JOURNAL OF PATHOLOGY, VOL. 180: 000–000 (1996)

APOPTOSIS OF HUMAN MONOCYTES/MACROPHAGES IN *MYCOBACTERIUM TUBERCULOSIS* INFECTION

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SUMMARY

Tuberculosis (TB) is still a major health problem, both as a single disease entity and as a cofactor in AIDS. The interaction between macrophage and *Mycobacterium tuberculosis* (MTB) is a critical step in the establishment of an early chronic infection. This study analyses the capacity of MTB to induce apoptosis in cells obtained by broncho-alveolar lavage (BAL) from patients with reactive pulmonary tuberculosis and from AIDS patients with disseminated pulmonary tuberculosis. Apoptosis was increased three-fold in BAL cells obtained from patients with pulmonary tuberculosis and even more markedly in alveolar macrophages of MTB-infected AIDS patients, compared with controls. Apoptosis was analysed and characterized by propidium iodide (PI) incorporation, terminal deoxy transferase (TDT)-mediated dUTP-biotin nick end labelling (TUNEL), and tissue transglutaminase (tTG) expression. The MTB–macrophage interaction was also investigated *in vitro* by infecting monocyte-derived macrophages (MDM) with MTB (virulent strain H37Rv). The induction of apoptosis by MTB required viable bacteria, was dose-dependent, and was restricted to H37Rv. Infection with either *Mycobacterium avium* complex (MAC) of HIV-1 and treatment with heat-killed MTB failed to induce apoptosis.

KEY WORDS—

INTRODUCTION

Mycobacterium tuberculosis (MTB) is responsible for the greatest number of deaths due to a single pathogen infection.[1] The interaction between MTB and monocytes/macrophages in the early phase of infection is crucial for the pathogenesis of the disease. Pathogens have developed specific mechanisms that allow them to establish a close interaction with host cells and to escape the immune response.[2] Infection occurs when the micro-organism evades the first cellular barrier of defence constituted by phagosome–lysosome fusion.[3] Macrophages that are unable to kill intracellularly replicating mycobacteria allow their multiplication until the cell bursts. In this first stage of tuberculosis (TB), necrosis is the cellular event responsible for macrophage death.[4]

Macrophages may also die by apoptosis[5] caused by various means, such as tumour necrosis factor α (TNF-α), oxidative stress, and exposure to silica,[6,7] or by intracellular pathogens, such as *Shigella flexneri* or *Bordetella pertussis*.[8,9] Intracellular infection with *Leishmania donovani*, on the other hand, inhibits macrophage apoptosis.[10] Interestingly, a strict association exists between apoptosis and intracellular killing of mycobacteria, as evidenced by the finding that ATP-induced apoptosis, but not necrosis, is responsible for inhibition of bacillus Calmette-Guérin (BCG) multiplication in monocytes.[11]

In order to investigate whether MTB infection induces apoptosis in monocytes/macrophage, cells present in broncho-alveolar lavages (BAL) obtained from patients with reactive pulmonary TB, or AIDS patients with disseminated MTB lung infection (AIDS-TB), were analysed for DNA fragmentation, as revealed by propidium iodide (PI) incorporation followed by cytofluorimetric analysis and also by the TdT-mediated dUTP-biotin nick end labelling (TUNEL) method. The expression of tissue transglutaminase (tTG), a cross-linking enzyme which acts as an effector element of the cell death programme, was also evaluated. tTG is a multifunctional protein which in its protein cross-linking configuration catalyses a Ca^{2+} and a thiol-dependent acyl-transfer reaction among polypeptide chains, forming $\varepsilon(\gamma$-glutamyl)lysine and N,N-bis(γ-glutamyl)polyamine isodipeptide linkages.[12] As far as the role of tTG in apoptosis is concerned, at least two potential interrelated functions can be hypothesized. tTG, in co-operation with other effector elements, might have a direct effect in killing and/or tTG-dependent cross-linking and might also stabilize the apoptotic cells before their clearance.[12]

BAL cells from the AIDS-TB group were examined because of the presence of numerous mycobacteria in the lungs of these patients, due to the impairment of the immune response, as compared with patients with reactive pulmonary tuberculosis.[13,14] Considering the complexity of apoptosis in the lungs of TB patients, monocyte-derived macrophages (MDM) from healthy donors were infected *in vitro* with MTB and the induction of apoptosis was characterized for the dose of bacteria, viability, virulence, and strain specificity required.

Received 27 March 1996

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CCC 0022-3417/96/000000-00 (0)

MATERIALS AND METHODS

Patients

BAL were obtained from three distinct groups of patients. The control group consisted of seven subjects (four males and three females with a mean age of 43 years) with non-MTB pulmonary diseases (one lung carcinoma, two chronic obstructive pulmonary disease, two pneumonia, two interstitial pneumonia). The TB group consisted of seven patients (all males with a mean age of 40 years) suffering from reactive pulmonary TB. The AIDS–TB group consisted of seven patients with AIDS (IV/C1 group of CDC classification, all males with a mean age of 35 years) affected by disseminated pulmonary TB. Direct microscopic identification of MTB was negative for TB patients and positive for AIDS–TB patients but the presence of MTB was confirmed in all patients by microbiological culture and PCR analysis.[15,16]

Broncho-alveolar lavage

The lavage solution (150 ml containing 0·9 per cent saline solution/2 per cent lidocaina) was introduced through the fibreoptic bronchoscope (Olympus BF 30, Lake Success, NY, U.S.A.). The chosen lobe was the ligula or the right middle, in cases with diffuse pathology, for optimum recovery. In cases with localized unilateral disease, the local lesion was the preferred site. Three aliquotes of 50 ml of 0·9 per cent saline solution were instilled. The instilled fluid was recovered (60–70 per cent of the initial solution) and filtered through a triple layer of sterile gauze. The fluid was centrifuged and the cells were then washed twice with RPMI 1640 (Hyclone, Cramlington, U.K.) medium, counted, and their viability was evaluated by trypan blue exclusion.

Purification of peripheral blood mononuclear cells and monocytes

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors by standard Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. Cells obtained from the interface were resuspended in Hanks balanced salt solution supplemented with 0·1 per cent bovine serum. Monocytes were isolated by countercurrent centrifugal elutriation (JE-6B-Elutriation System, Beckman, Palo Alto, CA, U.S.A.) as previously described.[17] Monocytes were 89–95 per cent pure as judged by staining with Leu M-3 monoclonal antibodies (MAbs) (Beckton Dickinson, Sane Jose, CA, U.S.A.).

Mycobacterial infection of MDM

Mycobacterium tuberculosis H37Rv strain and *Mycobacterium avium* were grown in 7H9 modified medium. The bacteria were then harvested, spun down, resuspended in 10 ml of RPMI 1640 medium without antibiotics, and sonicated for 10 min at low power output (Ultrasonic Cleaning Tank, UST, Milan, Italy). The concentration of bacilli was determined by densitometry at OD 580 nm, based on the standard curve established by correlation of OD with colony forming units (CFU). MDM (10^6/ml) were incubated in 24-well plates (Becton Dickinson, Lincoln Park, NJ, U.S.A.) for 3 h with MTB or MAC, using 1:1, or 10:1, or 100:1 bacillus/monocyte ratio in RPMI 1640 medium supplemented with 10 per cent FCS, gentamycin and 2 mM L-glutamine. MDM were then washed twice and cultured at 10^6/ml per well for various time intervals. The intracellular localization of MTB was followed with Kynoun modified staining for acid fast bacilli.[15] In some experiments, MDM were treated with heat-killed MTB.

HIV infection of MDM

The HIV-1 strain was derived from H9/HIV-1_{IIIB} cells, a chronically infected human tumour cell line. The virus stock was assayed for particle-associated reverse transcriptase activity.[18] Titration of the virus preparation showed $10^{3.25}$ tissue culture infectious doses 50 per cent ($TCID_{50}$) assayed in H9 cells and in primary monocyte cultures. HIV infection was performed by incubating MDM with 100 μl of an HIV-1_{IIIB} strain diluted 1:10 for 2 h at 37°C. To remove unabsorbed virus, the cells were washed three times in warm PBS. MDM were then cultured in 24-well plates at 10^6/ml. Infection was followed by monitoring of p24 antigen (Coulter, Miami, FL, U.S.A.) and for particle-associated reverse transcriptase activity[18] (data not shown).

Propidium iodide incorporation

The percentage of nuclei undergoing apoptosis was measured by propidium iodide (PI) staining.[19] Cells (1×10^6) were washed in PBS and the cell pellet was gently resuspended in 500 μl of hypotonic fluorochrome solution (PI 50 μg/ml in 0·1 per cent sodium citrate plus 0·1 per cent Triton X-100, Sigma Chemical Company, St Louis, MO, U.S.A.). The cells were incubated for 18 h at 4°C in the dark and analysed by a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, U.S.A.). Ten thousand cells of each sample were analysed.

DNA nick end labelling

TdT-mediated dUTP-biotin nick end labelling was performed on slide preparations using BAL cytosmears. The method used was that described by Gavrieli *et al.*[20] with a few modifications. Endogenous peroxidase was inactivated using 3 per cent H_2O_2 for 15 min at room temperature and then slides were incubated in TdT buffer containing TdT (terminal deoxynucleotidyl transferase 0·5 U/μl), biotinylated-dUTP (0·025 nmol/ml), and $CaCl_2$ (2·5 mM) for 60 min at 37°C in a moist atmosphere. The reaction was terminated by transferring the slides to TB buffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 min at room temperature. The cells were rinsed in distilled water, covered with a 2 per cent aqueous solution of bovine serum albumin (BSA) for 10 min, and then immersed in PBS for 5 min

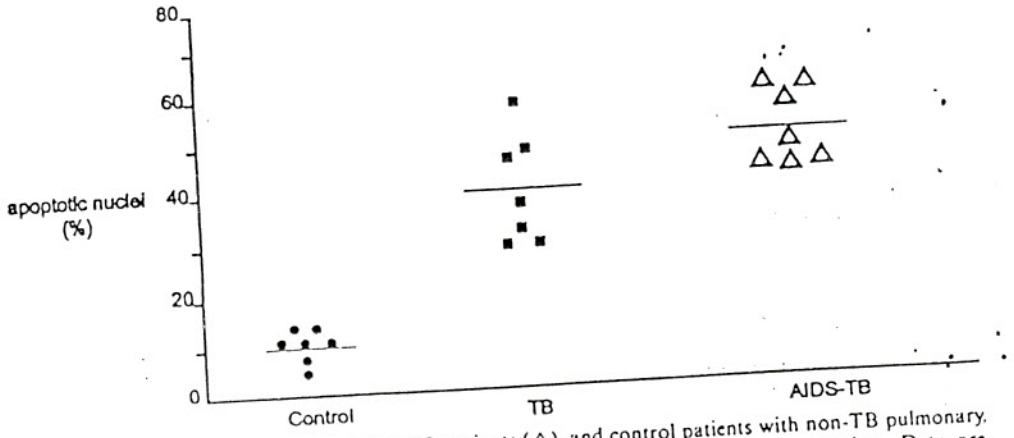


Fig. 1—BAL fromTB patients (■), AIDS-TB patients (△), and control patients with non-TB pulmonary diseases (●) were stained with propidium iodide and analysed for DNA fragmentation. Data are expressed as percentage (%) of apoptotic nuclei. Statistical analysis was performed by the Students *t*-test. The statistical significance was $P<0{\cdot}001$ between control and TB groups and $P<0{\cdot}001$ between control and AIDS-TB groups. The difference between TB and TB-AIDS groups was not significant

The slides were covered with peroxidase-conjugated streptavidin (BioGenex Lab., San Ramon, CA, U.S.A.), incubated for 20 min, and stained with 3,3'-diaminobenzidine tetrachloride (DAB) for 5–10 min. The percentage of apoptotic cells was evaluated using light microscopy (Laborlux D, Leitz) by counting the TUNEL-positive cells in a total of 1000 cells.

Tissue transglutaminase staining

The method previously described was used.[21] Briefly, BAL or cultured MDM were fixed on the slide and incubated with the affinity purified specific rabbit IgG against soluble tissue transglutaminase (tTG) of human red blood cells (a kind gift of L. Fesus, University Medical School, Debrecen, Hungary) in a wet chamber overnight at 4°C. As the secondary antibody, a biotinylated goat anti-rabbit IgG was used, followed by a preformed avidin–horseradish peroxidase complex (BioGenex Lab.). The reaction was developed using aminoethylcarbazole (AEC, BioGenex Lab.) as chromogen substrate and 0·01 per cent H_2O_2. Cells were counterstained in Mayer's haemalum. Endogenous peroxidase activity was blocked by methanol–H_2O_2. The percentage of apoptotic cells was evaluated using light microscopy (Laborlux D, Leitz, Wetzlar, Germany) by counting tTG-positive cells with the typical apoptotic morphology (shrunken cells with condensed chromatin) as well as apoptotic ghosts. Individual data for the percentage of tTG expression were compared by Student's *t*-test; composite treatments were analysed by two-way analysis of variance (ANOVA).

RESULTS

Apoptosis of BAL cells from patients with TB or AIDS-TB

Apoptosis of BAL cells was assessed by flow cytometry following staining with PI. Figure 1 shows that on average, 40–50 per cent of apoptotic cells were detected in TB and AIDS-TB patients, while on average, 12 per cent of apoptotic cells were observed in control patients with non-TB pulmonary diseases. To characterize the cells undergoing apoptosis, the same samples were also analysed for DNA fragmentation by the TUNEL method and for tTG expression.[20,21] Figure 2 illustrates that alveolar macrophages present in BAL from TB patients (panels B and C) showed the simultaneous presence of DNA fragmentation (nuclear staining) and tTG expression (cytoplasmatic red staining). Figure 3

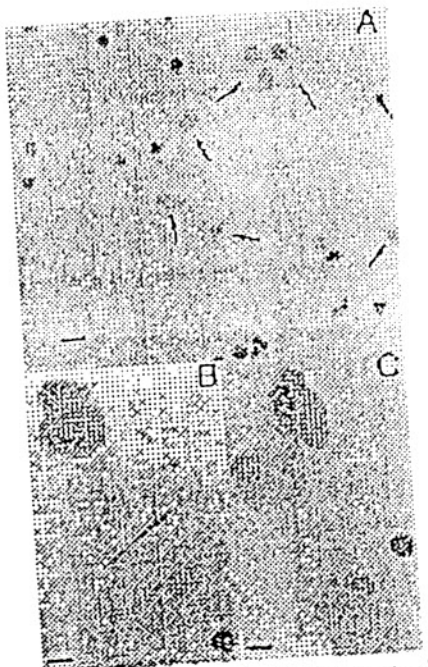


Fig. 2—Immunocytochemical analysis of BAL cells from patients with non-TB pulmonary diseases (panel A) and pulmonary TB (panels B and C). Cells were double-stained for expression of tTG (cytoplasmic red staining with AEC) and for DNA fragmentation by the TUNEL method (nuclei brown staining). Alveolar macrophages from TB patients are simultaneously positive by both stains, while they are negative in non-TB patients (arrows)

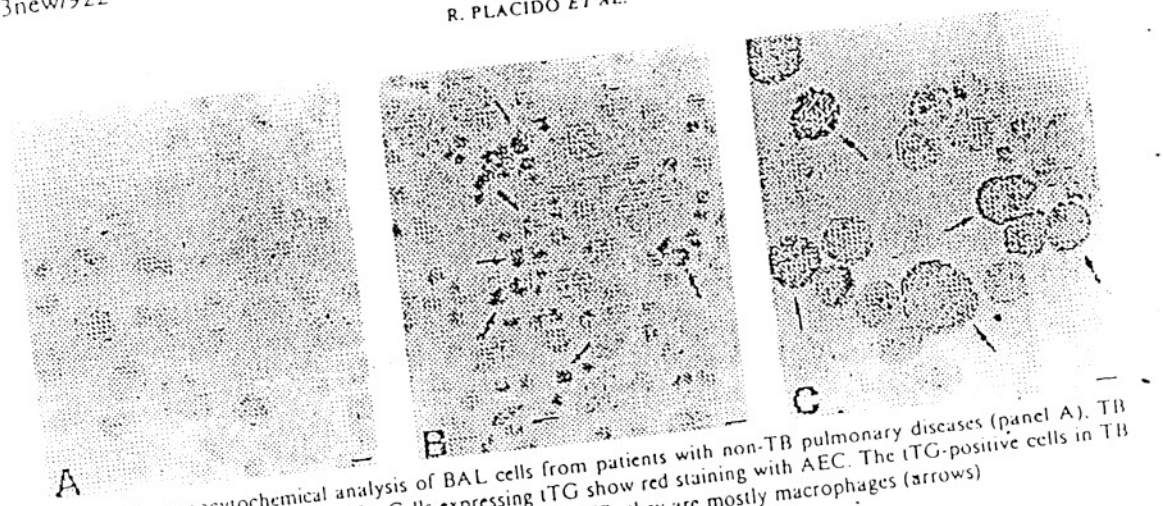


Fig. 3—Immunocytochemical analysis of BAL cells from patients with non-TB pulmonary diseases (panel A), TB (panel B), and AIDS-TB (panel C). Cells expressing tTG show red staining with AEC. The tTG-positive cells in TB patients are mainly neutrophils (arrows), while in AIDS-TB, they are mostly macrophages (arrows)

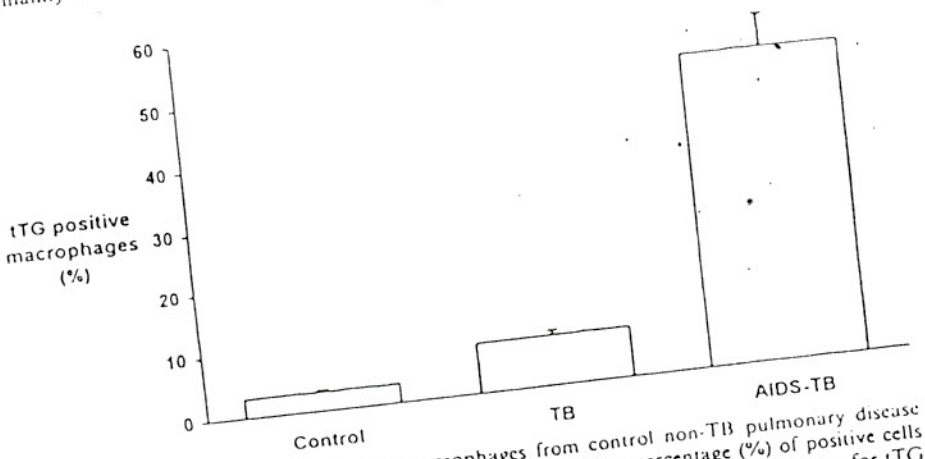


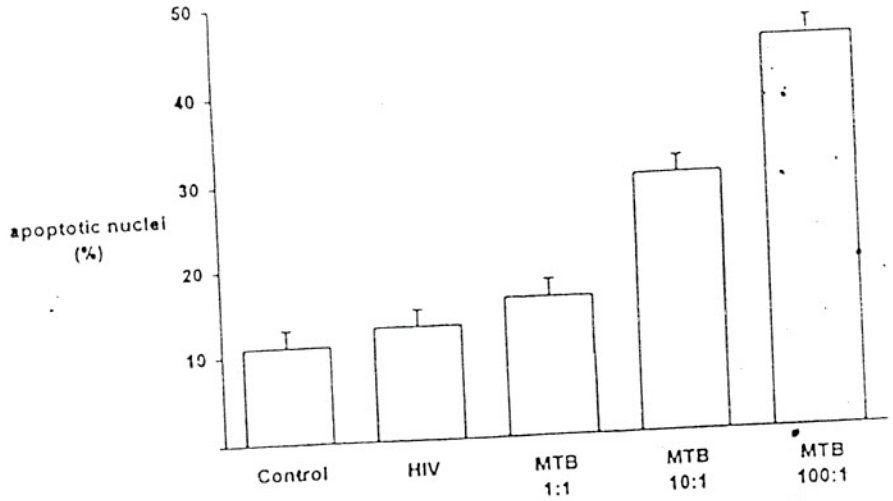
Fig. 4—Expression of tTG in alveolar macrophages from control non-TB pulmonary disease patients, TB patients, and AIDS-TB patients. Data expressed as percentage (%) of positive cells based on analysis of samples from five patients in each group. The statistical significance for tTG expression was $P<0{\cdot}001$ between control and TB groups, $P<0{\cdot}001$ between TB and AIDS-TB groups, and $P<0{\cdot}001$ between control and AIDS-TB groups

shows that in TB patients the majority of tTG-positive cells found in BAL samples were neutrophils, due perhaps to the high turnover of these cells during acute pulmonary inflammation[22] (panel B); by contrast, the majority of tTG-positive BAL cells in TB-AIDS patients were macrophages (panel C). Quantitative analysis of these data showed that 8–10 per cent of alveolar macrophages from TB patients were tTG-positive (Fig. 4). These data confirmed the statistical significance of tTG expression in alveolar macrophages of TB patients ($P<0{\cdot}001$ when compared with the control group). It is interesting to note that while the number of tTG-positive cells increased up to 50 per cent in AIDS-TB patients ($P<0{\cdot}001$), TUNEL and tTG staining was not observed in cells from BAL of patients with non-TB lung diseases.

Induction of apoptosis in MDM by MTB infection in vitro

To characterise the induction of apoptosis by MTB in macrophages, we developed an *in vitro* model in which MDM from healthy individuals were infected *in vitro* with MTB. Figure 5 (upper panel) shows that apoptosis, measured as PI incorporation, was dependent on the dose of MTB used for infection. The highest levels of apoptotic nuclei were observed at an MTB/monocyte ratio of 100:1, while background levels of apoptosis were found at a ratio of 1:1. Since an MTB/MDM ratio of 10:1 resulted in a statistically significant ($P<0{\cdot}001$) level of apoptosis, this ratio was used in subsequent experiments. No significant apoptosis was observed in MDM infected with HIV.[23] Figure 5 (lower panel) shows that MTB infection enhanced apoptosis from day 2 of culture and reached a maximum at day 7 of infection, when on average 40 per cent of MDM were apoptotic. At this time point, control MDM, showed a viability higher than 90 per cent, which is similar to that observed in MDM infected either with MAC or with HIV. Figure illustrates a representative experiment out of three which 31 per cent and 42 per cent of apoptotic nuc were observed in MDM cultured with silica[7] and w MTB, respectively.

The induction of tTG expression was also analyse MDM cultures *in vitro*. Figure 7 shows that higher le of tTG were expressed when MDM were infected MTB (41 per cent) or treated with silica (38 per c when compared with control of MAC-infected MI



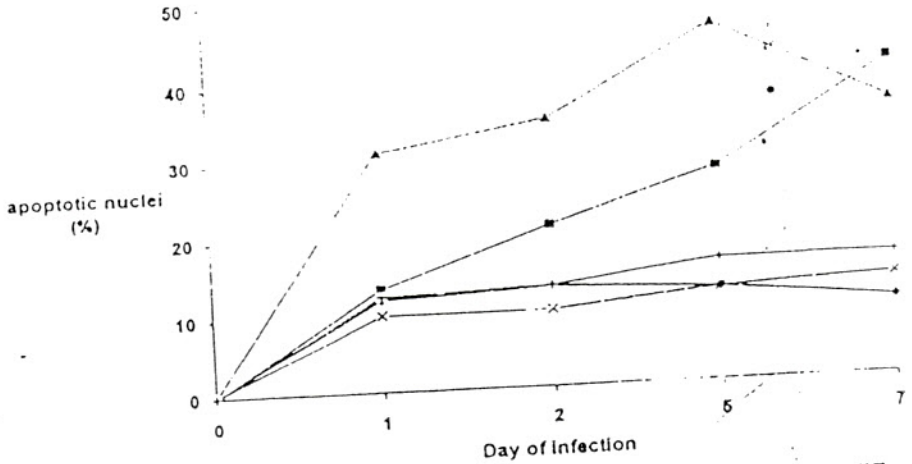


Fig. 5—Upper panel: MDM from healthy donors were infected with MTB H37Rv at different bacteria:cell ratios (1:1, 10:1, 100:1) or HIV and cultured for 7 days. Lower panel: MDM obtained from healthy donors were cultured for 12 days with medium alone (—+—), infected with MTB (—■—) or MAC (— × —) or HIV (—◆—), or treated with 50 μg/ml silica (—▲—). The statistical significance was $P<0{\cdot}001$ between MTB 10:1 or 100:1 versus control. Differences between MTB 1:1 or HIV versus control were not significant

DISCUSSION

Fifty years of research on the pathogenesis of tuberculosis have not yet clarified the relationship between the mechanism of intracellular killing of MTB and tissue destruction.

This paper shows that alveolar macrophages from patients with MTB pulmonary infection exhibit apoptosis, evaluated as PI incorporation, TUNEL labelling, and tTG expression. Indeed apoptosis, defined by histology, has been previously described in granulomas occurring in sarcoidosis, tuberculosis, and Crohn's disease.[9] The higher percentage of apoptotic macrophages in the lungs of AIDS-TB patients compared with TB alone could be explained by the finding that TB in AIDS is generally characterized by high numbers of mycobacteria in the tissue, due to the impaired T-cell immune response,[14] and to an increased attachment of MTB to alveolar macrophages, promoted by increased levels of surfactant protein A, during infection with HIV.[15] Moreover, direct microscopic identification of MTB was negative in TB patients, but positive in all AIDS patients, confirming the presence of numerous mycobacteria in the lungs of AIDS-TB patients. Thus, bacterial load and T-cell reactivity might explain the difference in the proportion of apoptotic alveolar macrophages between TB and AIDS-TB patients.

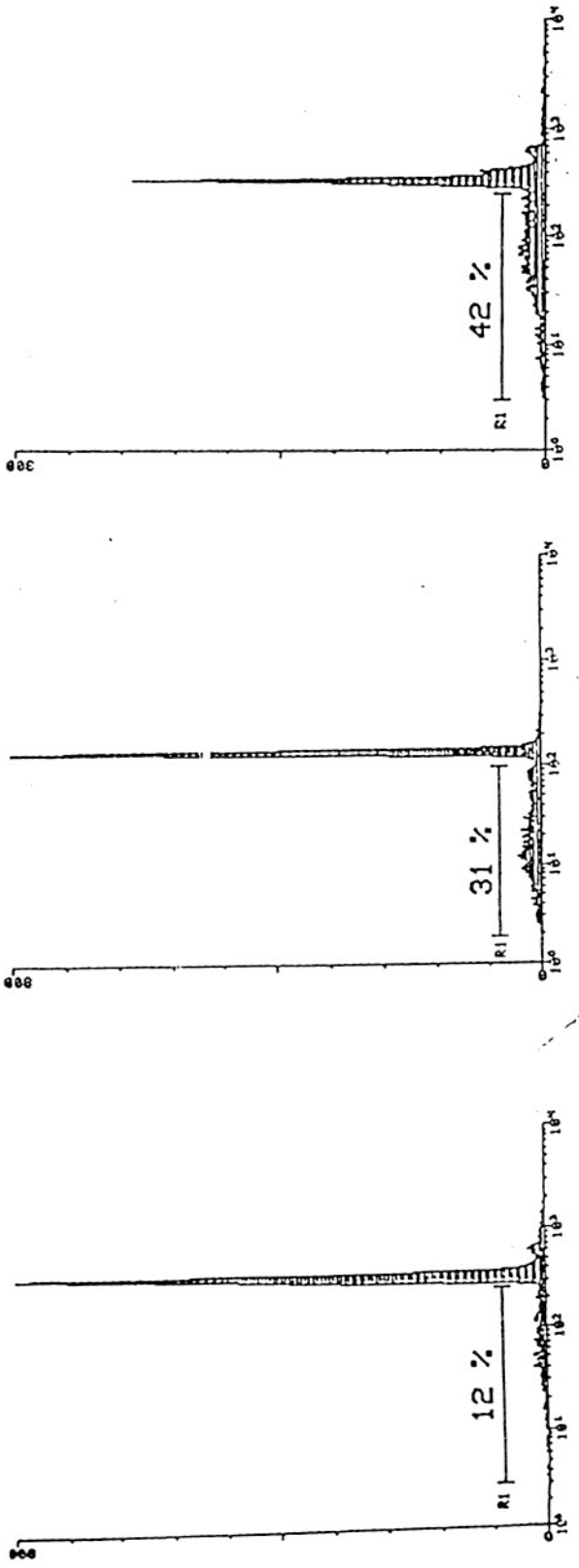


Fig. 6—Histograms from cytofluorimetric analysis after PI staining. Uninfected macrophages (A), silica-treated macrophages (B), and MTB-infected macrophages (C). Data are expressed as percentage (%) of apoptotic nuclei

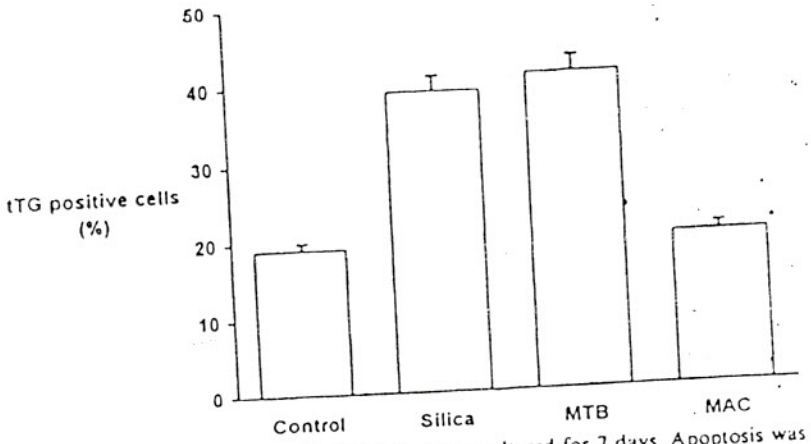


Fig. 7—MDM were infected with MTB and then cultured for 7 days. Apoptosis was evaluated by immunocytochemical analysis and expressed as percentage (%) of cells positive for tTG. The statistical significance was $P<0{\cdot}001$ between MTB or silica versus control The difference between control and MAC was not significant

The role of bacterial, load in MTB-induced apoptosis was confirmed by the *in vitro* data. A large proportion of human MDM showed apoptotic nuclei and expressed tTG when infected with high doses of MTB (bacterium/cell ratio 100:1), while no apoptosis was observed at a ratio of 1:1. Moreover, heat-killed MTB of *M. avium* failed to induce apoptosis, indicating that bacterial load, strain specificity, and virulence are key factors in the induction of apoptosis in macrophages. In contrast, the role of HIV in macrophage apoptosis is likely to be marginal, due to their relative resistance to HIV,[23] as formally demonstrated *in vitro*.

Direct evidence that programmed cell death or apoptosis is involved as a protective mechanism against MTB infection has been recently reported by Molloy *et al.*[11] These authors showed that ATP-induced apoptosis, but not necrosis, of infected monocytes is coupled with killing of intracellular BCG, as enumerated by colony forming units. Apoptosis renders the host environment more hostile for MTB and may block its replication. Furthermore, apoptotic cell death, in contrast to necrosis, is an immunologically silent event, not evoking any inflammatory response and leading to cell-controlled fragmentation into membrane-sealed apoptotic bodies, which may limit extracellular microbial dissemination. In these late events, an important role could be carried out by tTG, which is well expressed in apoptotic (TUNEL-positive) alveolar macrophages in the course of MTB infection. It has been demonstrated that tTG-catalysed intracellular protein polymerisation plays a crucial role in increasing the stability of apoptotic bodies and consequently in the prevention of leakage of their contents.[23] This phenomenon might contribute to preventing the release of harmful intracellular components into the extracellular space (enzymes, DNA, RNA, viruses) and could explain why apoptosis is an immunologically silent event, not associated with inflammation or scar formation in the surrounding tissues.

It has been shown that the anti-bacterial [illegible] be sufficient to kill MTB.[26] Many of the chemical and physical treatments that induce apoptosis are also known to evoke oxidative stress: these include ultraviolet radiation, ROI, H_2O_2, and OH^0. Exposure to low doses of H_2O_2 induces apoptosis, while high doses of this oxidant cause necrosis, suggesting that the severity of an insult determines the form of cell death which occurs. The regulation of ROI in macrophages is mediated, at least in part, by the 'anti-apoptotic' bcl-2 oncogene product, which prevents the induction of apoptosis by oxidative stress.[27] Preliminary experiments support a direct relationship between MTB infection and bcl-2 expression in a population of MTB-infected monocytes resistant to apoptosis and expressing several activation markers such as HLA-DR and ICAM-1 (Zembala *et al.*, in preparation).

In conclusion, this study provides evidence that MTB infection induces apoptosis in a proportion of alveolar macrophages *in vivo* and of monocyte-derived macrophages *in vitro*. Thus, the prompt death of macrophages after their engagement to kill a high load of bacilli may be teleologically considered as a response to deny surviving micro-organisms their intended sanctuary site. The question arises of whether MTB infection may lead to the selection of a population of cells able to harbour a large number of bacteria.

ACKNOWLEDGEMENTS

This work was supported by the Istituto Superiore di Sanità 'AIDS Project' and 'Tuberculosis Project', Consiglio Nazionale delle Ricerche P.F. FAT. MA, and E.C. BIOMED 1. RP thanks the ANLAIDS for the fellowship. MZ thanks ISS for support. We would like to thank Dr L. Fattorini, ISS, for providing the *M. tuberculosis* and *M. avium* strains used in this study, and Dr F. Poccia for a helpful discussion on this work.

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