Indonesian Journal of Biotechnology, December, 2006 Vol. 11, No. 2, pp. 914-920

Reactive Oxygen Intermediate (ROI) in Dog Macrophage Infected with *Mycobacterium tuberculosis*

Ida Tjahajati

Department of Internal Medicine,
Faculty of Veterinary Medicine, Gadjah Mada University, Indonesia.

Abstract

The experiment was conducted to measure macrophage ROI secretion in dogs infected with *M.tuberculosis*. The experiment used 24 healthy dogs, aged between 1 and 2 years, both male and female which were divided into two different groups consisting of 12 dogs each. The first group was the treatment group, that is they were infected with *M tuberculosis* and the second one was the control group. The activity of macrophages ROI secretion were measured at 1st, 2nd, 12th, and 24th after infection using *nitroblue tetrazolium (NBT) reduction assay*. Three cats were used to measure the macrophage activity in each period, using triplicate sample for each cat. The results of the experiment showed that ROI secretion increased in infected group compared with the control group, and this activity reached to the plateau level at 2 weeks after infection. Although these enhanced activities were gradually diminished thereafter, higher levels of these activities were consistently observed until the end of experiment compared with control group. The results of the experiment indicated that ROI played an important role to against *M.tuberculosis* infection in dogs.

Keyword: macrophage, ROI, *M.tuberculosis*, dogs

Introduction

To date, tuberculosis is still an international problem which recently becomes worse because the increasing risks, especially to the patient suffering from HIV and having problems with multi-drugs resistance *M.tuberculosis* (MDRTB) (WHO, 2004). *Mycobacterium tuberculosis* is the cause of tuberculosis which claims almost 2 millions death and there are 8 million new cases each year. According to WHO, one third of world population have been infected with *M.tuberculosis* and all are in the risk of being reactive (Dolin, 1994). In Indonesia, based on the Domestic Health Survey of Ministry of Health of Republic of Indonesia in 1992, it was estimated that 175,000 people died each year and there were around 500,000 new cases. Tuberculosis is the second cause of death in Indonesia after the cardiovascular and the first cause in the group of infection disease (Manaf, 1997).

Mycobacteria from the *M. tuberculosis* complex may cause pulmonary, gastrointestinal, or disseminated diseases in dogs and cats (Snider, 1971; Anonim, 1992; Bennet and Gaskell, 1996). Tuberculosis in dogs and cats have been much reported (Wilesmith, 1994; Aranaz *et al*., 1996; Janz, 1996; Monnies *et al.*, 2000). Pets can contract tuberculosis by living in intimate association with human beings or cattle that have the diseases (Acha and Szyfress, 1980; Aranaz *et al.*, 1996). The route of the infection is essentially through respiratory, but can also be alimentary and cutaneous (Aranaz, *et al*., 1996; Blunden and Smith, 1996). The dog is

*Corresponding author: Ida Tjahajati, Department of Internal Medicine, Faculty of Veterinary Medicine, Gadjah Mada University, Jl. Olahraga-Karangmalang, Yogyakarta 55281, Indonesia. HP: 081578890500; E-mail: ida_tjahajati@yahoo.com

equally sensitive to *M. tuberculosis* and *M. bovis* but most of the canine cases in urban areas by *M. tuberculosis* and are attributed to exposure to human carriers (Aranaz *et al.*, l996).

It was explained by Aranaz *et al.* (1996) that tuberculosis in dogs and cats were usually open lesion form so as to enable to spread organism to the surrounding environment. The animals might secrete organism through saliva, faeces, urine or from discharging wounds or sinuses in an infected animals (Farrow and Love, 1975; Aranaz *et al.*, 1996; Guun-Moore *et al.*, 1996). Tuberculosis in small animals have been a little attention although the disease must be considered as a danger for human health because of it potential role in the causation and maintenance of the disease (Aranaz *et al.*, 1996). Because of these reason, tuberculosis in both animals had to be aware of because of the risk of spreading it to people nearby (zoonotic disease) (Acha and Szyfress, 1980; Aranaz *et al.*, 1996).

In the immunity system of human beings and some experiment animals, it has been known that cell mediated immunity plays a very important role in overcoming *M. tuberculosis* infection. The immunity is mediated by T lymphocyte and macrophage (Janeway *et al.*, 2001) T cell of CD4 produce several cytokine, while T cell of CD8 is able to perform lyses in the targeted cell, that is macrophage infected with tuberculosis germ as well as interferon-gamma (IFN-g) production which activates macrophage. In T CD4 cell is found polarization of cell based on the profile of cytokine produced, namely Th 1 and Th 2 cells. Th 1 cell produces interleukin-2 (IL-2) and IFN-g which contain protective characteristic because they can activate macrophage to kill and digest phagocyted germ. Cytokine profiles produced by Th 2 group which include several interleukins, such as IL-4, IL-5,IL-6, IL-10 act as inhibitor for the activities of macrophage so that their role sometime harm to the body (Mosmann and Sad, 1996).

Although cases of tuberculosis in dogs have been reported, the immunity responses to *M. tuberculosis* infection in dogs have not been studied extensively. Several literature stated that dogs were more sensitive to the infection than cats (Francis, 1978; Acha and Szyfress, 1980; Francis cit. Thoen, 1994). However, the explanations of how and why dogs are more sensitive from cellular immunity responses which in this case are macrophage ROI secretion activities have never been reported. The previous experiment (Tjahajati *et al.*, 2003; 2004) revealed that relating to macrophage in cats infected by *M. tuberculosis*, the phagocyte and ROI secretion activities increased in folds which were assumed to have relationships with the mechanism of sensitivity to the infection. In this experiment, we study the ROI secretion activities in dogs macrophages infected with *M. tuberculosis*.

Based on the literature which argued that dogs were more sensitive to *M. tuberculosis* infection compared to cats and that macrophage was a phagocyte and ROI secretion which played an important role in the mechanism of killing Mycobacterium which entered the body, the hypothesis in this experiment is that dogs infected with *M. tuberculosis* have higher ROI secretion activities compared to control group in the attempt of killing *M. tuberculosis* in the body, the ROI secretion of dogs macrophage are lower compared to the results of similar activities in cats as have been undertaken in the previous experiment.

Material and Methods

Study groups

Twenty four healthy dogs, aged 1-2 years, male and female were divided into two groups, each consisting of 12. The firstgroup was infected by *M.tuberculosis*

1x10 cfu in a intraperitoneally while the second group being the control group were not given any treatment. Mycobacterium used in the experiment was *M. tuberculosis* strain H37Rv obtained from *Balai Laboratorium Kesehatan Daerah* (Regional Health Laboratory Clinic), Daerah Istimewa Yogyakarta (Yogyakarta Special Territory).

Period of examining macrophage activities

ROI secretion activity of peritoneum macrophage of the dogs were measured in week 1, 2, 12 and 24 after infection. For each period of measurement of macrophage activity, three dogs were used to measure ROI secretion, using triplicate sample for each cat.

Isolation and culture of peritoneum macrophage

As a schedule dogs were anesthetized using Anesject® (kethamine) with a dosage 0.1 mg/kg body weight, injected in an intramuscular. After the dogs were asleep, they were put in a dorso lateral position on place layered with sterile foil aluminum. Then the stomach skin was disinfected by 70% alcohol after which the abdomen skin was opened using sterile scissors so that mesenterium layer and peritoneum cavum as well as its contents could be seen clearly.

Cool medium RPMI around 100 ml was injected into peritoneum cavum and was massaged around 3 min. Then the medium was aspirated again. The obtained aspirate was put in sterile centrifuge glasses which was then centrifuge at the speed 1200 rpm in 4°C for 10 min. The supernatan was discarded and 3 ml complete medium RPMI containing FCS 10% was added to the pellet obtained (Coligan *et al*., 1997).

The number of macrophage was counted using haemocytometer which was then resuspended using complete RPMI medium so as to yield cells with 2.5x10 cells/ml density. The cell suspension that had been identified was then cultured in 24 well of microplate that had been given round cover slips. Each of well 200 µl (5x10 cells) was then incubated in 5% CO_2 incubator, 37°C for 30 min. After that, 1 ml of complete RPMI medium was added to each well which was then incubated for 2 h. Cells were suspended by RPMI twice. Additional 1 ml of complete RPMI medium was given to each well and put in incubator for 24 h (Coligan *et al*., 1997).

ROI secretions assay

The capability macrophage of dogs peritoneum in secreting ROI was measured by NBT reduction assay. To stimulate the secretion of anion superoxyde, cell culture was treated with PMA with final concentration 125 ng/ml. The chronology of treatment can be described in the following. Macrophage culture was suspended twice with RPMI which was then added 500 µl NBT liquid (1 mg/ml PBS) containing 125 ng/ml PMA for each well. The culture was then incubated in 5% CO_2 incubator in 37°C for 1 h. The cell was then suspended with PBS three times, dried up in the room temperature and fixated using absolute methanol for 30 s. After being dried up, the cell was colored with 2% Neutral Red Solution for 15 min and then was rinsed with aquades. After it was dry, cover slips were lifted from well of microplate to be examined under light microscope with 400x magnification (Leijh *et al*., 1986).

Macrophage activities to secrete ROI was measured by determining the percentage of macrophage which secretes ROI, that is by showing the formation of formazan (darker black colored), counted as 100 macrophage being under microscope, and score of level of formazan formation by each 100 macrophage was determined by adding the number of score gained by 100 macrophage. The score was 0 if no formazan is formed in the macrophages, score 1 when

formazan was formed in macrophage, but did not fulfill all cells, and score 2 if the formed formazan cover all cells (Leijh *et al.*, 1986).

Data Analysis

The difference of macrophage activities in ROI secreting between treatments and control groups were analyzed using T test (Kinnear and Gray, 1999). In interpreting and counting the number of cell, a second person was available to confirm the counting and objective results of interpretation.

Results and Discussion

The activities of peritoneum macrophage of dogs which secrete ROI were measured using NBT reduction assay. NBT reduction showed the existence of respiration burst followed by the formation of anion superoxide (O_2^-) which would reduce NBT in forming the undiluted blue/blackish formazan. The activities of peritoneum macrophage of dogs in secreting ROI could be determined from the percentage of macrophage which secreted ROI and from the score of the formation of formazan by each macrophage. The peritoneum macrophage of the dogs which secreted ROI in the control and infected groups after 2 week post infection can be seen in Figure 1.

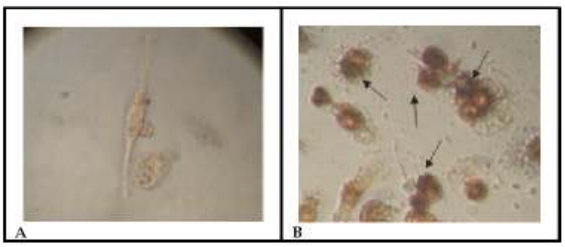

Figure 1. Peritoneum macrophage of dogs which secreted ROI (forming blackish formazan) in vitro in 2 weeks after 10 cf *M.tuberculosis* infection with Neutral Red coloring (400x magnification). A. On the control group, B. On the treatment group. The arrow showed ROI products which form blackish formazan.

From Figure 1, it appears that macrophage in the control group showed a picture where black formazan was not formed in macrophage and only few cells which secreted ROI while in the macrophage of treatment group, there appeared the formation of black formazan with a higher number. The average of percentage of macrophage which secreted ROI and the score of ROI in the control group and the one in the infected group (treatment group) can be seen in Figure 2.

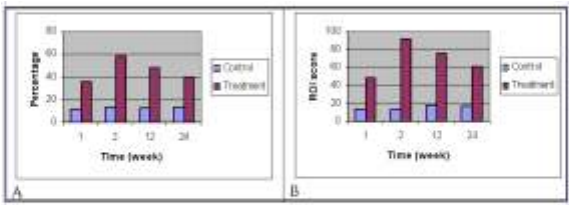

Figure 2. The average of percentage of macrophage which secreted ROI and the average of ROI score in the control and treatment groups.

Figure 2, it is revealed that the percentage of macrophage which secreted ROI and ROI score in the treatment group enhance and reached the peak in week 2 and was further in decrease until the end of the experiment. Different from the control group, ROI secretion activities did not relatively undergo any significant rise from time to time. The results of experiment demonstrated that the dogs infected by *M. tuberculosis* showed increases in their ROI secretion and ROI score by the microphage in the treatment group in comparison to the control group (which was different in significance in $P<0.05$). The result had a similar pattern as the previous experiment in cats (Tjahajati *et al.*, 2003; 2004), but the activities of macrophage in secret ing ROI were lower in comparison with those in cats. The result supported the notion that dogs were more susceptible to *M. tuberculosis* infection that cats were (Francis, 1978; Acha and Szyfress, 1980; Francis cit

Thoen, 1994).

The increase of ROI production was related to the attempts of the dogs in killing / eliminating *M. tuberculosis* which entered their bodies. It was explained by Chan and Kaufmann (1994) and Tizard (2000) that the activated macrophage would, among others, generate the increase of lysosome enzyme, phagocyte activities, and physically change the form and shape of macrophage as well as enhance the production of ROI and NO (Chan *et al.*, 2001). ROI and RNI were macrophage oxidative products which played important roles in the killing *M. tuberculosis* as antimicrobial (Akaki *et al.*, 2000) of which productions were induced together by cytokine IFN-g and TNF-a (Flynn and Chan, 2001).

Leijh *et al.* (1986) stated that the interaction between opsonim microorganism and phagocyte membrane did not only produce ingestion, but also yielded metabolic burst which was very unique with the increase in the need of oxygen and the production of anion superoxide (O_2) and hydrogen peroxide (H_2O_2); both of which had strong microbisidal activities. The importance of the system for eliminating microbacteria can be clearly seen in the patients having defects in the microbisidal activities in granulocyte and monocyte, such those who suffer from chronic granolumatous disease (Quie, 1972). The mechanism of elimination of *M. tuberculosis* done by cellular immune response in the dogs bodies can be shown in Figure 3.

Based on the above results and discussions, it can be concluded that the *M. tuberculosis* infection in dogs increased macrophage activities in the ROI secretion. The enhance in ROI secretion in the experiment indicated that ROI had an important role in the elimination of *M. tuberculosis* infection in the dogs' bodies.

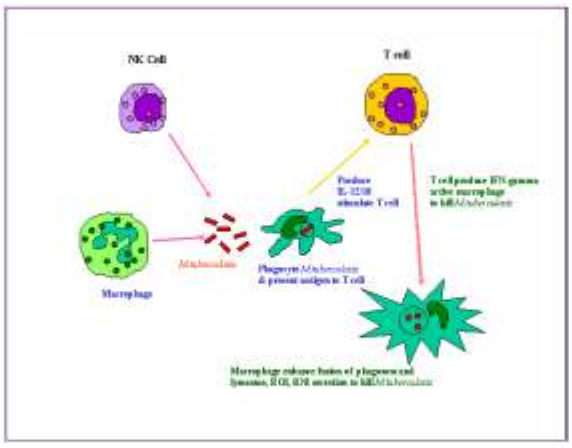

Figure 3. The roles of NK cell, macrophage, T cell, phagocytes and ROI secretion to against *M.tuberculosis* infection.

Acknowledgments

I would like to express my gratitude to Prof. dr. Marsetyawan HNE, Ph.D. and Prof. dr. Supargiono, Ph.D. from Faculty of Medicine, Gadjah Mada University Yogyakarta, Prof. drh. Widya Asmara, M.Sc., Ph.D. from Faculty of Veterinary Medicine, Gadjah Mada University Yogyakarta for their inputs and suggestions. The Head of Biology Laboratory of Gadjah Mada University and to drh. Wieklati M.Sc., as the Head of Microbiology Section of Regional Health Laboratory Clinics of Yogyakarta Special Territory for facilities during the experiment and also Suprihatin who has assisted me during the experiment process.

References

Acha, P.N., and Szyfres, B., l989. *Zoonoses and cummunicable diseases common to man and animal*. Pan American Health Organization, Wasington DC, USA. pp 7-19.

Akaki, T., Tomioka, H., Shimizu, Dekio, S., and Sato. K., 2000. Comparative roles of free fatty acids with reactive

nitrogen intermediates and reactive oxygen intermediates in expression of the anti-microbial activity of macrophages agains Mycobacterium tuberculosis. *Clin. & Exp. Immunol.*, 121, 320-332.

Anonim, l992. *The Merck Veterinary Manual A Handbook of Diagnosis, Therapy, and Disease Prevention and Control for the Veterinarian*. Merck & Co., Inc. Rhaway, N.J., USA.

Aranaz, A., Liebana, E., Pickering, X., Novoa, C., Mateos, A., and Dominguez, L., l996. Use of polymerase chain reaction in the diagnosis of tuberculosis in cat and dogs. *Vet. Rec.* 138, 53-58.

Bennet, M., and Gaskell, R.M., l996. *Feline and Canine infectious diseases*. Blackwell Wissenschafts-verlag GmbH Kurfurstendamm, Berlin, Germany. pp 130-132.

Blunden, A.S., and Smith, K.C., l996. A pathological study of mycobacterial infection in a cat caused by variant with cultural characteristics between *Mycobacterium tuberculosis* and *M. bovis*. *Vet. Rec.*, 138, 87-88.

Chan, J., Kaufmann, S.H.E., l994. Immune Mechanism of Protection. In *Tuberculosis Pathogenesis and Control*. Bloom, B.R. Editor. American Society for Microbiology, Washington, DC. 389-417.

Chan, E.D., Chan, J., and Schluger, 2001. What is the role of Nitric Oxide in murine and human host defense agains tuberculosis?. *Am. J. Res. Cell Mol. Biol.* 25 (5), 606-612.

Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M., and Strober, W., l997. *Current Protocols in Immunology*, Vol. III, John Wiley and Sons, Inc, United Stated of America.

Dolin, J.P., l994. Global Tuberculosis incidencs and mortality during 1990-2000. *Buletin World Health Organization*, 72 (2), 213-220.

Farrow, B.R.H., and Love, D.N., 1975. Infectious Diseases. In *Textbook of Veterinary Internal Medicine Diseases of Dog and Cat*. W.B. Saunders Company, Philadelphia, London, Toronto.

Flynn, J.L., and Chan, J., 2001. Immunology of Tuberculosis. *Annu. Rev. Immunol.*, 19, 93-129.

Francis, J., l978. *Tuberculosis in Animal and Man*. A Study Comparative Pathology. Cassel and company Limited, London.

Guun-Moore, D.A., Jenkins, P.A., and Lucke, V.M., l996. Feline tuberculosis: a literature review and discussion of 19 cases by unusual mycobacterial variant. *Vet. Rec.*, 138, 276-280.

Janerway, C.A., Walport, P.T.M., Schomchik, M., 2001. *Immuno Biology* 5th Ed. Granland Publishing, Francis.

Janz, R., l996. Tuberculosis in Cat. *Vet. Rec.*, 134, 395.

Kinnear, P.R., and Gray, C.D., l999. *SPSS for Window Made Simple* 3rd Ed. Lawrence Erbaum. ISBN 0-86377-827-5.

Leijh, P.C.J., Furh, R.V., and Zwet, T.L.V., l986. In vitro determination of phagocyte and intracellular killing by polymorphonuclear and mononuclear phagocyte. In Weir, D.M. Ed., *Cellular Immunology*, Blackwell Scientific Publication, London. pp 46.1-46.21.

Manaf, A., l997. Permasalahan pemberantasan Tuberkulosis di Indonesia. *Seminar Nasional Tuberkulosis dan Lepra*. Pusat Kedokteran Tropis Universitas Gadjah Mada, Yogyakarta.

Monies, R.J., Cranwell, M.P., Palmer, Inwald, J., Hewinson, R.G., Rule, B., 2000. Bovine tuberculosis in Domestic

Cats. *Vet. Rec.*, 146, 407-408.

Mosmann, T.R., and Sad, S., l996. The expanding universe of T-cell subset: Th1, Th2 and more. *Immunol. Today*, 17, 138-146.

Quie, P.Q., l972. Clinical manifestation of chronic in phagocyte function. In *Phagocyte Mechanisms in Health and Disease*, Eds. William, R.C. & Fudenburg, H.D.), Intercontinental, New York. p 139.

Snider, W.R., 1971. Tuberculosis in Canine and Feline: Review of the literature. *Am. Rev. Respir.*, 104, 877-887.

Thoen, C.O., l994. Tuberculosis in wild and domestic mammals. In *Tuberculosis Pathogenesis and Control. Bloom*, B.R. Editor. American Society for Microbiology, Washington, DC. 157-162.

Tizard, I., 2000. *Veterinary Immunology: An Introduction*, 6th Ed. W.B. Saunders Company, United State of America.

Tjahajati, I., Prodjoharjono, S., Soebono, H., Asmara, W., ans Harada, N., 2004. Reactive oxygen intermediate (ROI) in cat macrophage infected with *Mycobacterium tuberculosis. Journal Sain Veteriner*, Vol XXII (1), 46-53.

Tjahajati, I., Prodjoharjono, S., Soebono, H., Asmara, W., and Harada, N., 2004. Phagocyte activity in cat macrophage infected with *Mycobacterium tuberculosis. Journal Sain Veteriner, Vol* XXII (2), 61-65.

Tjahajati, I., Prodjoharjono, S., Soebono, H., Asmara, W., Subronto, Y.,W. dan Harada, N., 2003. The profile of interferon-gamma and IL-4 cytokine patterns production by peripheral blood mononuclear cell from cats infected with *M.tuberculosis*. *International Journal of Biotechnology*, 663-669.

Wilesmith, J.W., l994. Tuberculosis in Cat. *Vet. Rec.*, 134, 359.