ISONIAZID (INH) TREATMENT OF INH-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* INHIBITS INFECTED MACROPHAGE TO PRODUCE TNF-α

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Abstract. Macrophages undergo apoptosis after infected by *Mycobacterium tuberculosis* (M.tb), which is regulated by tumor necrosis factor *α* (TNF-*α*) and has a direct correlation with killing of intracellular bacilli. In order to clarify the role of isoniazid (INH) in the modulation of macrophages apoptosis and intracellular bacilli replication, we performed the following studies using an INH-resistant clinical M.tb isolate (INHres). Macrophages derived from peripheral blood were infected with INHres and treated or not treated with INH. Apoptosis was measured using an Ag-capture ELISA for histone and fragmented DNA. Production of TNF-*α* by INHres infected was assayed using ELISA and viability of intracellular M.tb was determined using bacterial culture of macrophage lysates on Löwenstein-Jensen (LJ) medium. INH pre-treatment to INHres reduced macrophages apoptosis, production of TNF-*α* and intracellular INHres viability. This study indicated that INH affected TNF-*α* release resulting in reduction of the extent macrophages apoptosis and of intracellular INH-resistant M.tb viability.

Keywords: *Mycobacterium tuberculosis*, apoptosis, isoniazid, macrophage

INTRODUCTION

Isoniazid (INH) is an effective most widely used anti-tuberculosis drugs which acts by interfering with nearly every metabolic pathway in *Mycobacterium tuberculosis* (M.tb). However, accumulating data suggest that mycolic acid synthesis is selectively inhibited by INH and is correlated with its lethal effect on M.tb (Takayama *et al*, 1972; Slayden and Barry, 2000). Interestingly, defective mycolic acid synthesis in *M. smegmatis* is not associated with the lethal effect of INH on this mutant strain (Liu and Nokaido, 1999). Thus it is an over simplification to believe that there is a single underlying mechanisms of M.tb killing by INH. Understanding the other targets of INH is very important to develop new strategies against INH-resistant M.tb.

M.tb is able to live and replicate within macrophages. Tumor necrosis factor (TNF)-*α* is capable of supporting the growth of intracellular virulent but not the attenuated M.tb (Engele *et al*, 2002). M.tb

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infection can induce human macrophage apoptosis (Danelishvili *et al*, 2003), which is regulated by the release of TNF-α (Patel *et al*, 2007) and has a direct correlation with the killing of intracellular bacilli (Oddo *et al*, 1998; Arcila *et al*, 2007). Macrophage apoptosis also is crucial for stimulating innate immunity against M.tb and eradication of intracellular M.tb. However, there is no satisfying explanation of the advantages of macrophage apoptosis for the infected host and the pathogen side effects that accrue. The latter phenomena may be a strategy for bacilli to overcome host defenses. On the other hand, the host might exploit apoptosis as a means to limit replication of intracellular pathogens.

INH modulates TNF α-induced apoptosis in murine macrophage cell line, which is a consequence of the metabolic blockade on M.tb by INH (Gil *et al*, 2003). In order to clarify the role of INH in the modulation of macrophage apoptosis and intracellular bacilli viability, we examined these properties using an INH-resistant clinical M.tb isolate cultured in human monocyte-derived macrophages.

MATERIALS AND METHODS

M.tb clinical isolate

M.tb clinical isolate was obtained from a patient in primary health care unit at a hospital in Yogyakarta, Indonesia. M.tb was identified using classical procedures based on such parameters as rate of growth, colony morphology and pigmentation, and also the biochemical properties (Wu *et al*, 2007). Susceptibility of the M.tb clinical isolate to INH was tested using the agar proportional method on Lőwenstein-Jensen (LJ) medium. One colony resistant to 1 μg ml^{-1} of INH INHres) was chosen. M.tb strain H37Rv was used as control.

Macrophage cell culture

Peripheral blood mononuclear cells were isolated using standard gradient centrifugation method of Hystopaque® (Sigma, St Louis, MO) from heparin-treated blood of healthy and non-smoker donors after informed consent was granted. Mononuclear cells were suspended in RPMI 1640 medium supplemented with L-glutamine (300 μg/ml), without sodium bicarbonate (GIBCO, Gaithersburg, MD), 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 10% L929 conditioned medium as source of macrophage colony-stimulating factor (M-CSF), penicillin (50 IU/ml) and streptomycin (50 μg/ml) (complete medium). The cells were incubated for 2 hours at 37°C under an atmosphere containing 5% CO_2. Then, non adherent cells were removed by washing with RPMI 1640 medium and the remaining adherent monocytes were collected and subjected to viability test and cell counting using trypan blue staining. Monocytes were suspended in complete medium, added to a 24-well plate at a concentration of $5x10^5$ cells ml^{-1} per well and incubated as describe above for 4 days, after which time cells were infected with M.tb. Prior to infection the complete medium was replaced with incomplete medium (without antibiotics).

M.tb infection of macrophages

M.tb INHres and H37Rv isolates were grown on LJ medium and then were suspended in Ringer lactate solution, vortexed and homogenized by repeated passages through a 27G syringe (Stokes *et al*, 2004). INH (0.1 μg ml^{-1} and 1 μg ml^{-1}) was added to the INHres suspension and incubated for 20 minutes, then the suspension was added to macrophage cell culture. Macrophages infected with INH-treated and untreated INHres and also H37Rv M.tb isolates for 4 hours at multiplicity of infection (MOI) of 1 to 10. F Extracellular M.tb was removed by washing with RPMI 1640. MOI was measured by counting the intracellular M.tb after staining with AcriFluor™ fluorescence dye.

Determination of apoptosis

M.tb-infected macrophages were incubated for 3 days as described above. Then, apoptosis was measured using an Ag-capture ELISA for histone and fragmented DNA (Cell Death Detection ELISA^{plus}, Roche, Indianapolis, IN) in cell lysates and absorbance was measured at 405 nm (Keane *et al*, 2000). Macrophage apoptosis also was confirmed by manually determining percent apoptotic cells after hematoxyline-eosin (HE) staining of parallel experiments. Cell counting was performed under a light microscope (400x magnification) for 300 fields.

Intracellular M.tb viability assay

Macrophages cell lysate used in the apoptosis measurements were applied to LJ medium and M.tb viability was measured (cfu ml^{-1}) by counting the colonies present after 4 weeks incubation at 37°C.

Measurement of macrophage TNF-α production

M.tb.-infected macrophages were incubated for 24 hours as described above and then TNF-α levels in the cell culture medium were measured using Tumour Necrosis Factor Alpha [(h)TNFα] Human Biotrak ELISA System (Amersham Biosciences, San Diego, CA).

Statistical analysis

Analysis of variance (ANOVA) was used together with Tukey HSD analysis to differentiate the effect of INH treatment.

RESULTS

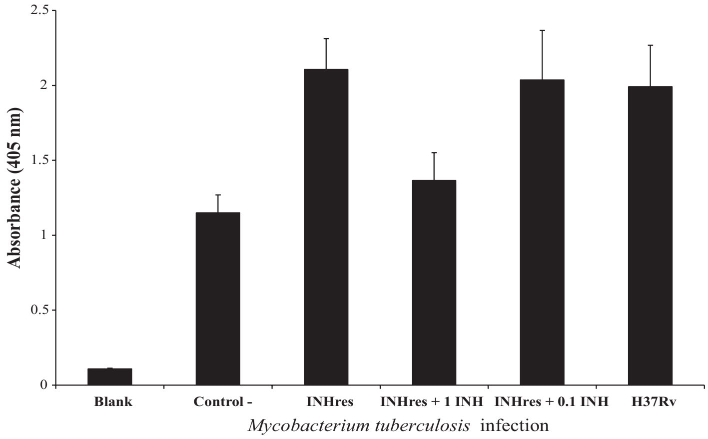
INHres M.tb-infection of human macrophages induced apoptosis, but this was attenuated nearly to uninfected (control) level when macrophages were infected with INH-treated INHres M.tb (Fig 1a). These findings were confirmed by manually counting of HE stained cells, showing a significant difference in apoptosis level between infection with INH-treated and untreated INHres M.tb ($p< 0.05$) (Fig 1b).

In order to elucidate the mechanism by which INH treatment decreased apoptosis of INHres M.tb-infected macrophages, production of TNF-α by such macrophages was investigated. No TNF-α was released from uninfected macrophages, but non INH treated INHres M.tb-infected macrophages released 2.2-folds more TNF-α compared to that NH treated INHres M.tb-infected cells (Fig 2a). It is worth noting that TNF-α production from INHres M.tb-infected macrophages is significantly lower than that from cells infected with H37Rv M.tb (Fig 2a).

The viability of intracellular INHres M.tb is significantly higher compared to that of intracellular H37Rv strain, but INH pre-treatment of INHres M.tb has no significant effect on the bacilli viability (Fig 2b).

DISCUSSION

The level of apoptosis of macrophages infected with INH-sensitive M.tb H37Rv strain has been reported to be indistinguishable from those infected with INH resistant strains (Rachmawaty *et al*, 2006). In the current study, a clinical INHres M.tb isolate was treated directly with INH prior to macrophage infection in order to avoid the direct effect of INH on the macrophage function (Gil *et al*, 2003). Our findings demonstrated that INH pre-treatment resulted in reduction of the capability of intracellular INHres M.tb to induce maca



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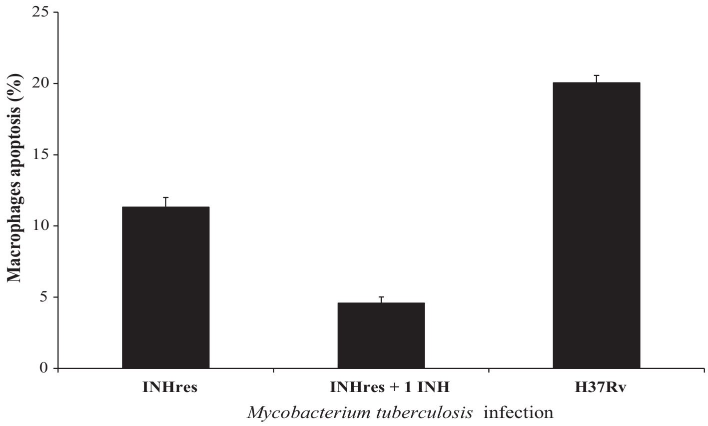

Fig 1–Apoptosis of M.tb-infected macrophages in culture. Macrophages cells were infected with H37Rv or INHres (untreated or pre-treated H with 0.1 μg ml^{-1} and 0.1 μg ml^{-1}) at MOI 1:10 and incubated at 37 °C under an atmosphere containing 5% CO_2 for 72 hours. Negative control macrophages were not infected with M.tb. Apoptosis was determined using Cell Death Detection ELISA^{plus} kit (Roche). Absorbance at 405 nm corresponds to the apoptosis level (a). Results are shown as mean ± SEM percent apoptosis of 5 independent experiments conducted in duplicate. The data were collected from duplicate measurements of five independent experiments and showed as means with SEM. In addition, infected macrophages were fixed, stained with hematoxyline-eosin and macrophages that underwent apoptosis were manually counted in 300 microscope fields (400x magnification) (b). Results are shown as mean ± SEM percent apoptosis of 3 independent experiments conducted in duplicate observations.

rophages apoptosis. The concentrations of INH used did not affect the viability of intracellular INHres M.tb. In any case, live M.tb (Keane *et al*, 1997; Placido *et al*, 1997) and also killed M.tb are able to induced macrophage apoptosis (Klingler *et al*, 1997). However, there is a significant decrease in TNF-α production by the INH-treated compared to non treated INHres M.tb-infected macrophages.

TNF-α is released by macrophages in response to either live or killed H37Rv M.tb (Lasco *et al*, 2003). A synthetic lipid-A like antagonist showed an ability to block TNF-α production and leads to human alveolar macrophage apoptosis arrest (Means *et al*, 2001). TNF-α production is crucial in mediating M.tb-induced macrophage apoptosis (Means *et al*, 2001). Injection of INH to mice has no direct effect on TNF-α production by monocytes (Urbascheck *et al*, 1991). There was a direct correlation of percent macrophage apoptosis and TNF-α production (pg/ml) ($r = 0.89$; $\alpha = 0.05$). Previous reports have indicated that apoptosis of macrophages leads to the intracellular M.tb elimination (Placido *et al*, 1997; Oddo *et al*, 1998). However, the induction of macrophage apoptosis by H_2O_2, ATP and stauro-

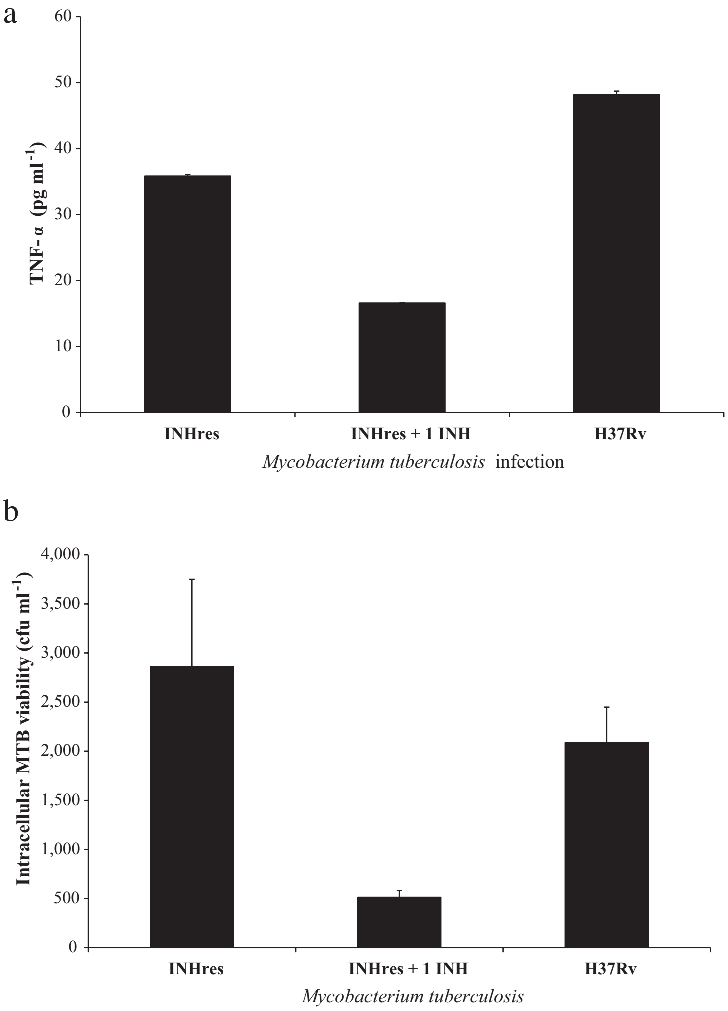

Fig 2–TNF-α production by M.tb-infected macrophage (a) and viability of intracellular M.tb (b). Macrophages were infected with either INH (1 μg ml^{-1}) pretreated INHres, untreated INHres or INHres M.tb strain, and incubated for 24 hours at 37ºC under an atmosphere containing 5% CO_2. TNF-α released into culture medium was measured using Tumour Necrosis Factor Alpha [(h)TNFα] Human Biotrak ELISA System (Amersham Biosciences). Results are shown as mean ± SEM of 3 independent experiments conducted in duplicate. Intracellular M.tb bacilli released from lyzed infected macrophages were grown on Lőwenstein-Jensen medium and colonies were counted after 4 weeks incubation at 37ºC. Results are shown as mean ± SEM of 4 independent experiments conducted in duplicate observations.

sporine has no significant effect on mycobacterial viability (Pais and Appelberg, 2004). TNF-α might support the growth of intracellular virulent but not attenuated M.tb (Engele *et al*, 2002). In addition, TNF-α antagonist is able to inhibit the replication of virulent M.tb (Haraguchi *et al*, 2006).

Intracellular M.tb has the ability to multiply during 48 hours in culture and the final intracellular M.tb population after 48 hours of incubation does not correspond to the MOI (Santucci *et al*, 2000). At low MOI, M.tb within macrophages replicates faster compare to the higher MOI, resulting in comparable numbers of intracellular M.tb after 48 hours. Similarly, our results showed that the viability of INH-treated intracellular INHres M.tb is not significantly different from untreated bacilli, even though the numbers of viable M.tb may not have been the same at the time of inoculation. The release of cytokines, including TNF-α, is a double-edged sword, contributing both to the protective immunity and the immunopathology of the tuberculosis. TNF-α antagonist treatment has been reported to activate latent tuberculosis (Carmona *et al*, 2005), but on the other hand decreasing local TNF-α levels may result in the control of intracellular M.tb replication (Engele *et al*, 2002). In this report we demonstrated another useful role of INH, instead of its mycobatericidal effect, it also is capable of inhibiting apoptosis and TNF-α production of INHres M.tb-infected macrophage.

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