubated in 24 well tissue culture plates.

Round cover glass slides (12 mm in diameter) were cleaned with detergent, thoroughly rinsed with sterile distilled water and then treated with absolute ethanol. Prior to use, slides were coated with 10 $\mu$g/ml poly-L-lysine (PLL) solution and incubated under a UV lamp for 20 min. PLL was then aspirated, and the slides were dried under a laminar flow.

Cells for AFM analysis were either grown directly on the glass slides placed in a 6 well tissue culture plate (Corning) or cultured in flasks, detached by scraping off, and put at various concentrations ($5 \times 10^4–5 \times 10^6$/ml) on the slides. In both cases, the slides were allowed to dry under a laminar flow, and fixed with 4% paraformaldehyde for 5 min. at room temperature. Slides were then washed 4 times with PBS, followed by 5 times washing with distilled water, and dried as before.

Samples with MTB only were prepared by centrifugation of approximately $8 \times 10^6$ bact/ml, washed once in complete medium, put on glass slides and prepared as described before.

**2**˙2. *Atomic force microscopy.* – The atomic force microscope is described in detail elsewhere [13]. Constant-force images have been obtained with the microscope working in the repulsive mode with forces less than 1nN from zero cantilever deflection. Friction images have been collected at the same time. Gold-coated $Si_3N_4$ MICRO-LEVERS, from Park Scientific Instruments with a spring constant of 0.023 N/m were used. All the images are unfiltered with only rigid plane subtraction.

## 3. – Results and discussion

**3**˙1. H9 *cells infected by* HIV. – Several experiments have been performed either by directly growing cells onto the glass slides or by putting on glass slides cell suspensions grown regularly in TC flasks. The latter method has been chosen since it provided more uniform monolayers. In all samples examined by AFM, uninfected H9 cells (white part of fig. 1a) showed smooth cell surface. Figure 1b is a cross-section of the H9 cell of fig. 1a, showing the round shape (diameter around 6 $\mu$m) of the H9 cell with a height of approximately 0.7 $\mu$m. Images of the HIV-infected H9 cells showed a different morphology (fig. 2a): a membrane structure with a great quantity of crater-like structures (see arrows in fig. 2a) and highly fragmented cell borders. Figure 2b is a cross-section of the H9/HIV cell of fig. 2a. Figure 3a is a 2 $\mu$m by 2 $\mu$m image of the cell border region: it was possible to detect round-shaped particles (arrows in fig. 3a) morphologically different from the cellular background. The height corrugation of the white line drawn over such particle is shown in fig. 3b: the particles have, approximately, a full width half-maximum (FWHM) of 120 nm and a height of 60 nm. These values are consistent with the expected dimensions of HIV, thus suggesting the viral origin of these particles. In this context it should again be underlined that investigation on dried and fixed cells by AFM can give images of processes at molecular level and may reduce scanning-force–induced deformations. Recently, Haeberle *et al.* [14], succeeded in getting AFM images of cultured cells from monkey kidneys infected with suspensions of pox viruses, demonstrating significant membrane modifications immediately after and several hours from infection. In accordance to these findings, our observation of crater-like holes and compromised microvilli, together with the identification of, presumably, viral particles around the microvilli, can be interpreted as the expression of an elevated endocytotic and exocytotic activity of the infected lymphocytic cell, considering in particular that the H9/HTLVIII-B represents a steadily proliferating cell line hosting a chronic viral infection. Generally, the exact percentage of actually infected lymphocytes in a chronically infected cell line can hardly be defined. Thus, one can expect a continuous process of endocytosis signaling new viral entry into proliferated yet uninfected cells, as well as exocytosis of viral proteins and progeny virus expelled after intracellular virus assembly which is known to occur after more than 8 hours from infection. In accordance with the findings of Haeberle *et al.* [14], we could observe the particles of round shape and 120–130 nm in size in all cases in a region where the cell membrane is dominated by finger-like structures (see fig. 3a). Since it is known from electron

microscope images that viruses exit the cell at the end of the microvilli formed on the membrane, the images shown in fig. 3a may represent progeny virus during exocytosis. Compared to studies on living cells, it is obviously less probable to identify a statistically significant quantity of cells in state of active exocytosis on dried and fixed

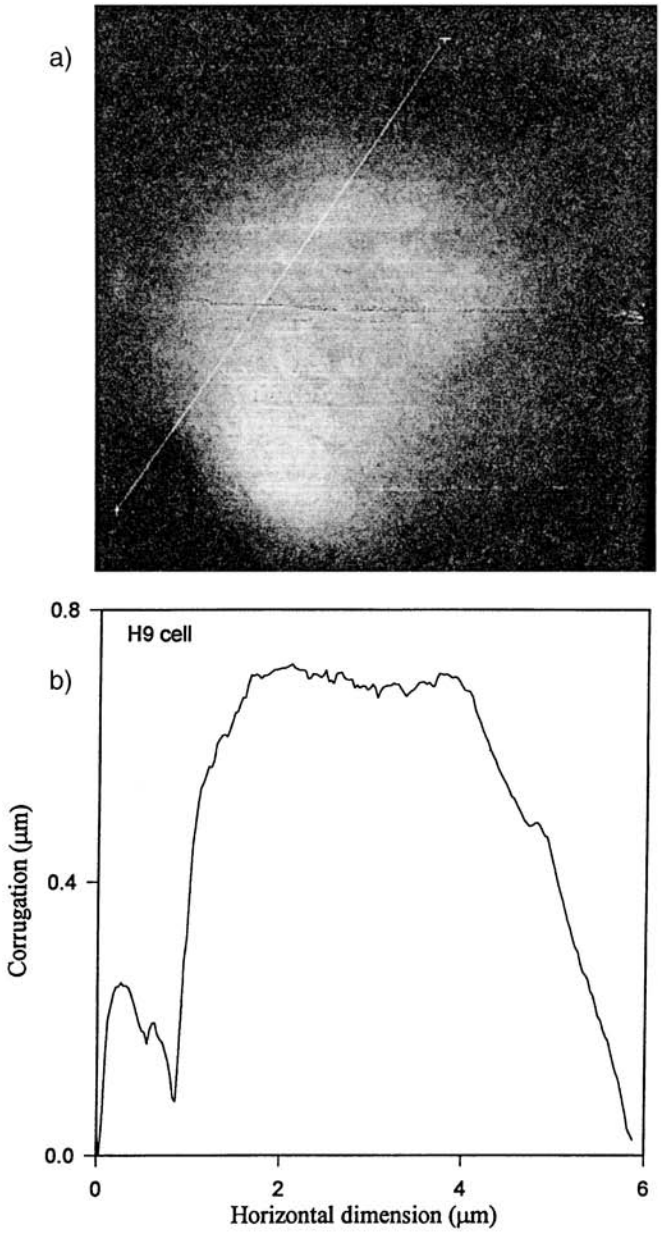


Fig. 1. – a) AFM image (6 $\mu$m by 6 $\mu$m) of an H9 cell taken in constant-force mode. The image is in a top-view representation with colors set white for the highest structures and black for the deepest ones. b) Cross-section taken along the H9 cell of panel a) showing its height corrugation.

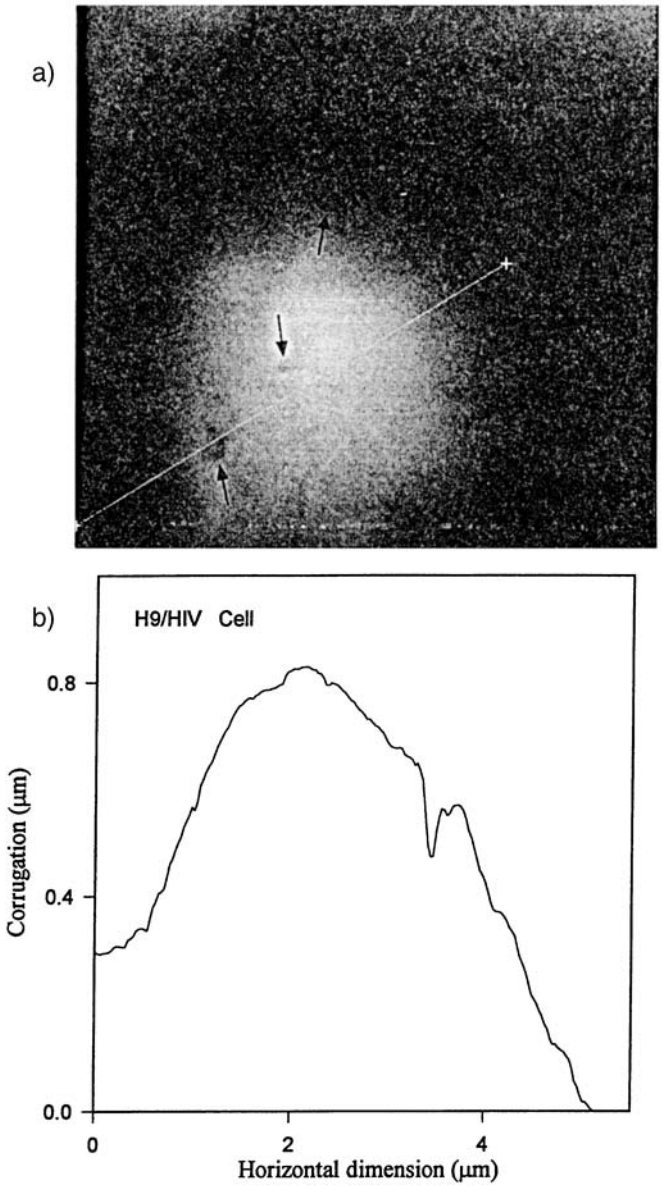


Fig. 2. – a) AFM image (6 $\mu$m by 6 $\mu$m) of an H9/HIV cell taken in constant-force mode. Crater-like structures and highly fragmented cell borders with compromised microvilli are indicated by arrows. Colors are set as in fig. 1. b) Cross-section taken along the H9/HIV cell of panel a) showing its height corrugation.

cells. However, by using a chronically infected cell line, we could show the ability of AFM to observe cell membrane modifications due to viral infection far below the micrometer scale in a three-dimensional view.

**3**·2. MTB *infected macrophages.* – Figure 4 shows an AFM image (6 $\mu$m by 6 $\mu$m) of MTB only after deposition on the substrate: it revealed, as the majority of AFM

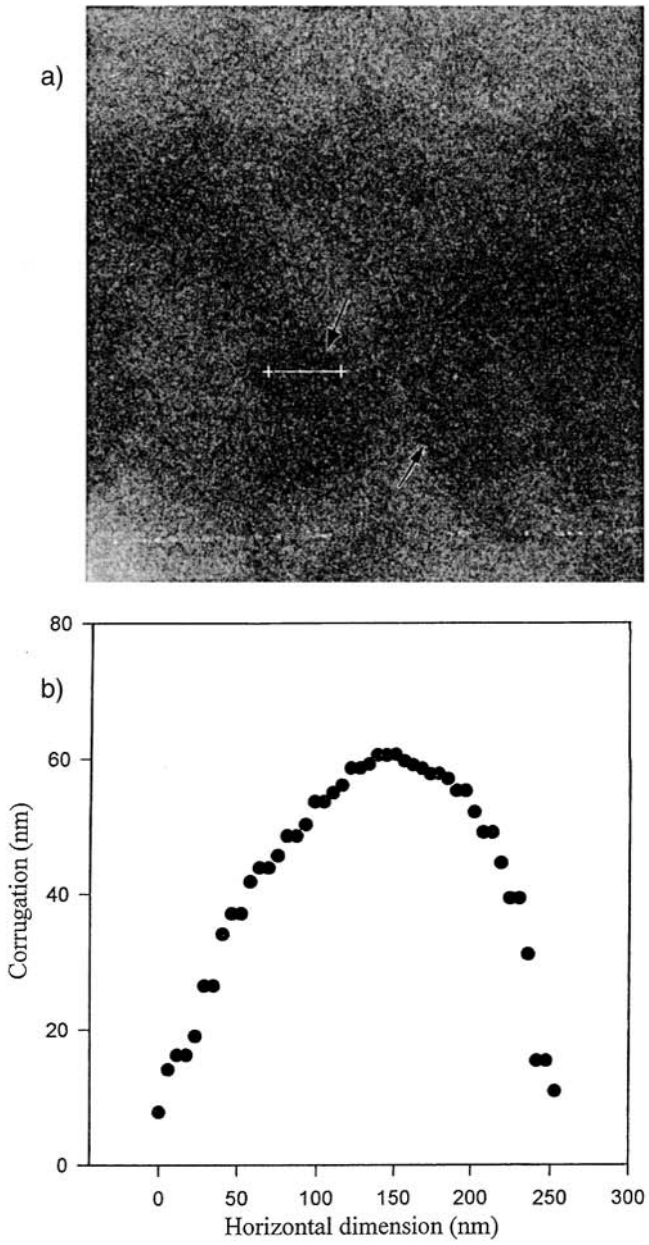


Fig. 3. – a) Constant-force AFM image (2 $\mu$m by 2 $\mu$m) of a highly fragmented cell border of the HIV-infected H9 cell. The image is in a top-view representation, and colors are set as in fig. 1. Round-shaped particles of approximately 120 nm size, presumably of viral origin, are indicated by arrows. b) Cross-section taken along the white line drawn in panel a).

images, aggregates of MTB particles with a single MTB size of approximately 2–3 $\mu$m length and 0.5 $\mu$m width.

Figure 5a shows a constant-force AFM image of a monocytic cell (lower part of the image) in a top-view representation, after fixation to the substrate. Figure 5b is a cross-section line taken along the cell showing that the macrophage has a width of 6 $\mu$m with a height of 0.9 $\mu$m. Figure 6 shows a constant-force AFM image of a monocytic cell (lower part of the image) in a three-dimensional representation, after infection with MTB. Generally, comparing AFM images from lymphocytic and monocytic cells, the stronger attachment of the macrophages to the PLL-treated glass slides resulted in less round-shaped borders of the monocytic cell, with in some cases a lower central area. The depression on top of the monocytic cell is clearly visible and resembles closely SEM images [15]. Sometimes in correspondence to the depression on top of the monocytic cell a mycobacterial particle of cigar-shaped structure with dimensions very similar to the MTB (see fig. 4) was identified. In some cases, AFM images taken in friction mode enhanced, by visualizing their different chemical nature, MTB particles just located in correspondence to the depression on top of the monocytic cell.

Studying phagocytotic activity of murine peritoneal macrophages, Beckmann *et al.* [16] were able to analyze size and distribution of phagocytized particles of different origin in living and fixed cells. Their results confirm that scan-induced deformations occur less often on fixed and/or dried macrophages; nevertheless, fixation procedures transform the topology of the cell surface, as shown by the characteristic relief structures due to particle phagocytosis [17]. In our case, samples of dried and fixed MTB-infected macrophages have been performed taking cells after 1-2 days from infection, assuming that part of the MTB particles were internalized by phagocytosis, and only a certain quantity could be found at the cell surface. Choosing this time

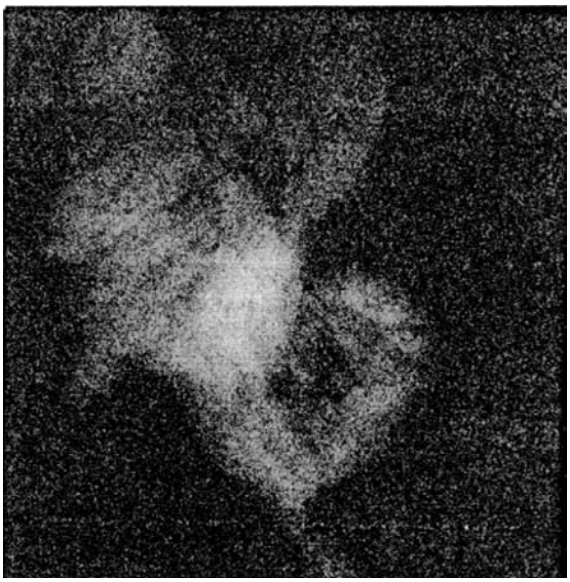

Fig. 4. – AFM image (6 $\mu$m by 6 $\mu$m), taken in constant-force mode, of MTB only. The image is in top-view representation, with colors set as in fig. 1. An aggregate of multiple mycobacteria oriented in cord formation is visible.

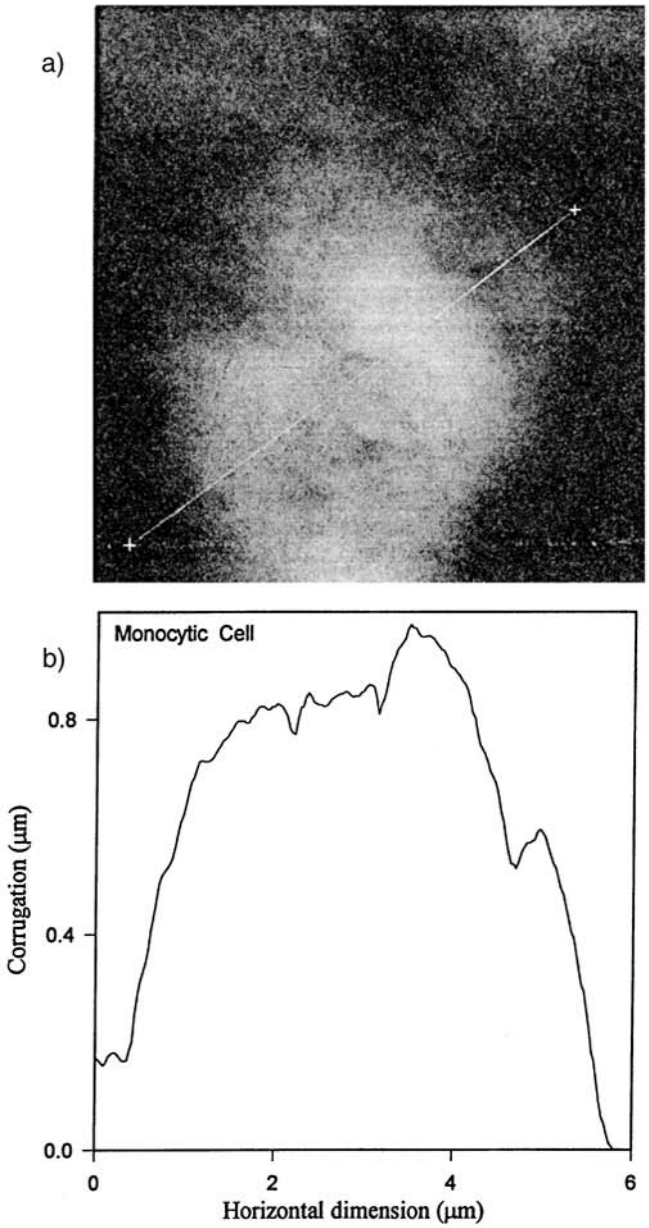


Fig. 5. – a) AFM image (6 $\mu$m by 6 $\mu$m), taken in constant-force mode, of a monocytic cell after fixation on the substrate. The image is a top-view representation with colors set as in fig. 1. b) Cross-section taken along the monocytic cell of panel a) showing its height corrugation.

interval, it was possible to confirm by AFM images our observations on the mechanism of *in vitro* MTB infection of human monocytes [18], which results usually in two different cell fractions: a non-adherent fraction, containing a majority of less vital cells undergoing programmed cell death (shown by trypan-blue dye exclusion and FACS

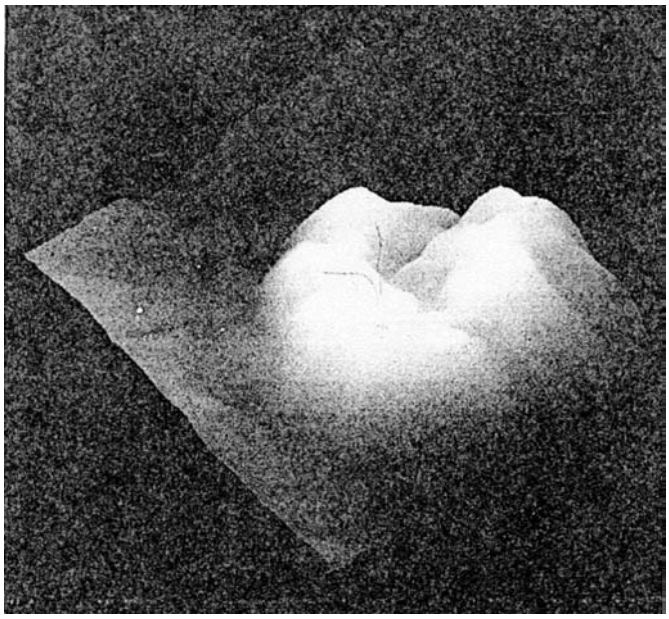

Fig. 6. – AFM image, taken in constant-force mode (6 $\mu$m by 6 $\mu$m) in a three-dimensional representation of a monocytic cell after infection with MTB.

analysis with propidium iodide staining), that detach from the plate after 12–24 h from infection, and an adherent population containing usually more than 70% viable cells characterized by maintenance of typical functional abilities, such as antigen presenting capacity, phagocytosis, and cytokine production. Accordingly, AFM analysis of the non-adherent fraction gave hardly interpretable images of cell fragments and destroyed membrane structures (data not shown), whereas the viable adherent cell fraction could be imaged normally by AFM.

Garcia *et al.* [19] have already demonstrated with their studies on BCG (Bacille Calmette-Guerain) that the AFM is very useful to obtain detailed morphology of mycobacteria, and to identify the presence of disk-like structures defined as extra-cellular microgranules or blebs which cannot be seen with light microscopy. Their study also confirmed the well-known cord formation of parallel-oriented mycobacteria, due to hydrophobic interactions. However, we want to point out that these studies were performed on BCG only, and not on bacteria in contact with the macrophage cell surface.

## 4. – Conclusions

This work confirms that the application of atomic-force microscopy in cell biology can provide new aspects for the study of human cells and distinct infection mechanisms. The three-dimensional AFM images gave detailed information on the surface interaction between microbes and host cells, and on the processes of phagocytosis and endo/exocytosis occurring in response to acute or chronical infection.

This information may be used to further study the mechanisms of susceptibility or resistance to infection by intracellular pathogens such as mycobacteria and HIV.

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## REFERENCES

[1] EDSTROM R. D., YANG X. R., LEE G. and EVANS D. F., *FASEB J.*, **4** (1990) 3144.
[2] HOH J. H. and HANSMA P. K., *Trends Cell Biol.*, **2** (1992) 208.
[3] BINNIG G., QUATE C. F. and GERBER CH., *Phys. Rev. Lett.*, **56** (1986) 930.
[4] PERSSON B. N. J., *Chem. Phys. Lett.*, **141** (1987) 366.
[5] LAL R. and JOHN S. A., *Am. J. Physiol.*, **266** (1994) C1.
[6] IMHOF B. A., VOLLMERS P., GOODMAN S. L. and BIRCHMEIER W., *Cell*, **35** (1983) 667.
[7] CRICENTI A., DE STASIO G., GENEROSI R., PERFETTI P., CIOTTI M. T. and MERCANTI D., *Scan. Microsc.*, **9** (1995) 695.
[8] HAEBERLE W., HOERBER J. K. H. and BINNIG G., *J. Vac. Sci. Technol. B*, **9** (1991) 1210.
[9] OHNESORGE F. and BINNIG G., *Science*, **260** (1993) 1451.
[10] BONFIGLIO A., PARODI M. T. and TONINI G. P., *Exp. Cell Res.*, **216** (1995) 73.
[11] DI MASSIMO A. M., PLACIDO R., BACH S., ANASTASI A. M., MASSIMO A., CAPOBIANCHI M. R. and COLIZZI V., *Immunology*, **76** (1992) 117.
[12] MARIANI F., PICCOLELLA E., COLIZZI V., RAPPUOLI R. and GROSS R., *J. Gen. Microbiol.*, **139** (1993) 1767.
[13] CRICENTI A. and GENEROSI R., *Rev. Sci. Instrum.*, **66** (1995) 2843.
[14] HAEBERLE W., HOERBER J. K. H., OHNESORGE F., SMITH D. P. E. and BINNIG G., *Ultramicroscopy*, **42** (1992) 1161.
[15] KAUFMANN S. H. E., *Immunology Today*, **16** (1995) 338.
[16] BECKMANN M., KOLB H. A. and LANG F., *J. Membr. Biol.*, **140** (1994) 197.
[17] KOLB H. A. and UBL J., *Biochim. Biophys. Acta*, **899** (1987) 239.
[18] ZEMBALA M., PRYIMA J., PLUCIENNICZAK A., SZCZEPANEK A., RUGGIERO I., JASINKI M. and COLIZZI V., *Immunol. Invest.*, **23** (1994) 189.
[19] GARCIA A. A., PETTIGREW W. C. and GRAHAM J., *Scanning Microsc.*, **7** (1993) 577.