Author: T. Spithill, D. Piedrafita, E. Estuningsih and J. P. R. R. H. M. E. Pleasance
Author Address: tspithill@csu.edu.au
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Abstract: Indonesian thin-tail (ITT) sheep resist infection by Fasciola gigantica by an immunological mechanism within 2 to 4 weeks of infection yet are susceptible to F. hepatica infection. Studies of ITT sheep show that little liver damage occurs following F. gigantica infection, suggesting that the invading parasites are killed within the peritoneum or shortly after reaching the liver. We investigated whether cells isolated from the peritoneums of ITT sheep could kill newly excysted juvenile F. gigantica in vitro and act as a potential mechanism of resistance against F. gigantica infection. Peritoneal cells from F. gigantica-infected sheep, rich in macrophages and eosinophils, mediated antibody-dependent cytotoxicity against juvenile F. gigantica in vitro. Cytotoxicity was dependent on contact between the parasite and effector cells. Isolated mammary gland eosinophils of F. gigantica-infected sheep, or resident peritoneal monocytes/macrophages from uninfected sheep, also killed the juvenile parasites in vitro. By using inhibitors, we show that the molecular mechanism of killing in these assays was dependent on the production of superoxide radicals by macrophages and eosinophils. In contrast, this cytotoxic mechanism was ineffective against juvenile F. hepatica parasites in vitro. Analysis of superoxide dismutase activity and mRNA levels showed that activity and gene expression were higher in F. hepatica than in F. gigantica, suggesting a possible role for this enzyme in the resistance of F. hepatica to superoxide-mediated killing. We suggest that ovine macrophages and eosinophils, acting in concert with a specific antibody, may be important effector cells involved in the resistance of ITT sheep to F. gigantica.

Peritoneal lavage cells of Indonesian thin tail sheep mediate antibody-dependent superoxide radical cytotoxicity *in vitro* against newly excysted juvenile *Fasciola gigantica* but not juvenile *Fasciola hepatica*

David Piedrafita[1,2,3*], Endah Estuningsih[4], Jill Pleasance[2], Rhoda Prowse[3], Herman W. Raadsma[5] , Els N.T. Meeusen[1,2] and Terry W. Spithill[3,6]

Department of Physiology, Monash University, Clayton, Australia[1]

School of Veterinary Science, Melbourne University, Victoria, 3010, Australia[2]

The Cooperative Research Center for Vaccine Technology, The Bancroft Centre, Royal Brisbane Hospital, Brisbane, Queensland, 4029, Australia[3]

Research Institute for Veterinary Science, Bogor, West Java, Indonesia[4]

Faculty of Veterinary Science, University of Sydney, NSW, 2570, Australia[5]

Institute of Parasitology and Centre for Host-Parasite Interactions, McGill University, Montreal, H9X 3V9, Canada[6]

Running Title: Superoxide-mediated killing of juvenile *Fasciola gigantica*

*Corresponding author. Mailing address: Department of Physiology, Monash University, Wellington Road, Clayton, 3800, Victoria, Auatralia. Phone: +61 3 9905 2593. Fax: +61 3 9905 2547. E-mail: david.piedrafita@med.monash.edu.au

ABSTRACT

Indonesian thin tail (ITT) sheep resist infection by *Fasciola gigantica* by an immunological mechanism within 2-4 weeks of infection yet are susceptible to *F. hepatica* infection. Studies in ITT sheep show little liver damage occurs following *F. gigantica* infection, suggesting that the invading parasites are killed within the peritoneum or shortly after reaching the liver. We investigated whether cells isolated from the peritoneum of ITT sheep could kill newly excysted juvenile *F. gigantica in vitro* and act as a potential mechanism of resistance against *F. gigantica* infection. Peritoneal cells from *F. gigantica*–infected sheep, rich in macrophages and eosinophils, mediated antibody-dependent cytotoxicity against juvenile *F. gigantica in vitro*. Cytotoxicity was dependent on contact between the parasite and effector cells. Isolated mammary gland eosinophils of *F. gigantica* infected sheep, or resident peritoneal monocyte/macrophages from uninfected sheep, also killed the juvenile parasite *in vitro*. Using inhibitors, we show that the molecular mechanism of killing in these assays was dependent on the production of superoxide radicals by macrophages and eosinophils. In contrast, this cytotoxic mechanism was ineffective against juvenile *F. hepatica* parasites *in vitro*. Analysis of superoxide dismutase activity and mRNA levels showed that activity and gene expression was higher in *F. hepatica* than *F. gigantica*, suggesting a possible role for this enzyme in the resistance of *F. hepatica* to superoxide-mediated killing. We suggest that ovine macrophages

and eosinophils acting in concert with specific antibody, may be important effector cells involved in the resistance of ITT sheep to *F. gigantica.*

INTRODUCTION

Fasciola gigantica is the most common *Fasciola* species infecting ruminants in Asia and Africa and is estimated to cause worldwide losses to the livestock industry of over US$3b per annum (44). Human infection with liver flukes is also recognised by WHO as an emerging human health problem with over 500m people at risk of infection with *Fasciola, Opisthorchis* or *Clonorchis* (11, 23, 44). There are at least 2.4m people infected with *Fasciola* and infection rates in children of up to 72% have been observed in Bolivia (23). Chemotherapy is not a sustainable method of control due to development of parasites resistant to available flukicides and the cost of treatment impedes its application within the rural areas of developing countries (7, 12, 23, 28, 43). Thus, other cost-effective control mechanisms such as vaccines need to be developed for control of fasciolosis (12, 43). However, the development of vaccines will require knowledge of the immune mechanisms involved in host resistance against *Fasciola* parasites since such knowledge may lead to the rational design of delivery methods for a vaccine.

There is no practical rodent model for studying immune responses to *F. gigantica*, since rodents are not permissive to infection (8, 15, 22, 34, 44). Consequently, little is known about the humoral or cell-mediated responses important for host immunity against *F. gigantica* (34, 42). However, studies in the natural hosts (sheep and cattle) provide evidence that ruminants do acquire resistance to *F. gigantica* infection (1, 34, 37, 38, 39, 44). When the susceptibility to *F. gigantica* is compared between sheep breeds, the

Indonesian Thin Tail sheep exhibits a high degree of resistance to infection relative to other breeds such as St. Croix and Merino (34, 42). For example, ITT sheep express high resistance to a primary infection with *F. gigantica* compared to Merino sheep and acquire further resistance to infection after exposure (34, 37, 38, 39, 49). Analysis of fluke burdens in sheep at varying times following infection showed that significant killing of parasites occurs between 2 and 4 weeks of challenge with little liver damage detected following infection, suggesting that many migrating flukes may not survive long enough to establish in the liver (39). Importantly, resistance to *F. gigantica* infection by ITT sheep is suppressed by the administration of dexamethasone, suggesting that the acquired resistance is immunologically based (39).

Taken together, these observations suggest that the peritoneal cavity may be an important site of immunological killing of migrating *F. gigantica* parasites in ITT sheep. They also suggest that the newly excysted immature juvenile (NEJ) parasite could be the primary target of the effective immune response expressed in ITT sheep. These observations are analogous to those seen in rats (a resistant host) during *F. hepatica* infection where resistance is immunologically based and occurs at both the gut wall and peritoneal cavity (13, 34, 46, 47). In the rat model, NEJ *F hepatica* parasites are susceptible to antibody-dependent cell mediated killing by reactive nitrogen intermediates released by peritoneal macrophages (33). Another recent study in rats confirmed that macrophage-mediated killing of *F. hepatica* was NO-dependent although an antibody dependence was not confirmed (41). Here, we have evaluated the possibility that a cell-mediated cytotoxicity mechanism is also expressed in the peritoneum of ITT sheep against the juvenile *F. gigantica* parasite. We show that juvenile *F. gigantica* are susceptible to

killing *in vitro* by superoxide radicals produced by macrophages, isolated from the peritoneum of ITT sheep, and mammary gland eosinophils: we suggest that this killing mechanism may be involved in determining the resistance of ITT sheep to *F. gigantica* infection.

MATERIALS AND METHODS

Animals, parasites, parasite extracts and reagents. *F. gigantica*-naive, male Indonesian Thin Tail (ITT) sheep, between 6 to 8 months old, were bred and raised in pens at Balitvet, Bogor, Indonesia. The naivety of the animals was confirmed by a negative reaction in an ELISA and a Western blot using *F. gigantica* whole worm extract as antigen (6). Throughout the experiments the sheep were maintained in pens on a diet of freshly cut *Pennisetum purpureum* and dairy concentrate (38, 39). Metaercariae for infections and parasite excystment were obtained from infected *Lymnaea rubiginosa* snails collected at Surade, West Java, Indonesia (for *F. gigantica*) or from *Lymnaea tomentosa* snails collected from laboratory snail cultures at Elizabeth Macarthur Agricultural Institute, Menangle, NSW Australia (for *F. hepatica*). Sheep were infected with 250 metacercariae of *F. gigantica* or *F. hepatica* by loading the required metacercariae on filter paper, which was placed inside gelatin capsules (Torpac Inc. Fairfield, NJ, USA) and delivered orally using a dosing gun. Sheep experiments in Indonesia were performed with approval by the Research Institute for Veterinary Science (Bogor, Indonesia) according to local guidelines and custom (38, 39). Adult *F. gigantica* and *F. hepatica* parasites were obtained from the

livers of infected ITT sheep and somatic fluke extracts were prepared as previously described (6).

Catalase, cytochrome C, gentamycin, mannitol, RPMI, superoxide dismutase (SOD), toluidine blue, trypan blue and the tetrazolium salt, MTT, were purchased from Sigma Chemical Co., USA. Amphotericin B was purchased from Life Technologies (Rockville, MD, USA). ELISA plates and 24-well tissue culture plates were purchased from Flow Laboratories Inc., USA and Greiner Labortechnik, Austria, respectively.

Cell populations. Resident monocyte/macrophage-rich cell populations from naïve sheep, or cell populations from ITT sheep infected for 4 weeks with *F gigantica* or *F. hepatica,* were collected from the peritoneal cavity using sterile PBS containing 6 mM EDTA. The recovered lavage fluid was collected, centrifuged at 1500 r.p.m. for 10 minutes and the cell pellet resuspended in sterile RPMI containing 10% heat-inactivated foetal calf serum, 2 μg/ml amphotericin and 10 μg/ml gentamycin.

Eosinophil-enriched cell populations were obtained from the mammary glands of infected ewes using *F. gigantica* parasite extract as previously described (4). Briefly, ITT ewes were infected with 100 metacercariae of *F. gigantica*: 14-16 weeks later, eosinophil recruitment into the teat canal was achieved using *F. gigantica* soluble adult fluke somatic extract. Briefly, 200 μg of somatic fluke extract was suspended in 5 ml of sterile saline and infused via a sterile, smooth-end 22-gauge needle into the teat canal of sensitized sheep, as previously described (35). Following isolation, a 5 μl sample of cells was diluted 10 fold with PBS, 50 μl of trypan blue (0.4% w/v in PBS) was then added and the total number of viable white blood cells determined using a Neubauer haemocytometer. For differential cell counts, cytospin preparations were made by centrifuging samples of

lavage cells at 400 r.p.m. for 5 minutes at 4°C in a Beckman TJ-6 benchtop centrifuge prior to differential staining (Diff-Quik®). 200-300 cells were identified microscopically and the relative percentage of lymphocytes, monocytes/macrophages, eosinophils, neutrophils, basophils and mast cells was determined.

Sheep sera. Sheep were infected with 250 metacercariae of *F. hepatica* or *F. gigantica* and blood was collected 8 weeks later by jugular venipuncture, using EDTA vacutainer tubes. The blood was allowed to clot at room temperature for 1 hour and centrifuged in a Beckman CS-6R centrifuge at 3000 g for 20 minutes. The serum was then removed and complement activity inactivated by heating at 56°C for 30 minutes prior to storage at -20°C.

Incubation of juvenile liver fluke with lavage cells. Metacercariae were excysted and newly excysted juvenile parasites (NEJ) were separated from empty cysts and debris by incubation overnight at 37°C as described (50). Cytotoxicity assays were carried out in 24 or 96-well tissue culture plates using up to 50 NEJ liver fluke per well. Due to the lower yield of peritoneal lavage cells from uninfected sheep, the incubation volumes were adjusted to 0.2 ml with this cell source. Plates were incubated for 3 days at 37°C in 5% CO_2 in 0.2 ml (cells from uninfected sheep) or 1 ml (cells from *Fasciola*-infected sheep) of RPMI media, containing 10% heat-inactivated FCS, 2 μg/ml amphotericin B, 10 μg/ml gentamycin and with or without the following additions: 10% sheep serum, 10 μg/ml superoxide dismutase, 10 μg/ml catalase, 10 μM mannitol or combinations of these reagents, and lavage cells at an effector:target ratio of 0.25-2 x 10^5 cells: NEJ liver fluke. NEJ liver fluke were defined as viable when they were determined microscopically as motile, having defined intestinal caecum and associated structures (lack of these structures

results in an opaque appearance) and a defined parasite shape with no tegumental damage (as determined by exclusion of the dye toluidine blue). Following the completion of the incubation period, NEJ liver fluke were incubated for 4 hours in a solution of 2 mg/ml MTT and viability was assessed as an ability to reduce the tetrazolium, MTT, as described (32). Comparative incubations with *F. hepatica* and *F. gigantica* parasites were performed on the same day using the same communal reagents in the same culture plates.

Assay of enzyme protein activity. Approximately 5,000 NEJ liver fluke or 20-50 adult parasites were manually homogenised in 100 mM Tris-HCL pH7.4 in an ice-cooled ground glass homogeniser for 5 minutes and the homogenate centrifuged at 4^{o}C at 1,000 g. The specific activity of superoxide dismutase (SOD) and glutathione *S*-transferase (GST) was measured in triplicate in the resultant supernatant. All reactions were carried out at 25^{o}C in a Shimadzu UV-160 spectrophotometer (Shimadzu Corporation, Japan) in a total reaction mixture of 1 ml, and the change in absorbance monitored continuously for 2 minutes. The specific activity of SOD was determined by the cytochrome c reduction method, using bovine erythrocyte SOD as a standard (32). Cytochrome c reduction was monitored at 550 nm. One unit of SOD activity was defined as the amount of enzyme necessary to inhibit the rate of reduction of cytochrome c by 50%. Superoxide dismutase (SOD) specific enzyme activity is expressed as U/mg protein. The GST assay measured the conversion of glutathione from the oxidised form to the reduced form and was monitored spectrophotometrically as an increase in absorbance at 340 nm (32, 40). The specific activity of GST was defined as the amount of 1-chloro-2, 4-dinitrobenzene conjugated per minute per mg of protein. Protein concentration was determined using the Bio-Rad DC colorimetric assay for protein concentration following detergent solubilisation.

Assay of enzyme mRNA expression. RNA was extracted in duplicate using the Lipid RNeasy© Mini Kit (Qiagen) according to manufacturer's guidelines. 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. The final reaction mixture was incubated at 37 °C for 70 minutes and stored at - 20 °C. Oligonucleotides used to amplify *Fasciola* β -actin, GST and SOD were designed from sequences obtained from the GenBank database using the text search program and the Primer3 software program (BioNavigator™; www.enitgen.com) under default parameters. Products from each primer set were sequenced verified to ensure correct amplification. Real-time PCR conditions for each primer set were optimised using pooled cDNA. Efficiencies were calculated from the average of three standard curves (RSq > 0.9) generated from separate experiments and serially diluted cDNA (standard deviation <5 %). Primers for B actin were sense 5'atcactgccacccagaagact and antisense 5'catgccagtgagcttcccgtt; primers for GST were sense 5' agaaatggttgggcgataaa and antisense 5' aacacgaacaaaacccatcc; primers for SOD were sense 5' gcgggacctcatttcaaccc and antisense 5' cacaagccaaacggcctccag. Primer sets used here were 100 % identical between *F. gigantica* and *F. hepatica* cDNA sequences for each target product and ensured the binding sites of the primers used in real-time PCR were identical for both *F. gigantica* and *F. hepatica* target genes and, hence, validate the real-time PCR efficiencies. Relative quantitation of SOD and GST mRNA were assayed on the MX3000P real-time PCR machine (Stratagene) using the SYBR green (with dissociation curve) experiment type. Each 20 µl reaction included 10 µl of 2 x Sybr Green Master Mix (QIAGEN) which incorporated the internal Rox Dye control, 0-1 µl of 50 mM $MgCl_2$ (Invitrogen), 0.3 µM of each primer, and 4 µl of cDNA (diluted 1:10).

Real-time conditions began with a 15 minute denaturation step at 95 °C followed by 40 cycles of 94 °C for 15 secs, primer specific temperature for 30 secs and 72 °C for 30 secs. A melt curve analysis (55-95°C at a heating rate of 0.01 °C/sec) was performed to ensure only the required PCR product at a specific melting temperature was measured. Each experiment was repeated three times and in each a designated calibrator was run in triplicate. Following amplification the experiment was converted to a Comparative Quantification (Calibrator) experiment type and analysed using the MX300P software. SOD and GST expression was normalised for each cDNA preparation using the respective β-actin housekeeping gene value and final values represent the expression relative to the calibrator. Averages represent the result of two RNA extractions and six real-time PCR experiments for each sample.

Statistical analysis. Significant differences between treatment groups was determined by the non-parametric Dunnett's multiple comparison test. Significant differences for specific anti-oxidant defence enzyme activities between adult *F. hepatica* and *F. gigantica* whole worm extracts were calculated using the unpaired alternative t-test. To analyze real-time PCR results crossing point values were used in the REST © (Relative Expression Software Tool) version 2 software program (29). Statistical analysis of gene regulation between groups was performed under the default parameters of the program using pairwise fixed reallocation randomisation tests.

RESULTS

Killing of NEJ *F gigantica* parasites *in vitro* by peritoneal lavage cells and immune sera from ITT sheep. Peritoneal lavage cells (PLCs) collected from ITT sheep infected for 4 weeks with *F. gigantica* consisted of (40-50%) monocyte/macrophages, 40-50% eosinophils, 2-20% lymphocytes and less than 5% neutrophils. When NEJ *F. gigantica* were incubated with these cells and sera from *F. gigantica*-infected ITT sheep, large numbers of cells adhered to the NEJ liver fluke tegument, as previously described for *F. hepatica* (33): this resulted in a reduction in the mean viability of NEJ *F gigantica* to 40% (Fig. 1). Dead NEJ *F. gigantica* were characterised by extensive cell attachment, immotility, loss of defined intestinal caecum, loss of parasite shape and extensive tegumental damage (as determined by toluidine blue staining) and an inability to reduce the tetrazolium salt MTT, as observed in the rat/*F hepatica* model (33). Killing of NEJ *F gigantica* parasites *in vitro* required both the presence of immune sera and PLCs. When NEJ *F gigantica* parasites were incubated in the absence of sera, or with sera from *F. gigantica*-naïve ITT sheep, a mean 85% of parasites were viable (Fig. 1). The viability of NEJ liver fluke was also not affected by incubation with sera from *F. gigantica*-infected ITT sheep in the absence of PLCs (90% viable NEJ liver fluke; Fig. 1).

Killing of NEJ *F. gigantica* parasites *in vitro* by PLCs requires direct cell contact. The mechanism of cytotoxicity to NEJ liver fluke by ITT sheep PLCs observed above required the inclusion of sera from *F. gigantica*-infected ITT sheep which resulted in extensive cell attachment, suggesting that intimate contact between the effector cells and the NEJ liver fluke tegument was required for parasite killing. In order to test this possibility, NEJ *F. gigantica* were incubated with sera from *F. gigantica*-infected ITT sheep in wells with tissue culture inserts in which the PLCs were separated from the NEJ

liver fluke by a 0.45 μm filter to inhibit direct contact between NEJ liver fluke and PLCs. Separation of NEJ liver fluke from PLCs, in the presence of sera from *F. gigantica*-infected ITT sheep, resulted in a mean parasite viability of 80% , when compared with a mean of only 20% viable parasites in incubations performed in the absence of tissue culture inserts (Fig. 2). This result suggests that killing of NEJ *F gigantica* by ITT PLCs requires intimate contact between the PLCs and the fluke tegument.

Identification of the cytotoxic mediator of parasite killing *in vitro* produced by PLCs. We attempted to identify which cytotoxic molecule(s) produced by ITT sheep PLCs was mediating the killing of NEJ *F. gigantica*. Cytotoxic molecules released by macrophages and eosinophils include reactive nitrogen (nitric oxide) and oxygen (superoxide, hydrogen peroxide) intermediates (3, 31). Inhibition of nitric oxide production by L-NMMA did not reverse the cytotoxic effects of ITT sheep PLCs as expected (data not shown), since we have previously shown that lavage cells of ITT sheep (and other sheep breeds) do not generate detectable levels of nitric oxide under our incubation conditions *in vitro* (33). However, addition of the inhibitor of superoxide radicals, superoxide dismutase (SOD), significantly reversed the killing of NEJ *F. gigantica* parasites from a mean 35% viable parasites in the absence of SOD to a mean 80% viability with SOD present (Fig. 3a). Superoxide radicals can give rise to other reactive oxygen species, including hydrogen peroxide and hydroxyl radicals. However, inhibitors of hydrogen peroxide (catalase) and the hydroxyl radical scavenger (mannitol) had no significant effect on the mean killing of NEJ *F. gigantica* incubated with ITT sheep PLCs and sera from *F. gigantica*-infected ITT sheep alone (Fig. 3a). This superoxide-mediated cytotoxicity against NEJ *F. gigantica* appeared to exert its effects early in the

incubation period with ITT PLCs and immune sera, as indicated by a reduction in mean parasite motility within 24 hours when compared to incubations with the addition of SOD (Fig. 3b).

Identification of effector cells in ITT PLCs mediating cytotoxicity *in vitro*. The *in vitro* cytotoxic assays used PLCs that consisted of two major immune cell types, monocyte/macrophages and eosinophils, which have been shown to have important roles in helminth parasite killing in other animal models (21, 35). We, therefore, obtained cell populations highly enriched for macrophages or eosinophils to test whether each cell type was capable of mediating superoxide-dependent cytotoxicity to NEJ *F gigantica*. Resident peritoneal cell populations from *F. gigantica*-naïve ITT sheep contained greater than 90% monocyte/macrophages with no eosinophils present (Fig. 4a). Cell populations collected from ITT mammary glands infused with a soluble somatic *F. gigantica* lysate contained a mean 90% of eosinophils with about 8-10% monocyte/macrophages (representing a E:T ratio of 2 x 10^4 monocyte/macrophages:1 NEJ parasite) (Fig 4b). As shown in Fig 1, this level of monocyte/macrophages would only reduce NEJ viability by about 15%. When NEJ *F gigantica* were incubated with either of these enriched cell populations and sera from *F. gigantica*-infected ITT sheep, a significant portion of parasites were killed with mean viability reduced to 30% in each case (Fig. 5): this is comparable to the level of killing of NEJ parasites seen with the whole peritoneal cell population from *F. gigantica*-infected ITT sheep described above. The cytotoxicity mediated to NEJ *F. gigantica* by the monocyte/macrophage-rich or eosinophil-rich populations was also abrogated by the addition of SOD to inhibit superoxide radical formation (Fig. 5).

ITT PLCs do not kill NEJ *F. hepatica* parasites *in vitro*. Our previous work showed that ITT sheep are susceptible to primary and secondary infections with the temperate liver fluke *F. hepatica* (38, 39). Interestingly, we have also shown that NEJ *F. hepatica* are highly resistant to oxygen free radical-mediated killing *in vitro* (32, 33). These observations suggested that *F. hepatica* could be resistant to the ITT effector mechanism(s) that is active against *F. gigantica*. In order to determine whether there are inherent differences between the susceptibility of *F. hepatica* and *F. gigantica* to killing by ITT effector mechanisms, we directly compared the killing of NEJ *F. hepatica* and NEJ *F. gigantica in vitro* by incubating PLCs isolated from the same *F gigantica*-naïve ITT sheep with each parasite in the presence of homologous immune sera. These incubations were carried out on the same day using cells from the same donor sheep. Only NEJ *F. gigantica* were susceptible to killing by PLCs of *F. gigantica*-naïve ITT sheep (mean 25% viability): no cytotoxic effect was observed against NEJ *F. hepatica* (Fig. 6). Extended incubations (10 days) of PLCs with NEJ *F. hepatica* did not result in an increase in parasite killing (data not shown).

A second question we addressed was whether the ability to kill NEJ *F. hepatica* was influenced by the source of immune sera or cells ie, whether homologous or heterologous immune sera and PLCs from exposed sheep would mediate *in vitro* cytoxicity against NEJ *F. hepatica*. Accordingly, NEJ *F. hepatica* or *F. gigantica* were incubated with homologous or heterologous immune sera or PLCs from *F. gigantica*-infected animals or *F. hepatica*-infected animals, respectively. As shown in Table 1, incubations of NEJ *F. gigantica* with homologous or heterologous sera, or PLCs from *F. gigantica*-infected animals or *F. hepatica*-infected animals, also resulted in killing of NEJ *F. gigantica* (mean

43-58% viable parasites). In contrast, the viability of NEJ *F. hepatica* was unaffected by incubation with either homologous or heterologous sera or PLCs (Table 1). These incubations were performed on the same day using communal sources of cells and sera.

Anti oxidant defence enzyme mRNA levels and protein activity in somatic extracts of NEJ and adult *Fasciola* parasites. The significant difference in susceptibility to killing by superoxide exhibited by NEJ of *F. gigantica* and *F. hepatica* could result from differences in the levels of expression of the superoxide radical defence enzyme SOD in the two parasite species. We also wanted to determine whether any putative differences in defence enzymes were a general trend between the parasites by measuring levels of the anti oxidant enzyme, glutathione *S*- transferase (GST), a general defence enzyme against most tissue damage arising from free radicals. We compared the specific enzyme activity of SOD and GST defence enzymes in whole worm somatic extracts (WWE) of NEJ and adult parasites of *F. hepatica* and *F. gigantica*: the relative gene expression levels in adult liver fluke mRNA were determined using RT-PCR. To assure that meaningful comparisons could be obtained, WWEs of *F. hepatica* and *F. gigantica* were prepared from 5000 NEJ parasites excysted on the same day, or adult fluke collected on the same day, and the assays for activity were performed immediately under identical conditions. Due to the limited amount of sample, only specific enzyme activities for GST and SOD were measured in NEJ liver fluke WWE.

GST and SOD specific enzyme activity was detected in WWE from two separate batches of NEJ of each liver fluke species (Table 2). The mean GST specific activity in two preparations of NEJ was similar in the two species. The mean SOD specific activity was 33% greater in WWE of NEJ *F. hepatica* compared to NEJ *F. gigantica.* SOD and

GST specific enzyme activity and gene expression levels were measured in adult *F. hepatica* and *F. gigantica* (Table 2; Fig. 7). Adult WWE of *F. hepatica* had significantly higher specific enzyme activity ($p<0.001$) of SOD (5-fold) compared to adult WWE of *F. gigantica* whereas specific GST enzyme activity was similar in WWE of both *Fasciola sp* (Table 2). These findings were validated by significantly higher gene expression levels of SOD in *F. hepatica* relative to *F. gigantica* adult parasites while gene expression levels of GST were equivalent in the two parasite species (Fig. 7).

DISCUSSION

This study demonstrates for the first time a cytotoxic immune effector mechanism expressed by sheep against a major trematode parasite, *F. gigantica,* and has revealed fundamental differences between *F. gigantica* and *F. hepatica* parasites in their susceptibility to this effector mechanism *in vitro*. Our results show that peritoneal lavage cells from ITT sheep are able to kill NEJ *F gigantica in vitro* by a dose-dependent cell mediated mechanism that exhibits several features. This cell-mediated killing is antibody-dependent since parasite death does not occur in the absence of immune sera, strongly suggesting that killing requires direct attachment of cells to the parasite's surface: this is similar to observations in rats where antibody-dependent cytotoxicity against NEJ *F hepatica* was observed (33). The cytotoxic mechanism expressed by ITT sheep appears to be mediated by superoxide radicals since killing is blocked by the addition of SOD and is unaffected by the addition of L-NMMA, catalase or mannitol, known inhibitors of nitric oxide production, hydrogen peroxide and hydroxyl radicals, respectively. Both

monocyte/macrophages and eosinophils appear to be able to mediate this effector mechanism since cell populations enriched (>90%) for these cells are effective at killing NEJ *F. gigantica* parasites. Most importantly, NEJ parasites of the related species *F. hepatica* are resistant to this *in vitro* effector mechanism which is active against NEJ *F. gigantica.*

The demonstration of an effector mechanism that is active *in vitro* against juvenile *F. gigantica* suggests the possibility that superoxide-mediated killing of migrating parasites by peritoneal cells could be occurring *in vivo* in ITT sheep and that this may be at least one mechanism of resistance expressed by this sheep breed against tropical fasciolosis. Indeed, the experimental data *in vivo* supports this hypothesis. ITT sheep exhibit a rapid induction of eosinophilia, IgG and IgE within 8-14 days of infection with *F. gigantica* (9) and significant killing of the invading parasites in ITT sheep occurs within 2-4 weeks of infection and before significant damage to the liver occurs (38, 39, 44). This lack of damage to the liver observed within 2-4 weeks of infection suggests that many invading parasites are killed in the peritoneal cavity or shortly after reaching the liver. The fact that peritoneal cells (mainly monocyte/macrophages) from naïve ITT sheep can also elicit killing of NEJ *F. gigantica in vitro* suggests that ITT sheep possess a resident population that is competent to act against the invading juvenile parasite. Our results suggest that such resident cells can be effective, provided there is sufficient specific antibody present to promote the attachment of the effector cells to the surface of the parasite, as observed within 8-14 days in infected ITT sheep (9). Interestingly, rats mediate effective immunity to *F. hepatica* and also have resident cells (monocyte/marophages) which can kill parasites *in vitro* in the presence of parasite

specific antibody, albeit the effector mechanism is nitric oxide not superoxide radicals (33, 34, 41, 46, 47). Furthermore, intraperitoneal injection of this parasite-specific antibody on the day of *F. hepatica* challenge in uninfected rats results in parasite killing (10, 13, 34, 36). The requirement for specific antibody in this killing mechanism in ITT sheep may reflect the need to focus a concentrated attack by free radicals at the tegumental surface of the relatively large target such as *F. gigantica* in order to achieve a lethal hit, as observed in the rat/*F hepatica* model (33). Such a rapidly-induced killing mechanism is analogous to that proposed to act against *F. hepatica* in exposed rats where killing occurs in the gut wall or peritoneum within 24-48 of challenge and before significant damage to the liver occurs (34, 45, 46, 47, 48).

A curious and important aspect of our observations is that ITT sheep are fully susceptible to infection with *F. hepatica* (38, 39) and, in parallel, NEJ *F hepatica* are resistant to the superoxide-mediated *in vitro* killing mechanism expressed by peritoneal cells of ITT sheep. Such a correlation suggests that the inability of ITT cells to kill NEJ *F. hepatica in vitro* and the inability of ITT sheep to resist *F. hepatica* infection are related phenomena. The resistance of NEJ *F. hepatica* to superoxide-mediated killing *in vitro* is clearly a property of the parasite since our killing assays with both parasite species were conducted on the same day using the same source of sheep cells, sera and media. This suggests that NEJ *F. hepatica* and *F. gigantica* differ in some fundamental, biochemical parameter which renders *F. hepatica* resistant to superoxide radicals. We have previously reported that NEJ *F. hepatica* are indeed relatively resistant to killing by chemically-generated free radicals, compared with schistosomula of *S. mansoni,* and this correlated with an up to 10 fold higher specific enzyme activity of SOD and GSH-Px in NEJ *F.*

hepatica relative to schistosomula (25, 26, 27, 32). We therefore examined the possibility that NEJ *F. hepatica* and *F. gigantica* differ in their specific enzyme activities of the superoxide defence enzyme SOD or had higher general anti oxidant enzyme defenses such as GST. Using somatic extracts of NEJ from these two species we found that the mean SOD specific activity in two NEJ preparations is 33% higher in *F. hepatica* relative to *F. gigantica*, whereas GST specific activity was comparable. We also assayed specific defence enzyme activities in adult parasites exposed to the ITT sheep immune response. Adult parasite SOD gene expression and specific enzyme activity levels was significantly higher in *F hepatica.* Thus, although our initial results are limited, the data reveal a trend toward higher SOD defence enzyme levels in NEJ and adult *F. hepatica* parasites relative to *F. gigantica* and suggest that *F. hepatica* has the potential to mount a more effective defence against superoxide free radical attack by host immune cells: whether this trend is sufficient to account for the difference in susceptibility to killing by the superoxide-mediated mechanisms expressed by PLCs from ITT sheep is unclear.

Another possibility is that the relative resistance of NEJ *F hepatica* to superoxide-mediated killing *in vitro* results from the active suppression by the parasite of superoxide production by peritoneal cells *in vivo* or the expression of a non-enzymatic mechanism for absorbing superoxide (eg. via a molecule expressed in the tegument). Jefferies and colleagues (16) showed *in vitro* that increasing concentrations of excretory-secretory (ESP) molecules released by adult *F. hepatica* correlated with increasing suppression of superoxide and hydrogen peroxide production from sheep neutrophils. Adult *F. gigantica* ESP was also shown to inhibit reactive oxygen radical production from sheep neutrophils *in vitro* (5). SOD activity and protein has been detected in adult *F. hepatica* ESP (17, 30)

and a cDNA encoding *F. hepatica* SOD is reported (20). Interestingly, in our study PLCs isolated from *F. hepatica*-infected ITT sheep were still able to mediate killing of NEJ *F. gigantica in vitro*, suggesting that, if suppression of superoxide production occurs *in vivo* during *F. hepatica* infection in sheep, it is transient or ineffective under our experimental conditions. We are currently examining the effect of NEJ of the two *Fasciola sp.* on superoxide production by ITT PLCs.

Our results raise broader issues relating to the nature of the host and parasite factors that determine the host specificity of a parasite. If biochemical differences can occur between parasite species such that resistance to a host effector mechanism is expressed, then clearly the host-specificity of a particular parasite is a dynamic interplay between the evolution, and/or level of expression of, a parasite's defences and the evolution of a host's effector armory. NEJ *F. hepatica* are susceptible to antibody-dependent NO-mediated killing by rat monocyte-macrophages and rat monocyte/macrophages make a robust inducible NO response which is associated with resistance to *F. hepatica* (32, 33, 41).. Rats express an even higher resistance to *F. gigantica* infection (15, 22, 44) and we have shown that NEJ *F. gigantica* are highly susceptible to NO killing in the absence of anti-parasite antibodies (unpublished data). In complete contrast, monocyte/ macrophages from sheep, including ITT sheep, do not generate significant levels of inducible NO *in vitro* (2, 18, 19, 33)**:** accordingly, sheep are fully susceptible to *F. hepatica.* Thus, rats and sheep represent two ends of the spectrum with respect to both inducible NO production and susceptibility to *F. hepatica.* From the parasite's perspective, *F. hepatica* appears to express higher levels of certain defence enzymes relative to *F. gigantica.* Interestingly, Miller *et al*. (24) demonstrated variations in isoenzyme expression and activity of GSTs in

adult *F. hepatica* recovered from different hosts. Lower GST activity levels were observed for flukes removed from resistant hosts (cattle and rats) as opposed to susceptible hosts (sheep and mice), confirming that defence enzyme levels can vary depending on the host in which the parasite resides. Such observations show that the outcome of infection by *Fasciola sp*. is determined by both host and parasite factors. It should be noted that *F. hepatica* and *F. gigantica* diverged about 19m years ago which is sufficient time for variation in the level of expression of defence enzymes to evolve in these two parasite species (14).

In conclusion, our results suggest that a mechanism of antibody-dependent cell-mediated cytotoxicity involving superoxide-mediated killing may play a role in the control of *F. gigantica* infection in ITT sheep. This killing appears to be mediated, at least *in vitro*, by monocyte/macrophages and eosinophils and such cells are known to be present, or rapidly induced following infection, in the peritoneal cavity of ITT sheep. Studies are in progress to further define the effector mechanisms involved in determining the resistance of sheep to *F. gigantica* as well as the parasite factors involved in subverting this resistance.

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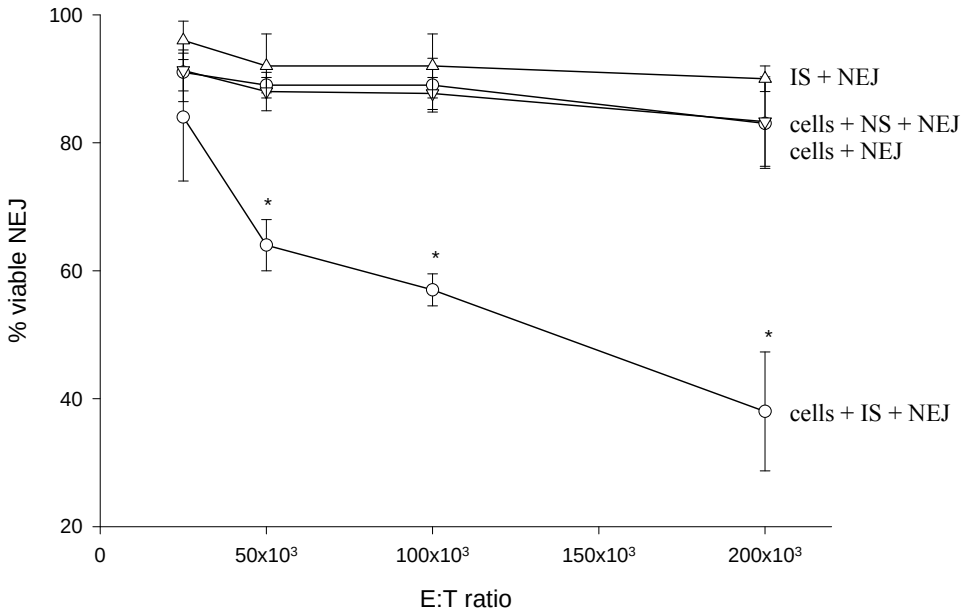
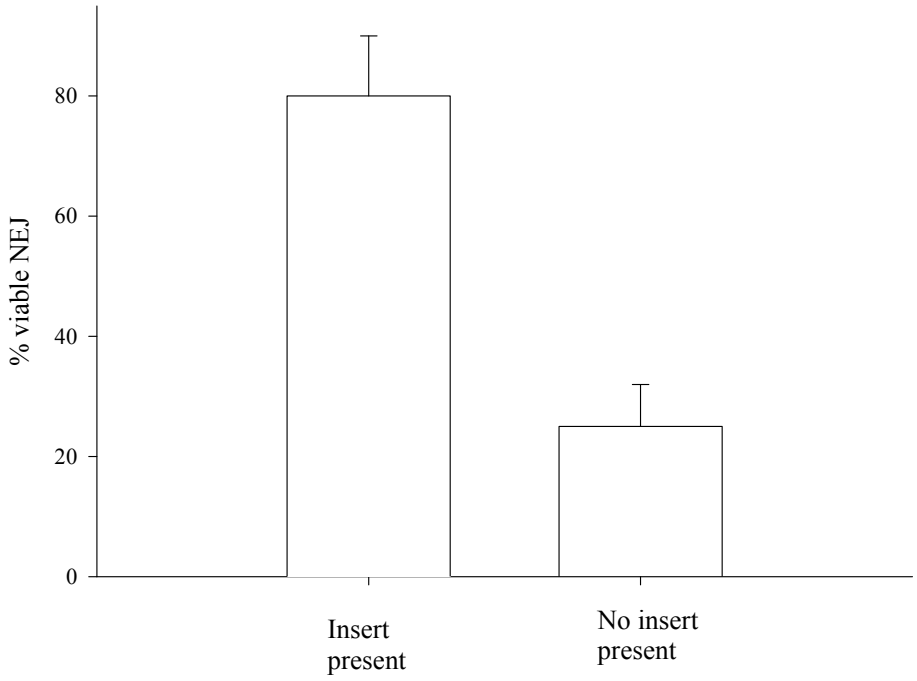
Figure 1. Effect of increasing numbers of peritoneal lavage cells from *F. gigantica*-infected ITT sheep on the killing of newly excysted juvenile (NEJ) *F. gigantic*a.



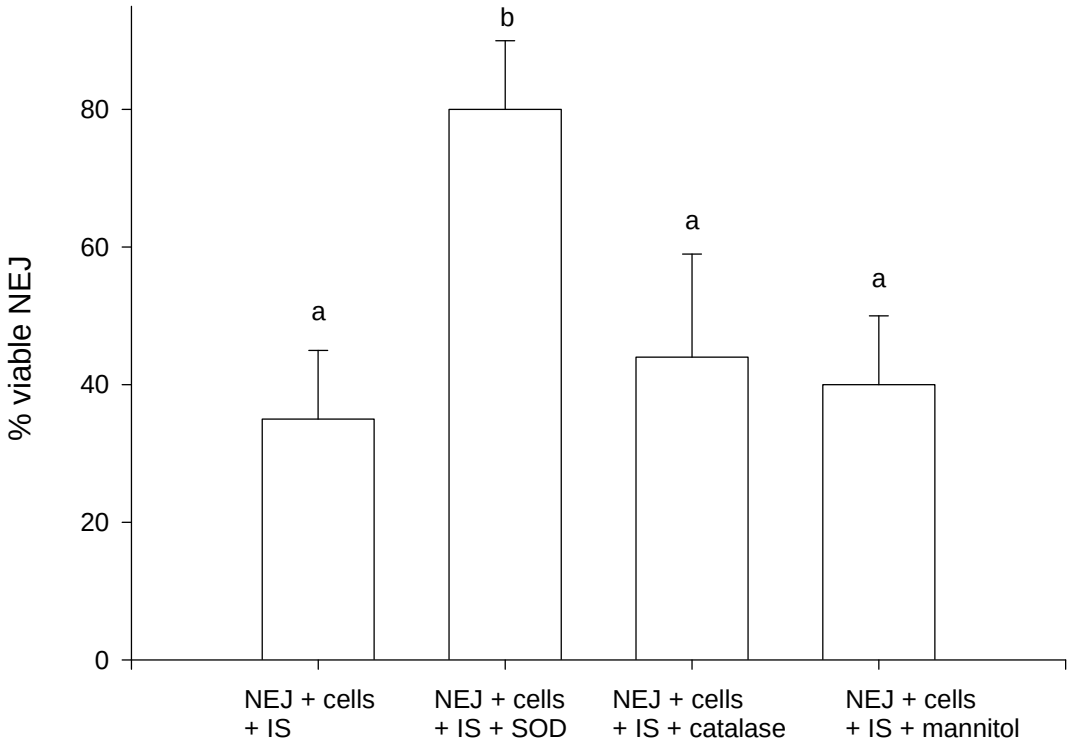
Each of three replicate wells containing 20-30 NEJ parasites and peritoneal lavage cells (E:T ratio of 0.25-2 x 10^5 cells:1 NEJ) in 1 ml of media were incubated for 3 days. The viability of the NEJ parasites was then assessed as an ability to reduce the tetrazolium, MTT . Results are the mean ± SD of three experiments. *Represents mean values that were significantly different by the Dunnett's multiple comparison test at $P < 0.05$, relative to control incubations (cells + NEJ parasites alone). NS: sera from *F. gigantica*-naïve ITT sheep; IS: immune sera from ITT sheep infected for 8 weeks with *F. gigantica.*

Figure 2. Effect of physical separation of newly excysted juvenile (NEJ) *F. gigantica* and peritoneal lavage cells of *F. gigantica*-infected ITT sheep on the subsequent killing of the NEJ parasites.



Each of three replicate wells containing 25-50 NEJ parasites were placed in 24-well tissue culture plates with immune sera from *F. gigantica*-infected ITT sheep. In those incubation wells containing the insert, the peritoneal cells were placed inside the insert at an E:T ratio of $2x10^5$ cells:1 NEJ parasite; the sheep peritoneal cells were thus separated from the NEJ parasites by a 0.45 μm membrane. Following incubation for 3 days, the viability of the NEJ parasites was then assessed as an ability to reduce the tetrazolium, MTT. Results are representative of 3 experiments.

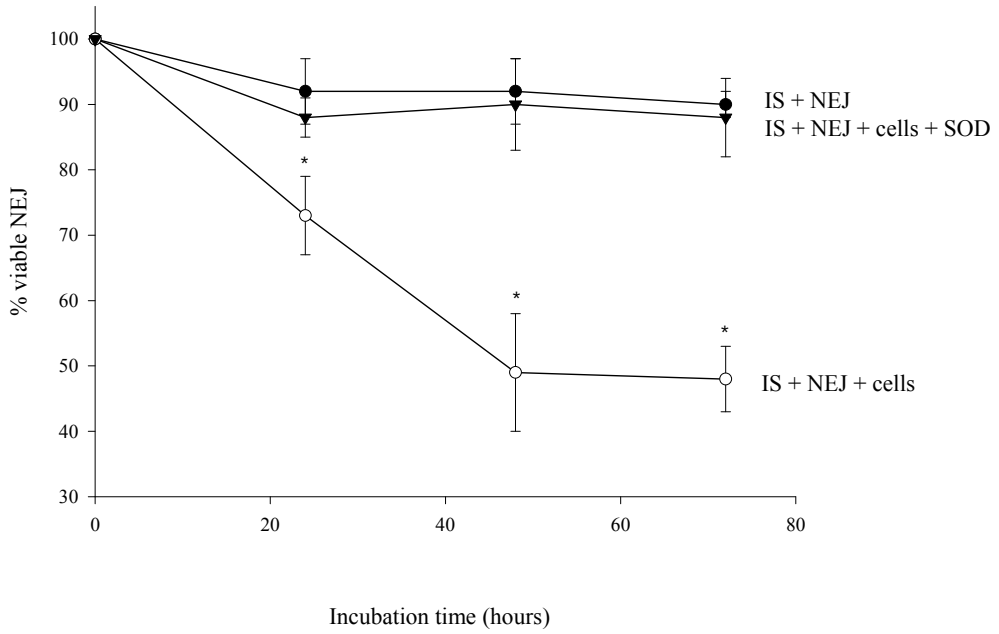
Figure 3(a). Effect of adding exogenous superoxide dismutase (SOD), catalase or mannitol to culture incubations on the viability of newly excysted juvenile (NEJ) *F gigantica* following exposure to immune sera (IS) and peritoneal lavage cells from *F. gigantica*-infected ITT sheep.



Each of three replicate wells containing 20-30 NEJ parasites, cells (E:T ratio of 2 x 10^5 cells:1 NEJ parasite) and immune sera in 1 ml of media were incubated for 3 days with or without exogenous SOD (10 μg/ml), catalase (10 μg/ml) or mannitol (10 μM). The viability of the NEJ parasites was then assessed as an ability to reduce the tetrazolium, MTT. Results are the mean ± SD of three experiments. For each incubation, mean values

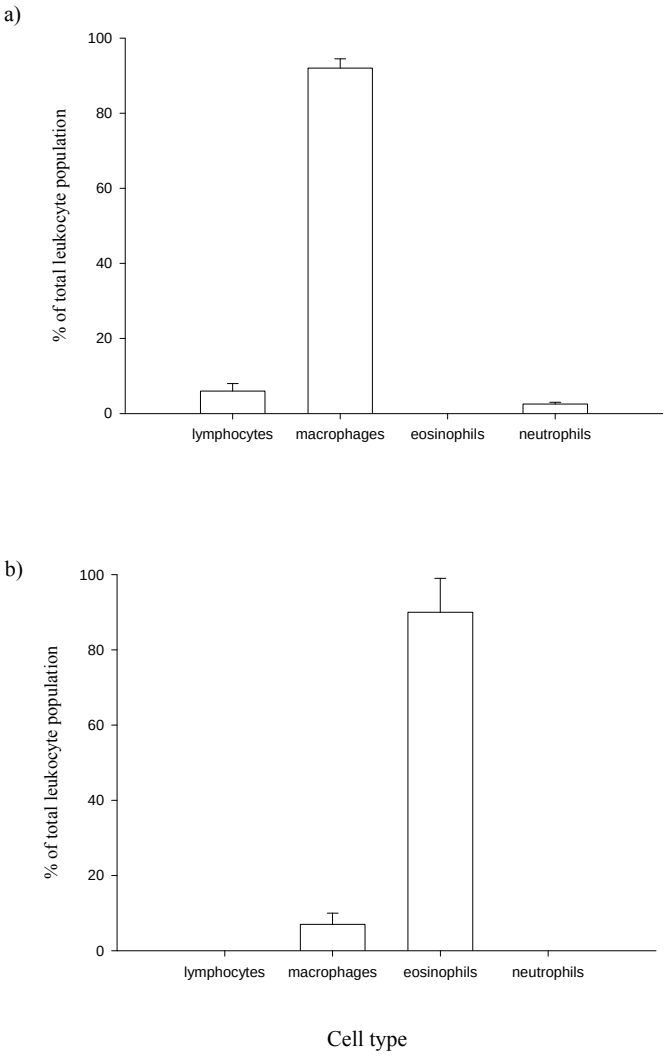
with the same superscript (a or b) could not be significantly differentiated by the Dunnett's multiple comparison test at $P < 0.05$**.** IS: immune sera from ITT sheep infected for 8 weeks with *F. gigantica.*

Figure 3(b). Effect of adding exogenous superoxide dismutase (SOD) to culture incubations on the motility of newly excysted juvenile (NEJ) *F. gigantica* in the presence of immune sera (IS) and peritoneal lavage cells from *F. gigantica*-infected ITT sheep.



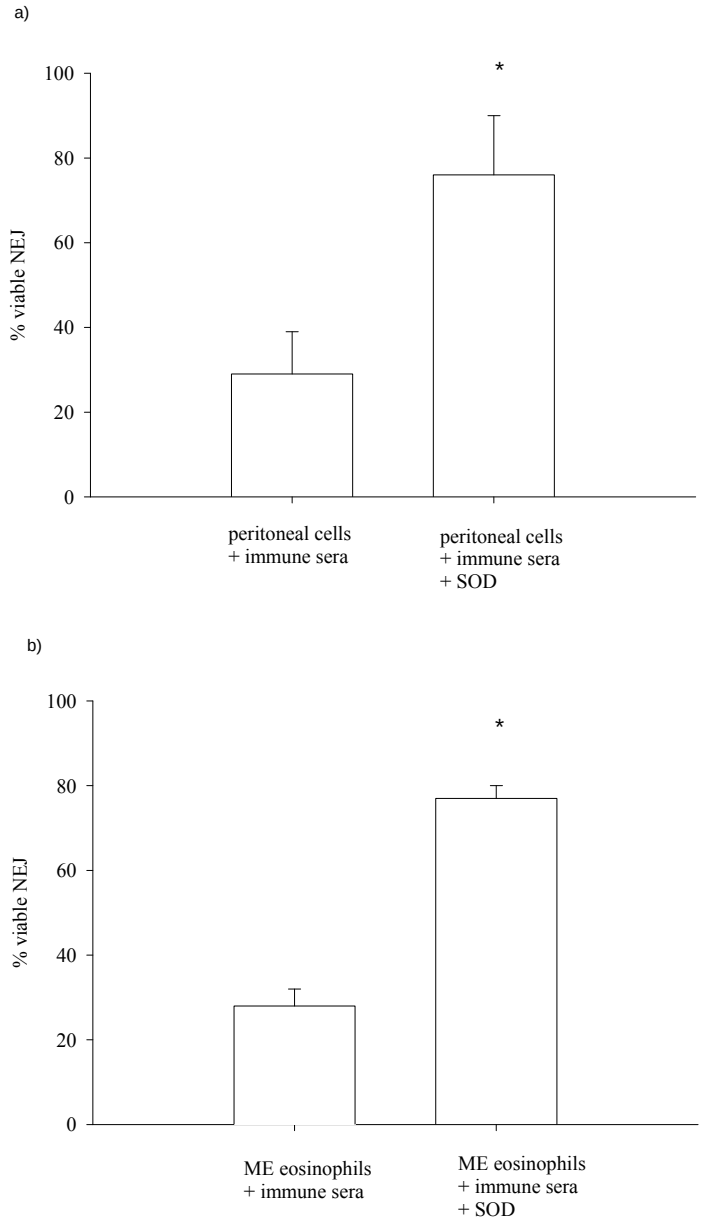
Each of three replicate wells containing 20-30 NEJ liver fluke, cells (E:T ratio of 2 x 10^5 cells:1 NEJ parasite) and immune sera in 1 ml of media were incubated for 3 days with or without exogenous SOD (10 μg/ml). At 24 hours and 48 hours viability was assessed as the the motility and structural integrity of NEJ: at 72 hours, viability was assessed as an ability to reduce the tetrazolium, MTT. Results are the mean ± SD of three experiments. *Represents means that were significantly different by the Dunnett's multiple comparison test at $P < 0.05$, to incubations with cells + NEJ parasites. IS: immune sera from ITT sheep infected for 8 weeks with *F. gigantica.*

Figure 4. Differential cell counts of (a) peritoneal lavage cells from *F. gigantica*-naïve ITT sheep and (b) mammary-elicited lavage cell samples from *F. gigantica*-infected ITT sheep.



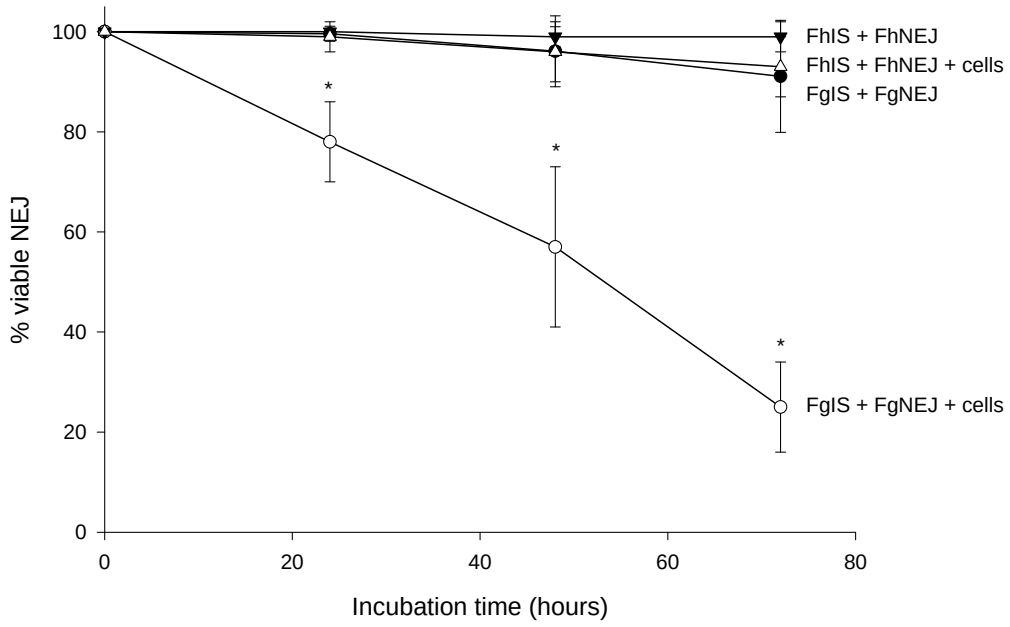
Cytospin preparations were stained using the Diff Quik® system. Each cell type was identified and expressed as a percentage of the total number of leukocytes counted. Values represent the mean ± SD from 3 separate animals

Figure 5. Effect of adding exogenous superoxide dismutase (SOD) to incubations of (a) peritoneal lavage cells of *F. gigantica*-naive ITT sheep and (b) mammary elicited (ME) lavage cells from *F. gigantica*-infected ITT sheep on the subsequent killing of newly excysted juvenile (NEJ) *F. gigantica* in the presence of ITT immune sera.



Each of three replicate wells containing 20-30 NEJ parasites, cells (E:T ratio of 2 x 10^5 cells:1 NEJ parasite) and immune sera in 1 ml of media were incubated for 3 days with or without exogenous SOD (10 μg/ml). The viability of the NEJ parasites was then assessed as an ability to reduce the tetrazolium, MTT. Results are the mean ± SD of three experiments. *Represents means that were significantly different ($P < 0.05$) to incubations with NEJ parasites and lavage cells only (data not shown). Immune sera are from ITT sheep infected for 8 weeks with *F. gigantica.*

Figure. 6. Comparative susceptibility of newly excysted juvenile *F. gigantica* (FgNEJ) and *F. hepatica* (FhNEJ) to killing by peritoneal lavage cells of *F. gigantica*-naive ITT sheep following incubation with homologous *Fasciola*-immune sera (IS).



Each of 10 replicate wells containing 4 NEJ parasites and cells (E:T ratio of 2 x 10^5 cells:1 NEJ parasite) and homologous immune sera in 0.2 ml of media were incubated for 3 days. At 24 hours and 48 hours viability was assessed as the motility and structural integrity of NEJ: at 72 hours, viability was assessed as an ability to reduce the tetrazolium, MTT. Results are the mean ± SD of 5 experiments. *Represents means that were significantly different by the Dunnett's multiple comparison test at $P < 0.05$, to incubations with IS + Fg NEJ parasites alone. FgIS: immune sera from ITT sheep infected for 8 weeks with *F. gigantica*; FhIS: immune sera from ITT sheep infected for 8 weeks with *F. hepatica.*

Table 1 Comparative susceptibility of newly excysted juvenile *F. gigantica* (Fg NEJ) and *F. hepatica* (Fh NEJ) to killing by peritoneal lavage cells of *F. hepatica*-infected (Fh PLCs) or *F. gigantica*-infected (Fg PLCs) ITT sheep following incubation with homologous and heterologous *Fasciola*-immune sera (Fh IS, Fg IS).

Incubation	% viable NEJ
1. Fh PLCs + Fh NEJ + NS	95 ± 4^a
2. Fh PLCs + Fh NEJ + Fh IS	95 ± 4^a
3. Fh PLCs + Fh NEJ + Fg IS	93 ± 4^a
4. Fg PLCs + Fh NEJ + NS	92 ± 4^a
5. Fg PLCs + Fh NEJ + Fh IS	93 ± 4^a
6. Fg PLCs + Fh NEJ + Fg IS	94 ± 4^a
7. Fg PLCs + Fg NEJ + NS	88 ± 8^a
8. Fg PLCs + Fg NEJ + Fh IS	58 ± 15^b
9. Fg PLCs + Fg NEJ + Fg IS	43 ± 17^b
10. Fh PLCs + Fg NEJ + NS	92 ± 3^a
11. Fh PLCs + Fg NEJ + Fh IS	49 ± 13^b
12. Fh PLCs + Fg NEJ + Fg IS	48 ± 4^b

Each of three replicate wells containing 20-30 NEJ *of F. gigantica* or *F. hepatica* and peritobeal lavage cells (E:T ratio of 2 x 10^5 cells:1 NEJ parasite) in 1 ml of media were incubated for 3 days with either sera from *Fasciola*-naïve ITT sheep (NS) or with immune

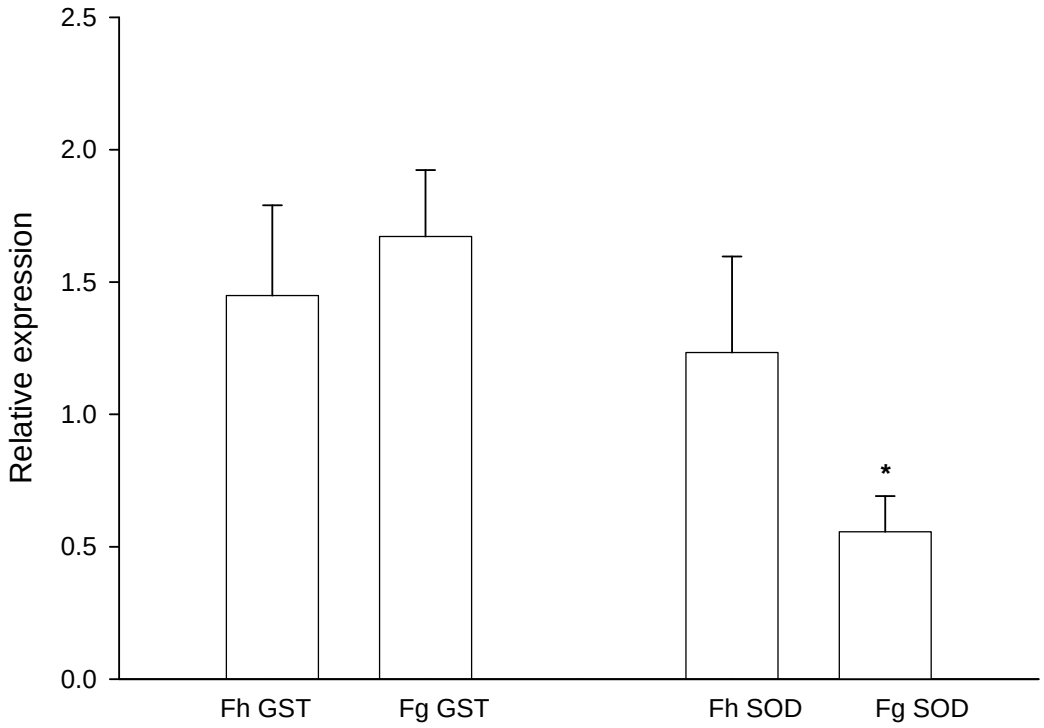
sera from *Fasciola*-infected (Fh IS, Fg IS) ITT sheep. The viability of the NEJ parasites was then assessed at 72 hours as an ability to reduce the tetrazolium, MTT. Results are the mean ± SD using PLCs from five ITT sheep. For each incubation, mean values with the same superscript (a or b) could not be significantly differentiated by the Dunnett's multiple comparison test at $P < 0.05$**.** No significant killing was observed with Fg or Fh NEJ liver fluke incubated with naïve sera (NS) or immune sera (IS) alone (data not shown). PLCs: peritoneal lavage cells; Fg PLCs were obtained from *F. gigantica*-infected ITT sheep; Fh PLCs were obtained from *F. hepatica* infected ITT sheep.

Table 2 Anti-oxidant defence enzyme activities in whole worm extracts of adult and newly excysted juvenile (NEJ) *F. hepatica* and *F. gigantica.*

Enzyme Activity	NEJ parasite[1]		Adult parasite[2]	
	F. gigantica	*F. hepatica*	*F. gigantica*	*F. hepatica*
SOD	33, 32	44, 46	21 ± 5	117 ± 44*
GST	512, 491	486, 503	4214 ± 1084	6508 ± 959

[1]Values represent the mean enzyme activity of 3 determinations from each of 2 separate preparations of 5000 NEJ parasites; [2]Values represent the mean ± SD from separate preparations of 20-50 adult parasites collected from 10 age-matched ITT donor sheep infected with 250 metacercariae: 5 sheep were infected with *F. gigantica* and 5 sheep were infected with *F. hepatica* parasites. Superoxide dismutase (SOD) specific enzyme activity is expressed as U/mg protein. Glutathione *S*-transferase (GST) specific enzyme activity is expressed as nmoles of 1-chloro-2,4-dinitrobenzene conjugated/min/mg protein. Significant differences ($p<0.01$) for anti-oxidant defence enzyme activities between adult *F. hepatica* and *F. gigantica* whole worm extracts (WWE) were calculated using the unpaired alternative t-test.

Figure. 7. Relative expression levels of superoxide dismutase (SOD) and glutathione *S*-transferase (GST) mRNA in adult *F. hepatica* (Fh) and *F. gigantica* (Fg) parasites isolated from *Fasciola*-infected ITT sheep.



Ten ITT sheep were infected with 250 metacercariae of either *F. gigantica* or *F. hepatica* (five animals per group) for twelve weeks and whole adult parasites were recovered from the livers. Parasites were washed with 1 x PBS, incubated for 1 hr at 37 °C and preserved in RNAlater (Qiagen). Parasites from each group were combined and 50 parasites of each *Fasciola* species were homogenised in Qiazol (Qiagen). Relative mRNA expression levels

were determined by RT-PCR. Significant differences ($p<0.01$) for anti-oxidant defence enzyme mRNA levels between adult *F. hepatica* and *F. gigantica* parasites were calculated using the unpaired alternative t-test.