

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*[∇]

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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*.

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 more than 3 billion U.S. dollars per annum (44). Human infection with liver flukes is also recognized by the World Health Organization as an emerging human health problem, with more than 500 million people at risk of infection with *Fasciola*, *Opisthorchis*, or *Clonorchis* (11, 23, 44). There are at least 2.4 million 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 because of the 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 requires 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 of 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 susceptibilities of sheep breeds to *F. gigantica* are compared, the Indonesian thin-tail (ITT) 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 various 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 themselves 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).

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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 immature newly excysted juvenile (NEJ) parasite could be the primary target of the effective immune response expressed in ITT sheep. These observations are analogous to those obtained with 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 with 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 peritoneums of ITT sheep against the juvenile *F. gigantica* parasite. We show that juvenile *F. gigantica* parasites are susceptible to killing in vitro by superoxide radicals produced by macrophages isolated from the peritoneum of ITT sheep and by 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 ITT sheep 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 enzyme-linked immunosorbent assay and a Western blot assay with *F. gigantica* whole worm extract (WWE) as the antigen (6). Throughout the experiments, the sheep were maintained in pens on a diet of freshly cut *Pennisetum purpureum* and dairy concentrate (38, 39). Metacercariae 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 the Elizabeth Macarthur Agricultural Institute, Menangle, New South Wales, Australia (for *F. hepatica*). Sheep were infected with 250 metacercariae of *F. gigantica* or *F. hepatica* by loading the required metacercariae onto filter paper, which was placed inside gelatin capsules (Torpac Inc., Fairfield, NJ) and delivered orally with 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*, gentamicin, mannitol, RPMI, superoxide dismutase (SOD), toluidine blue, trypan blue, and the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma Chemical Co. Amphotericin B was purchased from Life Technologies (Rockville, MD). Enzyme-linked immunosorbent assay plates and 24-well tissue culture plates were purchased from Flow Laboratories Inc. and Greiner Labortechnik, Frickenhausen, Austria, respectively.

Cell populations. Resident monocyte/macrophage-rich cell populations from naive sheep or cell populations from ITT sheep infected for 4 weeks with *F. gigantica* or *F. hepatica* were collected from the peritoneal cavity with sterile phosphate-buffered saline (PBS) containing 6 mM EDTA. The recovered lavage fluid was collected and centrifuged at 1,500 rpm for 10 min, and the cell pellet was resuspended in sterile RPMI containing 10% heat-inactivated fetal calf serum, 2 µg/ml amphotericin, and 10 µg/ml gentamicin.

Eosinophil-enriched cell populations were obtained from the mammary glands of infected ewes with *F. gigantica* parasite extract as previously described (4). Briefly, ITT ewes were infected with 100 metacercariae of *F. gigantica* and 14 to 16 weeks later, eosinophil recruitment into the teat canal was achieved with *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 canals 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% [wt/vol] in PBS) was then added, and the total number of viable white blood cells was determined with a Neubauer hemocytometer. For differential cell counts, Cytospin preparations were made by centrifuging samples of lavage cells at 400 rpm for 5 min at 4°C in a Beckman TJ-6 bench-top centrifuge prior to differential staining (Diff-Quik). Two hundred to 300 cells were identified microscopically, and the relative percentages of lymphocytes, monocytes/macrophages, eosinophils, neutrophils, basophils, and mast cells were determined.

Sheep serum. Sheep were infected with 250 metacercariae of *F. hepatica* or *F. gigantica*, and blood was collected 8 weeks later by jugular venipuncture, with EDTA Vacutainer tubes. The blood was allowed to clot at room temperature for 1 h and centrifuged in a Beckman CS-6R centrifuge at 3,000 × *g* for 20 min. The serum was then removed, and complement activity was inactivated by heating at 56°C for 30 min prior to storage at -20°C.

Incubation of juvenile liver flukes with lavage cells. Metacercariae were excysted, and NEJ flukes were separated from empty cysts and debris by incubation overnight at 37°C as previously described (50). Cytotoxicity assays were carried out with 24- or 96-well tissue culture plates with up to 50 NEJ liver flukes per well. Because of the lower yield of peritoneal lavage cells (PLCs) from uninfected sheep, the incubation volume was adjusted to 0.2 ml with this cell source. Plates were incubated for 3 days at 37°C in 5% CO₂ in 0.2 ml (cells from uninfected sheep) or 1 ml (cells from *Fasciola*-infected sheep) of RPMI medium containing 10% heat-inactivated fetal calf serum, 2 µg/ml amphotericin B, and 10 µg/ml gentamicin, with or without the addition of 10% sheep serum, 10 µg/ml SOD, 10 µg/ml catalase, 10 µM mannitol, or combinations of these reagents and lavage cells at an effector-to-target (E/T) ratio of 0.25 × 10⁵ to 2 × 10⁵ cells per NEJ liver fluke. NEJ liver flukes were defined as viable when they were determined microscopically as motile and having a defined intestinal cecum 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 completion of the incubation period, NEJ liver flukes were incubated for 4 h in a solution of 2 mg/ml MTT and viability was assessed as the ability to reduce the tetrazolium salt MTT as previously described (32). Comparative incubations with *F. hepatica* and *F. gigantica* parasites were performed on the same day with the same communal reagents in the same culture plates.

Assay of enzyme protein activity. Approximately 5,000 NEJ liver flukes or 20 to 50 adult parasites were manually homogenized in 100 mM Tris-HCl (pH 7.4) in an ice-cooled ground glass homogenizer for 5 min, and the homogenate was centrifuged at 4°C and 1,000 × *g*. The specific activities of SOD and glutathione *S*-transferase (GST) were measured in triplicate in the resultant supernatant. All reactions were carried out at 25°C in a Shimadzu UV-160 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) in a total reaction mixture of 1 ml, and the change in absorbance was monitored continuously for 2 min. The specific activity of SOD was determined by the cytochrome *c* reduction method with bovine erythrocyte SOD as the 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%. SOD specific enzyme activity is expressed as units per milligram of protein. The GST assay measured the conversion of glutathione from the oxidized 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 milligram of protein. Protein concentration was determined with the Bio-Rad DC colorimetric assay for protein concentration following detergent solubilization.

Assay of enzyme mRNA expression. RNA was extracted in duplicate with the Lipid RNeasy Mini Kit (QIAGEN) according to the manufacturer's guidelines. Total RNA (2 µg) was reverse transcribed with an Omniscript reverse transcription kit (QIAGEN) according to the manufacturer's guidelines in a total volume of 20 µl. The final reaction mixture was incubated at 37°C for 70 min and stored at -20°C. Oligonucleotides used to amplify *Fasciola* β-actin, GST, and SOD were designed from sequences obtained from the GenBank database with the text search program and the Primer3 software program (BioNavigator; www.ingen.com) under default parameters. Products from each primer set were sequence verified to ensure correct amplification. Real-time PCR conditions for each primer set were optimized with pooled cDNA. Efficiencies were calculated from the average of three standard curves (coefficient of determination, >0.9) generated from separate experiments and serially diluted cDNA (standard deviation, <5%). The primers for β-actin were sense primer 5'ATCACTGCCAC CCAGAAGACT and antisense primer 5'CATGCCAGTGAGCTTCCCGTT, the primers for GST were sense primer 5'AGAAATGGTTGGCGATAAA and antisense primer 5'AACACGAACAAAACCCATCC, and the primers for SOD were sense primer 5'GCGGGACCTATTCAACCC and antisense

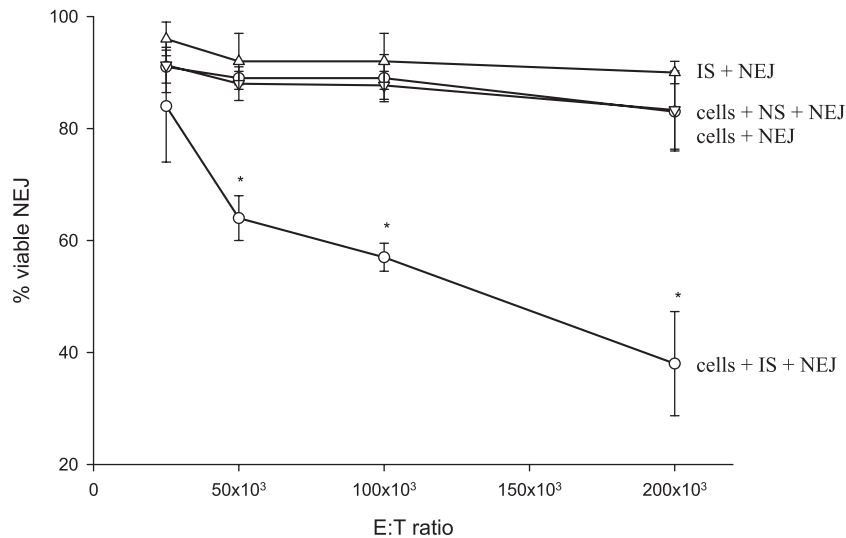


FIG. 1. Effect of increasing numbers of PLCs from *F. gigantica*-infected ITT sheep on the killing of NEJ *F. gigantica*. Each of three replicate wells containing 20 to 30 NEJ parasites and PLCs (E:T ratio of 0.25×10^5 to 2×10^5 cells to 1 NEJ parasite) in 1 ml of medium was incubated for 3 days. The viability of the NEJ parasites was then assessed as the ability to reduce the tetrazolium salt MTT. Results are the mean \pm the standard deviation of three experiments. Asterisks indicate mean values that are significantly different by the Dunnett's multiple comparison test at $P < 0.05$, relative to control incubations (cells plus NEJ parasites alone). NS, serum from *F. gigantica*-naive ITT sheep; IS, immune serum from ITT sheep infected for 8 weeks with *F. gigantica*.

primer 5' CACAAGCCAAACGCTCCAG. The primer sets used here were 100% identical between the *F. gigantica* and *F. hepatica* cDNA sequences for each target product and ensured that the binding sites of the primers used in the real-time PCRs 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 mRNAs was done with an MX3000P real-time PCR machine (Stratagene) with the SYBR green (with dissociation curve) experiment type. Each 20- μ l reaction mixture included 10 μ l of $2 \times$ SYBR green master mix (QIAGEN) which incorporated the internal Rox Dye control, 0 to 1 μ l of 50 mM MgCl₂ (Invitrogen), 0.3 μ M each primer, and 4 μ l of cDNA (diluted 1:10). Real-time conditions began with a 15-min denaturation step at 95°C, followed by 40 cycles of 94°C for 15 s, the primer-specific temperature for 30 s, and 72°C for 30 s. A melting curve analysis (55 to 95°C at a heating rate of 0.01°C/s) was performed to ensure that 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 analyzed with the MX3000P software. SOD and GST expression was normalized for each cDNA preparation with the respective β -actin housekeeping gene value, and final values represent the expression relative to the calibrator. Averages represent the results of two RNA extractions and six real-time PCR experiments for each sample.

Statistical analysis. Significant differences between treatment groups were determined by the nonparametric Dunnett multiple-comparison test. Significant differences for specific antioxidant defense enzyme activities between adult *F. hepatica* and *F. gigantica* WWEs were calculated with 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 with pairwise fixed reallocation randomization tests.

RESULTS

Killing of NEJ *F. gigantica* parasites in vitro by PLCs and immune serum from ITT sheep. PLCs collected from ITT sheep infected for 4 weeks with *F. gigantica* consisted of 40 to 50% monocytes/macrophages, 40 to 50% eosinophils, 2 to 20% lymphocytes, and less than 5% neutrophils. When NEJ *F. gigantica* was incubated with these cells and serum 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* parasites were characterized by extensive cell attachment, immotility, loss of a defined intestinal cecum, loss of parasite shape, 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 the presence of both immune serum and PLCs. When NEJ *F. gigantica* parasites were incubated in the absence of serum, or with serum from *F. gigantica*-naive ITT sheep, a mean of 85% of the parasites were viable (Fig. 1). The viability of NEJ liver flukes was also not affected by incubation with serum from *F. gigantica*-infected ITT sheep in the absence of PLCs (90% viable NEJ liver flukes; Fig. 1).

Killing of NEJ *F. gigantica* parasites in vitro by PLCs requires direct cell contact. The mechanism of cytotoxicity to NEJ liver flukes by ITT sheep PLCs observed above required the inclusion of serum 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* parasites were incubated with serum from *F. gigantica*-infected ITT sheep in wells with tissue culture inserts in which the PLCs were separated from the NEJ liver flukes by a 0.45- μ m-pore-size filter to inhibit direct contact between NEJ liver flukes and PLCs. Separation of NEJ liver flukes from PLCs in the presence of serum from *F. gigantica*-infected ITT sheep resulted in a mean parasite viability of 80%, 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

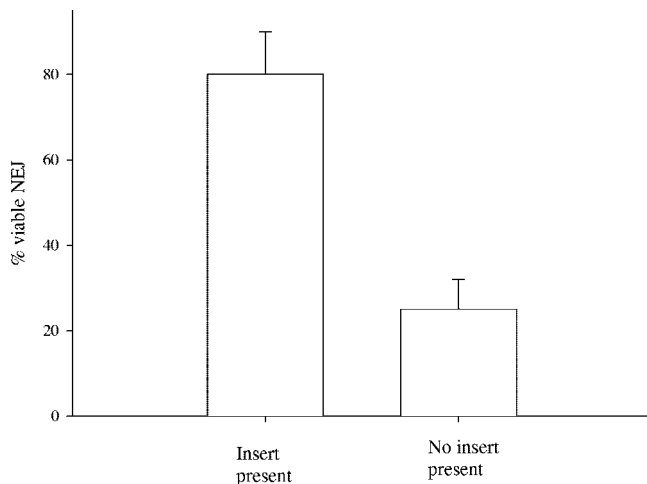


FIG. 2. Effect of physical separation of NEJ *F. gigantica* and PLCs of *F. gigantica*-infected ITT sheep on the subsequent killing of the NEJ parasites. Each of three replicate wells containing 25 to 50 NEJ parasites was placed in 24-well tissue culture plates with immune serum from *F. gigantica*-infected ITT sheep. In those incubation wells containing the insert, the peritoneal cells were placed inside the insert at an E/T ratio of 2×10^5 cells to one NEJ parasite; the sheep peritoneal cells were thus separated from the NEJ parasites by a 0.45- μ m-pore-size membrane. Following incubation for 3 days, the viability of the NEJ parasites was assessed as the ability to reduce the tetrazolium salt MTT. Results are representative of three experiments.

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 *N*^G-monomethyl-L-arginine 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 superoxide radical inhibitor SOD significantly reversed the killing of NEJ *F. gigantica* parasites from a mean of 35% viable parasites in the absence of SOD to a mean of 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 serum 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 serum, as indicated by a reduction in mean parasite motility within 24 h compared to incubations with the addition of SOD (Fig. 3b).

Identification of effector cells in ITT PLCs mediating cytotoxicity in vitro. The in vitro cytotoxicity assays used PLCs that consisted of two major immune cell types, monocytes/macro-

phages 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*-naive ITT sheep contained greater than 90% monocytes/macrophages with no eosinophils present. Cell populations collected from ITT mammary glands infused with a soluble somatic *F. gigantica* lysate contained a mean of 90% eosinophils with about 8 to 10% monocytes/macrophages (representing an E/T ratio of 2×10^4 monocytes/macrophages to one NEJ parasite [Fig. 4b]). As shown in Fig. 1, this level of monocytes/macrophages would only reduce NEJ viability by about 15%. When NEJ *F. gigantica* parasites were incubated with either of these enriched cell populations and serum from *F. gigantica*-infected ITT sheep, a significant portion of the parasites was killed, with the mean viability reduced to 30% in each case (Fig. 4); 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 to NEJ *F. gigantica* mediated by the monocyte/macrophage-rich or eosinophil-rich population was also abrogated by the addition of SOD to inhibit superoxide radical formation (Fig. 4).

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* parasites 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 susceptibilities of *F. hepatica* and *F. gigantica* to killing by ITT effector mechanisms, we directly compared the killing of NEJ *F. hepatica* and that of NEJ *F. gigantica* in vitro by incubating PLCs isolated from the same *F. gigantica*-naive ITT sheep with each parasite in the presence of homologous immune serum. These incubations were carried out on the same day with cells from the same donor sheep. Only NEJ *F. gigantica* parasites were susceptible to killing by PLCs of *F. gigantica*-naive ITT sheep (mean, 25% viability); no cytotoxic effect against NEJ *F. hepatica* was observed (Fig. 5). 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* is influenced by the source of immune serum or cells, i.e., whether homologous or heterologous immune serum and PLCs from exposed sheep mediate in vitro cytotoxicity against NEJ *F. hepatica*. Accordingly, NEJ *F. hepatica* or *F. gigantica* were incubated with homologous or heterologous immune serum or PLCs from *F. gigantica*-infected or *F. hepatica*-infected animals, respectively. As shown in Table 1, incubations of NEJ *F. gigantica* with homologous or heterologous serum or with PLCs from *F. gigantica*-infected animals or *F. hepatica*-infected animals also resulted in killing of NEJ *F. gigantica* (mean, 43 to 58% viable parasites). In contrast, the viability of NEJ *F. hepatica* was unaffected by incubation with either homologous or heterologous serum or PLCs (Table 1).

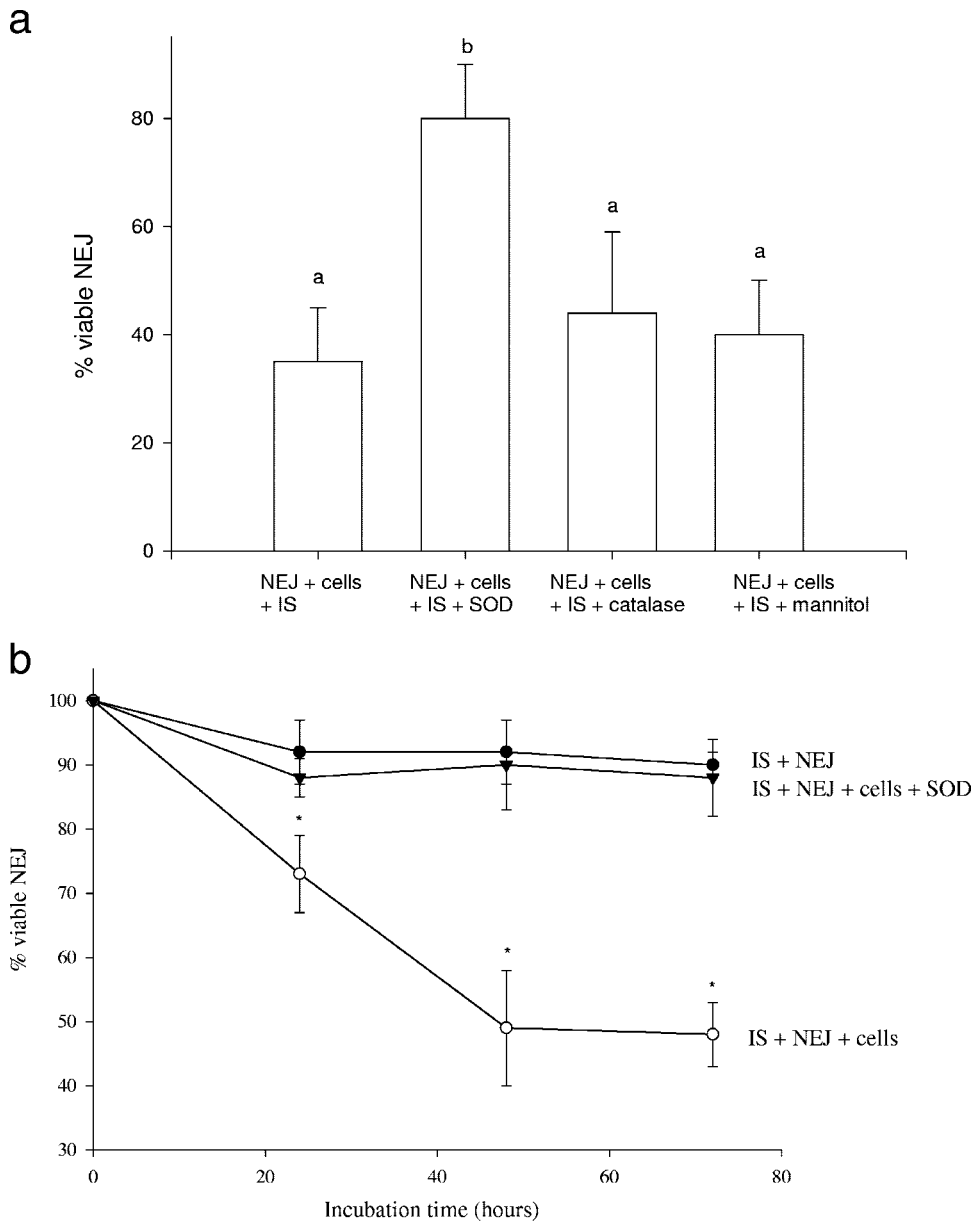


FIG. 3. (a) Effect of adding exogenous SOD, catalase, or mannitol to culture incubations on the viability of NEJ *F. gigantica* following exposure to immune serum and PLCs from *F. gigantica*-infected ITT sheep. Each of three replicate wells containing 20 to 30 NEJ parasites, cells (E/T ratio of 2×10^5 cells to 1 NEJ parasite), and immune serum in 1 ml of medium was incubated for 3 days with or without exogenous SOD (10 $\mu\text{g/ml}$), catalase (10 $\mu\text{g/ml}$), or mannitol (10 μM). The viability of the NEJ parasites was then assessed as the ability to reduce the tetrazolium salt MTT. Results are the means \pm standard deviations of three experiments. For each incubation, mean values with the same superscript (a or b) could not be significantly differentiated by the Dunnett multiple-comparison test at $P < 0.05$. IS, immune serum from ITT sheep infected for 8 weeks with *F. gigantica*. (b) Effect of adding exogenous SOD to culture incubations on the motility of NEJ *F. gigantica* in the presence of immune serum and PLCs from *F. gigantica*-infected ITT sheep. Each of three replicate wells containing 20 to 30 NEJ liver flukes, cells (E/T ratio of 2×10^5 cells to 1 NEJ parasite), and immune serum in 1 ml of medium was incubated for 3 days with or without exogenous SOD (10 $\mu\text{g/ml}$). At 24 h and 48 h, viability was assessed as the motility and structural integrity of NEJ; at 72 h, viability was assessed as the ability to reduce the tetrazolium salt MTT. Results are the means \pm standard deviations of three experiments. Asterisks indicate means that were significantly different by the Dunnett multiple-comparison test at $P < 0.05$ from incubations with cells plus NEJ parasites.

These incubations were performed on the same day with communal sources of cells and serum.

Antioxidant defense enzyme mRNA levels and protein activities 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 defense enzyme SOD in the two parasite species. We also wanted to determine whether any putative differences in defense enzymes were a general trend between the parasites by measuring levels of the antioxidant enzyme GST, a general defense enzyme against most tissue damage

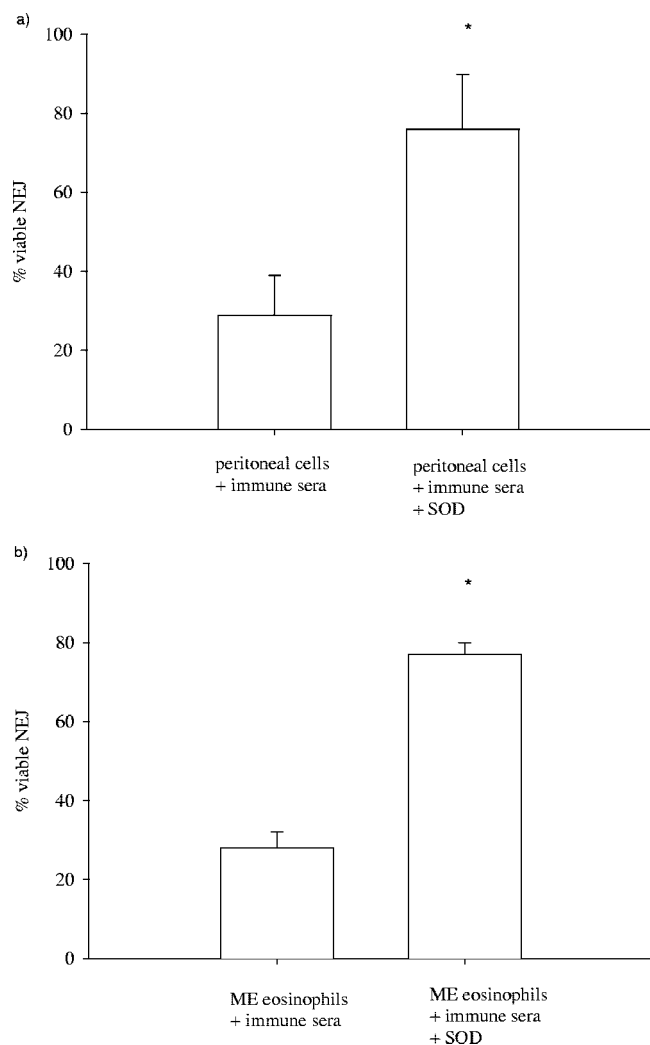


FIG. 4. Effect of adding exogenous SOD to incubations of (a) PLCs of *F. gigantica*-naive ITT sheep and (b) mammary elicited (ME) lavage cells from *F. gigantica*-infected ITT sheep on the subsequent killing of NEJ *F. gigantica* in the presence of ITT immune serum. Each of three replicate wells containing 20 to 30 NEJ parasites, cells (E/T ratio of 2×10^5 cells to 1 NEJ parasite), and immune serum in 1 ml of medium were incubated for 3 days with or without exogenous SOD (10 μ g/ml). The viability of the NEJ parasites was then assessed as the ability to reduce the tetrazolium salt MTT. Results are the means \pm standard deviations of three experiments. Asterisks indicate means that were significantly different ($P < 0.05$) from incubations with NEJ parasites and lavage cells only (data not shown). The immune serum used was from ITT sheep infected for 8 weeks with *F. gigantica*.

arising from free radicals. We compared the specific activities of the SOD and GST defense enzymes in somatic WWEs of NEJ and adult parasites of *F. hepatica* and *F. gigantica*; the relative gene expression levels in adult liver fluke mRNA were determined by reverse transcription-PCR. To ensure that meaningful comparisons could be obtained, WWEs of *F. hepatica* and *F. gigantica* were prepared from 5,000 NEJ parasites excysted on the same day or adult flukes collected on the same day, and the assays for activity were performed immediately under identical conditions. Because of the limited amount of

sample, only specific enzyme activities of GST and SOD were measured in NEJ liver fluke WWEs.

GST and SOD specific enzyme activities were detected in WWEs from two separate batches of NEJ of each liver fluke species (Table 2). The mean GST specific activities in two preparations of NEJ were 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* parasites (Table 2; Fig. 6). Adult WWE of *F. hepatica* had significantly higher specific enzyme activity ($P < 0.001$) of SOD (fivefold) compared to adult WWE of *F. gigantica*, whereas GST specific enzyme activities were similar in WWEs of both *Fasciola* spp. (Table 2). These findings were validated by significantly higher SOD-encoding gene expression levels in *F. hepatica* relative to *F. gigantica* adult parasites, while the GST-encoding gene expression levels were equivalent in the two parasite species (Fig. 6).

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 PLCs 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 serum, strongly suggesting that killing requires direct attachment of cells to the parasite's surface; this is similar to results obtained with 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 N^G -monomethyl-L-arginine, catalase, or mannitol, known inhibitors of nitric oxide production, hydrogen peroxide, and hydroxyl radicals, respectively. Both monocytes/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 obtained in vivo support this hypothesis. ITT sheep exhibit a rapid induction of eosinophilia and immunoglobulins G and E within 8 to 14 days of infection with *F. gigantica* (9), and significant killing of the invading parasites in ITT sheep occurs within 2 to 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 to 4 weeks of infection suggests that many invading parasites are killed in the perito-

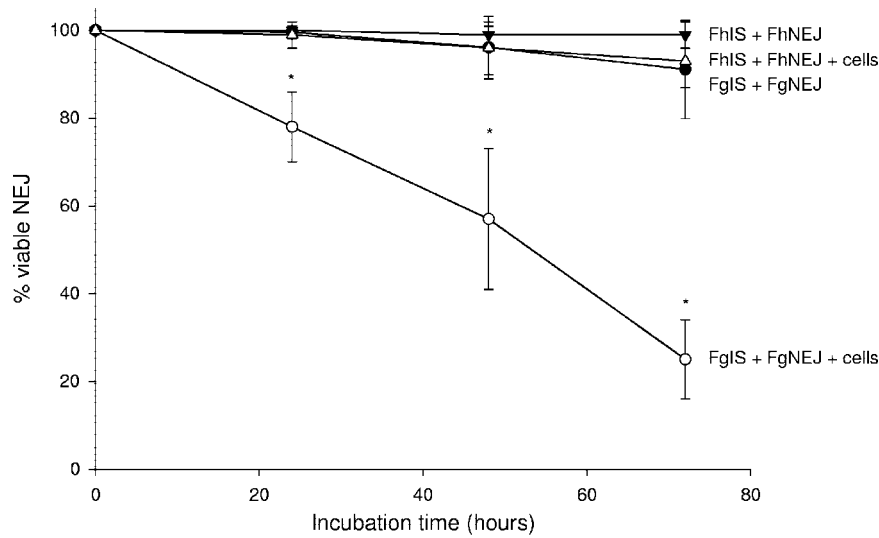


FIG. 5. Comparative susceptibility of NEJ *F. gigantica* (FgNEJ) and *F. hepatica* (FhNEJ) to killing by PLCs of *F. gigantica*-naive ITT sheep following incubation with homologous *Fasciola*-immune serum (IS). Each of 10 replicate wells containing four NEJ parasites and cells (E/T ratio of 2×10^5 cells to one NEJ parasite) and homologous immune serum in 0.2 ml of medium was incubated for 3 days. At 24 h and 48 h, viability was assessed as the motility and structural integrity of NEJ; at 72 h, viability was assessed as the ability to reduce the tetrazolium salt MTT. Results are the means \pm standard deviations of five experiments. Asterisks indicate means that were significantly different by the Dunnett multiple-comparison test at $P < 0.05$ from incubations with immune serum plus FgNEJ parasites alone. FgIS, immune serum from ITT sheep infected for 8 weeks with *F. gigantica*; FhIS, immune serum from ITT sheep infected for 8 weeks with *F. hepatica*.

neal cavity or shortly after reaching the liver. The fact that peritoneal cells (mainly monocytes/macrophages) from naive 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, pro-

vided there is sufficient specific antibody present to promote the attachment of the effector cells to the surface of the parasite, as observed within 8 to 14 days in infected ITT sheep (9). Interestingly, rats mediate effective immunity to *F. hepatica* and also have resident cells (monocytes/macrophages) which can kill parasites in vitro in the presence of a parasite-specific antibody, albeit the effector mechanism is nitric oxide and not superoxide radicals (33, 34, 41, 46, 47). Furthermore, intraperitoneal injection of this parasite-specific antibody into uninfected rats on the day of *F. hepatica* challenge results in parasite killing (10, 13, 34, 36). The requirement for a specific antibody in this killing mechanism in ITT sheep may reflect the need to focus a concentrated attack by free radicals at the

TABLE 1. Comparative susceptibilities of NEJ *F. gigantica* and *F. hepatica* to killing by PLCs of *F. hepatica*-infected or *F. gigantica*-infected ITT sheep following incubation with homologous or heterologous *Fasciola*-immune serum^a

Incubation	% Viable NEJ parasites
Fh PLCs + Fh NEJ + NS.....	95 \pm 4†
Fh PLCs + Fh NEJ + Fh IS.....	95 \pm 4†
Fh PLCs + Fh NEJ + Fg IS.....	93 \pm 4†
Fg PLCs + Fh NEJ + NS.....	92 \pm 4†
Fg PLCs + Fh NEJ + Fh IS.....	93 \pm 4†
Fg PLCs + Fh NEJ + Fg IS.....	94 \pm 4†
Fg PLCs + Fg NEJ + NS.....	88 \pm 8†
Fg PLCs + Fg NEJ + Fh IS.....	58 \pm 15‡
Fg PLCs + Fg NEJ + Fg IS.....	43 \pm 17‡
Fh PLCs + Fg NEJ + NS.....	92 \pm 3†
Fh PLCs + Fg NEJ + Fh IS.....	49 \pm 13‡
Fh PLCs + Fg NEJ + Fg IS.....	48 \pm 4‡

^a Each of three replicate wells containing 20 to 30 NEJ *F. gigantica* (Fg) or *F. hepatica* (Fh) parasites and PLCs (E/T ratio of 2×10^5 cells to 1 NEJ parasite) in 1 ml of medium was incubated for 3 days with either serum from *Fasciola*-naive ITT sheep (NS) or immune serum (IS) from *Fasciola*-infected ITT sheep. The viability of the NEJ parasites was then assessed at 72 h as the ability to reduce the tetrazolium salt MTT. Results are the mean \pm standard deviation obtained with PLCs from five ITT sheep. For each incubation, mean values followed by the same symbol († or ‡) could not be significantly differentiated by the Dunnett multiple-comparison test at $P < 0.05$. No significant killing was observed with *F. gigantica* or *F. hepatica* NEJ liver flukes incubated with naive-sheep serum or immune serum alone (data not shown). *F. gigantica* PLCs were obtained from *F. gigantica*-infected ITT sheep; *F. hepatica* PLCs were obtained from *F. hepatica*-infected ITT sheep.

TABLE 2. Antioxidant defense enzyme activities in WWEs of adult and NEJ *F. hepatica* and *F. gigantica*

Enzyme	Sp act ^a			
	NEJ parasites ^b of:		Adult parasites ^c of:	
	<i>F. gigantica</i>	<i>F. hepatica</i>	<i>F. gigantica</i>	<i>F. hepatica</i>
SOD	33, 32	44, 46	21 \pm 5	117 \pm 44 ^d
GST	512, 491	486, 503	4,214 \pm 1,084	6,508 \pm 959

^a Five sheep were infected with *F. gigantica* parasites, and five sheep were infected with *F. hepatica* parasites. SOD specific enzyme activity is expressed as units per milligram of protein. GST specific enzyme activity is expressed as nanomoles of 1-chloro-2,4-dinitrobenzene conjugated per minute per milligram of protein. Significant differences ($P < 0.01$) for antioxidant defense enzyme activities between adult *F. hepatica* and *F. gigantica* WWEs were calculated with the unpaired alternative *t* test.

^b Values represent the mean enzyme activities of three determinations from two separate preparations of 5,000 NEJ parasites.

^c Values represent the mean \pm standard deviation from separate preparations of 20 to 50 adult parasites collected from 10 age-matched ITT donor sheep infected with 250 metacercariae.

^d Statistically significantly different.

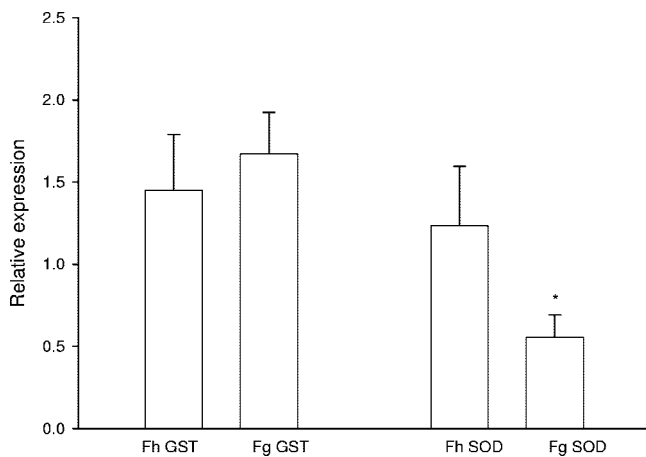


FIG. 6. Relative expression levels of SOD and GST mRNAs in adult *F. hepatica* (Fh) and *F. gigantica* (Fg) parasites isolated from *Fasciola*-infected ITT sheep. Ten ITT sheep were infected with 250 metacercariae of either *F. gigantica* or *F. hepatica* (five animals per group) for 12 weeks, and whole adult parasites were recovered from their livers. Parasites were washed with 1× PBS, incubated for 1 h at 37°C, and preserved in RNAlater (QIAGEN). Parasites from each group were combined, and 50 parasites of each *Fasciola* species were homogenized in Qiazol (QIAGEN). Relative mRNA expression levels were determined by real-time PCR. Significant differences ($P < 0.01$) in antioxidant defense enzyme mRNA levels between adult *F. hepatica* and *F. gigantica* parasites were calculated with the unpaired alternative t test.

tegumental surface of a 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 mechanism of rapidly induced killing is analogous to that proposed to act against *F. hepatica* in exposed rats, where killing occurs in the gut wall or peritoneum within 24 to 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* parasites 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 with the same sheep cell source, serum, and medium. 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* parasites are indeed relatively resistant to killing by chemically generated free radicals, compared with schistosomula of *S. mansoni* parasites, and this correlated with an up-to-10-fold higher specific enzyme activity of SOD and glutathione peroxidase 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 activities of the superoxide defense enzyme SOD or have higher general antioxidant enzyme defenses such as GST. With somatic extracts

of NEJ from these two species, we found that the mean SOD specific activity in two NEJ preparations was 33% higher in *F. hepatica* relative to *F. gigantica*, whereas the GST specific activities were comparable. We also assayed specific defense enzyme activities in adult parasites exposed to the ITT sheep immune response. Adult parasite SOD gene expression and specific enzyme activity levels were significantly higher in *F. hepatica*. Thus, although our initial results are limited, the data reveal a trend toward higher SOD defense 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 defense against a 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 expression of a nonenzymatic mechanism for absorbing superoxide (e.g., via a molecule expressed in the tegument). Jefferies and colleagues (16) showed in vitro that increasing concentrations of excretory-secretory product (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 have been detected in adult *F. hepatica* ESP (17, 30), and a cDNA encoding *F. hepatica* SOD has been 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 flukes of the two *Fasciola* spp. 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 defenses and the evolution of a host's effector armory. NEJ *F. hepatica* parasites are susceptible to antibody-dependent NO-mediated killing by rat monocytes/macrophages, and rat monocytes/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* parasites are highly susceptible to NO killing in the absence of antiparasite antibodies (unpublished data). In complete contrast, monocytes/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 defense enzymes relative to *F. gigantica*. In-

terestingly, Miller et al. (24) demonstrated variations in isoenzyme expression and activity of GSTs in adult *F. hepatica* parasites recovered from different hosts. Lower GST activity levels were observed in flukes removed from resistant hosts (cattle and rats) as opposed to susceptible hosts (sheep and mice), confirming that defense 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 19 million years ago, which is sufficient time for variation in the level of expression of defense 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 monocytes/macrophages and eosinophils, and such cells are known to be present, or rapidly induced following infection, in the peritoneal cavities 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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