Comparison of Cytokine Profile between Indonesian Thin-Tailed and Merino Sheep during A Primary Infection with Fasciola gigantica

(PERBANDINGAN PROFIL SITOKIN ANTARA DOMBA EKOR TIPIS DAN DOMBA MERINO YANG DIINFEKSI PERTAMA DENGAN FASCIOLA GIGANTICA)

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ABSTRACT

The aim of this study was to investigate the expression of cytokines profiles interferon-γ (IFN-γ), interleukin-5 (IL-5) and IL-10 in Rasciola gigantica resistant Indonesian thin-tailed (ITT) sheep compared to susceptible merino sheep infected with Fasciola gigantica. A total of ten ITT and merino sheep were randomly allocated into infected (n=5) and control (n=5) groups, sheep were infected with 250 viable metacercaiae of F. gigantica. The cytokines were determined by isolated mRNA from hepatic lymph node by semi-quantitative RT-PCR (Reverse Transcriptase -Polymerase Chain Reaction). The result showed ITT sheep produced significantly higher IL-5 and 10 (P<0.05) than merino sheep, while ITT sheep produced less IFN-γ (P<0.05) than merino sheep at 10 weeks post infection. It could be concluded that merino sheep tend to develop T type 1 cells, while the ITT sheep tend to develop T type 2 cells which effectively killed F. gigantica.

Key Words: Fasciola gigantica; Indonesian thin-tailed sheep; merino sheep; IL-5; IL-10; IFN-γ

INTRODUCTION

Tropical fasciolosis is a significant disease in ruminants causing losses to agricultural and livestock productions. It is caused by the tropical liver fluke F. gigantica. F. gigantica is a digenetic leaf-like trematode that requires a lymnaeid snail, Lymnae rubiginosa auricularia, as an intermediate host (Torgeson and Claxton, 1999). The parasite invades the liver and maturity occurs in the bile ducts. F. gigantica is generally restricted to the tropics and having been recorded in Africa, the Middle East, eastern Europe, and south and eastern Asia (Mas-Coma et al., 2005).

F. gigantica causes a significant reduction in meat and milk production, growth rate,
fertility and draught power in infected animals. Other causes of economic losses are condemnation of infected livers at slaughter and the cost of control measures (Spithill et al., 1999). Recent reports indicate that fasciolosis is also an important emerging pathogen in humans, with approximately 2.5 million people infected worldwide (WHO, 1995).

Immune responses to parasitic infections are regulated by T helper cells via their production of cytokines. Development of protective immunity to infections is influenced by these cells and their cytokines (Abbas et al., 1996). It is well accepted that CD4+ Th (helper) cells can be separated into two major subsets, Th1 and Th2, on the basis of their cytokine production patterns and function (Fitch et al., 1993). T-helper1 cells produce interferon (IFN)-γ, interleukin (IL)-2 and IL-12 and promote the activation of macrophages and mediate delayed type hypersensitivity. In contrast, Th2 cells produce IL-4, IL-5, IL-6, and IL-10 and promote immediate type hypersensitivity reactions involving IgE, eosinophils and mast cells. The cytokines of each T cell subtype are mutually inhibitory for the differentiation and effector functions of the reciprocal subset resulting in the polarization of the immune response to either type 1 or type 2.

The previous studies had shown that ITT sheep are more resistant to infection with F. gigantica than the merino sheep (Wiedosari and Copeman, 1990). The innate cytokine response occurred during infection is a critical component which can control the production of cytokines in the microenvironment of the responding lymph node (Abbas et al., 1996). This study was conducted by measuring IFN-γ, IL-5, and IL-10 which were determined by isolated mRNA from hepatic lymph node following F. gigantica infection by semi-quantitative RT-PCR. The purpose of this study is to investigate the cytokine expression in tissues of ITT and merino sheep during F. gigantica infection which will show critical differences between the T cells from the two sheep breeds allowing to understand why ITT sheep are better at killing this parasite.

**RESEARCH METHODS**

**Experimental Animals and Design**

Ten of each ITT and merino sheep, approximately nine months old at the time of infection with no detectable eggs of F. gigantica in faeces, were used in this experiment. Animals in each breed were allocated at random into infected (n=5) and control (n=5) groups.

The trial was carried out at Research Centre for Biotechnology Indonesian Institute of Sciences (LIPI), Bogor, West Java, Indonesia where sheep were maintained in open pens on a diet of freshly cut Pennistium purpureum and concentrate. Metacercariae for infections were obtained from infected L. rubiginosa snails collected at Surade, West Java, Indonesia. Sheep were infected with 250 viable metacercariae of F. gigantica by loading the required metacercariae on filter paper which was placed inside gelatin capsules and delivered orally using a dosing gun (Wiedosari and Copeman, 1990).

**Necropsy and Lymph Node Collection**

Sheep were sacrificed at 10 weeks postinfection by severing the jugular vein. Immediately following slaughter, the hepatic lymph node was dissected from each animal and rinsed in 100% ethanol to remove excess blood and tissue. Approximately 0.5 - 1 g of tissue was cut into slices of 0.5 cm diameter, rinsed in cold PBS and immersed in 5 ml of RNA later (Ambion). Samples were kept at 4°C for 12 hours before long term storage at -20°C.

**Homogenisation, RNA Extraction and cDNA Synthesis** (Clery and Mulcahy, 1998):

Samples were thawed by gentle rotation at 4°C and approximately 400-500mg of tissue was removed, rinsed in cold PBS and homogenised in 5 ml of QIAzol (Qiagen) for 30 seconds using a sterilized polytron homogenizer. Samples were stored in 1ml aliquots at -70°C until RNA was extracted.

RNA was extracted using the Lipid RNeasy© Mini Kit (Qiagen) according to manufacturer's guidelines. Genomic DNA contamination was eliminated by treating each sample with 27 Kunitz of RNase free DNase I (Qiagen) during the extraction procedure as recommended by the manufacturer. High quality RNA (OD 260nm/280nm > 1.9) was eluted from the columns using RNase-free water and quantitated using a Biophotometer (Eppendorf).

Total RNA (2 μg) was reversed transcribed using Omniscript® Reverse Transcription kit (Qiagen) according to manufacturer’s guidelines in a total volume of 20 μl containing 2 μl of 10 x Reverse Transcriptase Buffer (Qiagen), 2 μl dNTP mix (Qiagen), 0.5 μg Oligo-dT primer (Invitrogen), 10 units RNase Inhibitor (Promega),
4 Units of Omniscript Reverse Transcriptase (Qiagen), Rnase free water, and 2 μg RNA. The final reaction mixture was incubated at 37 ºC for 70 minutes and aliquots stored at −20 ºC or use directly in PCR reaction. A 10 μl of the RT reaction was used in a 100 μl PCR reaction which contained 0.5 μl of Taq DNA polymerase (Qiagen), 10 μl of 10 x PCR buffer, 0.2 μl of 10 mM dNTP, 4 μl of 50 mM MgCl, 1 μl of each appropriate forward and reverse primers. Oligonucleotides were used to amplify ovine IL-5, IL-10 and IFN-γ and the reference gene, GAPDH. Oligonucleotides were synthesised by GeneWorks Pty Ltd (Adelaide, Australia) and were used at a concentration of 10μM. Prior to PCR amplification a denaturation step (95°C for 15 minutes) was used followed by 34 cycles of denaturation (95°C for 0.5 min); annealing (53°C for 1 min); and elongation (72°C for 1 min). A final extension step was performed by heating to 72°C for 8 min. The number of PCR cycles and the total RNA input in the RT reaction were determined in a preliminary experiment to ensure the amplification was well below the saturation under this assay condition.

Primer sequence IFN- g:
IFN- g (F): AGGGTGGGCCCTTCTTCTC (262bp)
IFN- g (R): ATTAGATGGCCTTGTGCTG
Primer sequence IL-10:
IL-10 (F): AGCTGTACCCACTTCCCA (305bp)
IL-10 (R): GAAAACGATGACAGCGCC
Primer sequence IL-5:
IL-5 (F): CCTTGACACTGCTCTACGC (303bp)
IL-5 (R): CTTTCCATCGTCCACTGC
Primer sequence GAPDH:
GAPDH (F): ATCACTGCCACCCAGAAGACT (200bp)
GAPDH (R): CATGCCAGTGAGCTTCCCGTT

After PCR amplification, a 10μl aliquot was mixed with 2 x loading buffer and electrophoresed on a 1.2 % agarose gel containing ethidium bromide (0.25 μg/ml). The results of DNA bands in the gel were photographed (Figure 1, 2 and 3) while the quantitation of cytokine mRNA was accomplished by 1 D Image Analysis Software. The mRNA level of each cytokine was normalized to GAPDH (control) and expressed as ratio to GAPDH mRNA for each sample.

Statistical Analysis
Statistical analysis was performed using SAS system (1998), the ANOVA and the Duncan test were used to evaluate the result. Differences at the P<0.05 level were considered significant.

RESULTS AND DISCUSSION
The result showed that hepatic lymph node cells from ITT sheep produced significantly more IL-5 (P<0.05) than cells from merino sheep at 10 weeks post infections with F. gigantica (Figure 4). The presence of IL-5 indicates a stimulation of a Th2-type of response and it participates in the polarization of the immune response towards a Th2 response. The main activities of IL-5 are to promote the growth and terminal differentiation of eosinophils, to attract eosinophils to sites of inflammation, and to enhance IgG1 induced eosinophil degranulation (Abbas et al., 1996). Interleukin-5 has also been implicated in eosinophil activation and shown to enhance the killing of nematode larval in sheep (Rainbird et al., 1998).

The role of eosinophils during infection with F. gigantica has been suggested as one of the main effector cells involved in the killing of young flukes. Van Milligen et al., (1998) reported that eosinophils may be associated with the expression of resistance to F. hepatica as these cells infiltrate the intestine and selectively adhere to newly-excysted juveniles, where their degranulation causing severe damage and perhaps death to the young flukes (Burden et al., 1983). Immunological analysis of response in ITT and merino sheep following a primary infection showed ITT sheep exhibited a significantly higher eosinophilia than merino sheep (Wiedosari, 2005). A study by Piedrafita (2001) revealed that eosinophils were identified as the major immune cell-types within the peritoneum during the early migration of F. gigantica parasites in ITT sheep. These immune cells from the ITT sheep could indeed effectively kill immature F. gigantica but this ability was critically dependent on the presence of sera from F. gigantica-infected sheep (Piedrafita, 2001) By using a series of inhibitors, the author was further able to determine that the major cytotoxic molecules mediating parasite killing, which were produced by these cells, were superoxide radicals. Consequently, the enhanced resistance of ITT sheep to F. gigantica is probably associated with IL-5, as indicated in these observations that IL-5 of ITT sheep was higher compared to merino sheep.
A Th2 response was also indicated by IL-10 which was observed at 10 weeks postinfection and the production was higher (P<0.05) in ITT sheep than merino sheep (Figure 5). A recognized role of IL-10 in mediating Th2 responses is its inhibition of Th1 cell differentiation via suppression of IFN-γ production by Th1-committed cells. Interleukin-10 does not act directly on Th1 cells but impairs the capacity of splenic and peritoneal macrophages to stimulate Th1 cytokine synthesis by Th1 cells. This effect is partly due to the ability of IL-10 to down-regulated the expression of class II MHC on APCs that interact with antigen-activated Th1 cells to induce cytokine synthesis (Tizard, 2000). Schopf (2002) demonstrated that a deficiency in IL-10 can lead to increased susceptibility to Trichuris muris and even death. Interestingly, this lethal outcome is not only to Trichuris muris infections, because mortality of IL-10 deficient mice has also been observed during Toxoplasma gondii, Trypanosoma cruzi, Plasmodium chabaudi-chabaudi, and Schistosoma mansoni infection. It is, therefore, likely that the increased production of IL-10 in the ITT sheep have an enhanced capacity for the sheep to kill F. gigantica in vivo.

In present study, the production of IFN-γ was higher (P<0.05) in merino sheep than ITT sheep (Figure 6). Interferon-γ is an important Th1 cytokine that serves primarily to activate natural killer cells and mononuclear phagocytes (e.g. macrophages) which are responsible for cytotoxic activity against tumor cells and microbes (Abbas et al., 1996). Research suggests that IFN-γ inhibits or delays protective immunity to nematodes by suppressing the growth and expansion of Th2 cells via a down-
as *Trichuris muris* in rodents (Schopf, 2002)). Wiedosari (2005) reported that ITT sheep produced significantly lower levels of IgG2 antibody relatives to the high level detected in merino sheep. The production of IgG2 has been shown to be strongly up-regulated by IFN-γ in mice, humans and cattle (Abbas et al., 1996). It is therefore likely that elevated IFN-γ levels are related to elevated IgG2 levels in the susceptible merino sheep. This observation is intriguing given by Bielefeldt and Babiuk (1985) that IFN-γ, a typical Th1-type cytokine, decreases superoxide radicals production by sheep immune cells.

Thus, the immunological pathways which could inhibit an effector mechanism in the resistant ITT sheep, appear to be up-regulated in the susceptible merino host. Fitch et al., (1993) reported that characteristic cytokine products of Th1 and Th2 cells are mutually antagonistic for the differentiation and activity of effectors.

regulation of IL-5, inhibiting production of IgG1, blocking eosinophilia (Urban et al., 1993). These inhibitory effects of IFN-γ on Th2 effector responses have been associated with prolonged survival of intestinal nematode parasites, such

**Figure 3.** Lane 1-9 were an example of PCR products using specific primers for sheep IFN-γ and the housekeeping gene GAPDH from all of 20 sheep, were visualised by electrophoresis using a 1.2% agarose gel containing ethidium bromide.

**Figure 4.** IL-5 profile in Indonesian Thin-tailed and Merino sheep at 10 weeks post infection with *Fasciola gigantica* ■ ITT ■ Merino

**Figure 5.** IL-10 profile in Indonesian Thin-tailed and Merino sheep at 10 weeks post infection with *Fasciola gigantica* ■ ITT ■ Merino

**Figure 6.** IFN-γ profile in Indonesian Thin-tailed and Merino sheep at 10 weeks post infection with *Fasciola gigantica* ■ ITT ■ Merino

**Figure 7.** IL-5 profile in Indonesian Thin-tailed and Merino sheep at 10 weeks post infection with *Fasciola gigantica* ■ ITT ■ Merino
belonging to the reciprocal phenotype. Hence, IL-10 inhibits cytokine synthesis by Th1 cells and macrophage function, whereas IFN-γ suppresses the production of Th2-type effectors and cytokines. These results strongly support that the protective immune response in ITT sheep to F. gigantica infection involves the selective expansion of Th2-type subset cells with strong IL-5 and IL-10-secreting activities, with minimal activation (and possibly active down-regulation) of Th1-type IFN-γ-secreting activity.

CONCLUSION

The resistant ITT sheep tends to develop Th2-cells, while susceptible merino sheep develop Th1-cells. Fasciola gigantica infection in ITT sheep could be prevented by producing IL-10, IL-5 and activating eosinophils expressed by Th2-cells that effectively killed F. gigantica. The development of significant IFN-γ response by Th1 which inhibited superoxide production may be the primary explanation for the increased susceptibility of merino sheep.

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