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Antibody-dependent cell-mediated cytotoxicity to newly excysted juvenile *Fasciola hepatica in vitro* is mediated by reactive nitrogen intermediates

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SUMMARY

Passive intraperitoneal transfer of sera from Fasciola hepatica*-infected sheep, cattle or rats can protect naive rats from* F. hepatica *infection, suggesting a parasite killing mechanism within the peritoneal cavity that is dependent on the presence of parasite-specific antibody. We investigated antibody-dependent cell-mediated cytotoxicity by resident peritoneal lavage cell populations, containing large numbers of monocytes/macrophages, as a potential host resistance mechanism by which juvenile flukes could be killed within the peritoneal cavity of naive rats. Comparative studies were conducted using cell populations containing large numbers of monocytes/macrophages from sheep. The results demonstrate that monocyte/macrophage-rich lavage cell populations from rat and sheep differ substantially in their ability to generate nitric oxide . Only resident rat peritoneal lavage cells were able to mediate antibody-dependent cell-mediated cytotoxicity against newly excysted juvenile liver fluke. The mechanism of cytotoxicity was dependent on, and directly proportional to, the production of nitric oxide and required attachment of effector cells to the newly excysted juvenile liver fluke tegument, which occurred following the addition of sera from* F. hepatica*-infected animals. This is the first report demonstrating a mechanism of cell-mediated cytotoxicity to newly excysted juvenile liver fluke.*

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INTRODUCTION

The immune response to *Fasciola hepatica* infection has been examined in a wide variety of mammalian hosts including laboratory animals and domestic ruminants (1–3). The rat is a unique model in that this species develops an immunologically based resistance to reinfection with *F. hepatica* and resistance is expressed within 24–48 h of reinfection with two sites involved in parasite killing, namely the gut wall and peritoneal cavity (2,4–9). In ruminants, sheep do not acquire resistance to *F. hepatica* whereas cattle acquire resistance >20 weeks postinfection (2,10). Comparative studies in ruminants have been unable to demonstrate any damage to juvenile *F. hepatica* at the gut or peritoneal cavity (11).

We investigated potential host resistance mechanism(s) by which juvenile flukes migrating to the liver could be killed within the peritoneal cavity of rats. Newly excysted juvenile (NEJ) liver fluke of *F. hepatica* are killed when injected intraperitoneally into resistant rats, suggesting effector mechanism(s) present in the peritoneal cavity alone are able to kill the migrating parasite (12). Excysted juveniles of *F. hepatica* which penetrate the gut wall of resistant rats are, prior to their destruction within the peritoneal cavity, coated with antibody and host cells including eosinophils, neutrophils, macrophages and mast cells (2,9,12). However, studies have been unable to demonstrate irreversible damage to NEJ liver fluke by sera and eosinophils or neutrophils (7,8,13–15). Monocytes/macrophages have not been specifically studied in the killing of NEJ liver fluke but are one of the major effector

cells involved in the killing of parasites by the production of free radicals (16–19). Resident peritoneal lavage cells (PLCs) of naive rats are a source rich in monocytes/macrophages and passive transfer of sera from *F. hepatica*-infected sheep, cattle or rats protect naive rats from *F. hepatica* infection when injected intraperitoneally (20–25). Most juvenile parasites are killed within the gut and peritoneum before reaching the liver, suggesting that resident PLCs such as monocytes/macrophages could be involved in the killing of NEJ liver fluke by a mechanism dependent on parasite-specific antibody (2,20–23). We therefore studied antibody-dependent cell cytotoxicity based on the production of free radicals by resident rat cell populations containing large numbers of monocytes/macrophages as a potential host resistance mechanism by which juvenile flukes could be killed in the peritoneal cavity of rats. Comparative studies were conducted using cell populations containing large numbers of monocytes/macrophages from sheep.

MATERIALS AND METHODS

Animals, parasites and reagents

Metacercariae of *F. hepatica* were purchased from Compton Paddock Laboratories (Compton, UK) and stored on cellophane at 4°C until excystment of NEJ liver fluke (26). *F. hepatica*-naive, Merino-cross wether sheep, between 6 and 18 months of age, were obtained from local farms in Victoria, Australia, and used as donors of lung and peritoneal lavage cells. *F. hepatica*- and *F. gigantica*-naive, male Indonesian Thin Tail sheep, aged 6–8 months, were obtained from local farms in Bogor, West Java, Indonesia, and used as donors for lung lavage cells (LLCs). *F. hepatica*-naive white male Wistar rats, aged 7–12 weeks, were obtained from Monash University Animal Services, Victoria, Australia, and used as donors of lung and peritoneal lavage cells. Rats were euthanized by asphyxiation with CO_2 gas in a semiclosed container, while *F. hepatica*-naive sheep used to donate lung and peritoneal lavage cells were killed by an intravenous injection of Lethobarb. Catalase, cytochrome C, gentamycin, naphthylenediamine dihydrochloride, nitrate, nitrite, phenol red-free RPMI, sulphanilamide, superoxide dismutase, toluidine blue, trypan blue and the tetrazolium salt, MTT, were purchased from Sigma Chemical Co. (St Louis, MO, USA). Amphotericin B was purchased from Life Technologies (Rockville, MD, USA). Monomethyl L-arginine (L-NMMA) was purchased from Calbiochem Behring Corp. (La Jolla, CA, USA). ELISA plates and 24-well tissue culture plates were purchased from Flow Laboratories Inc. (Rockville, MD, USA) and Greiner Labortechnik (Kremsmuenster, Austria), respectively. Recombinant bovine rBoIFN-γ (rBoIFN-γ, $2{\cdot}2 \times 10^6$ U/mg), recombinant ovine rOvTNF-α (rOvTNF-α), approximately 1×10^7 U/mg) and lipopolysaccharide (LPS) of *Escherichia coli* were kindly provided by CSIRO Division of Animal Health, Melbourne, Australia. The bioactivity of each cytokine was confirmed prior to use and expressed as units/mg protein.

Cell populations

Resident monocyte/macrophage-rich cell populations were collected by either bronchoalveolar or peritoneal cavity lavage using sterile PBS containing 6 mM EDTA. Lungs from apparently normal sheep were discarded if either pneumonic lesions or lungworm larvae were observed or if blood was present in the lavage fluid. The recovered lavage fluid was collected, centrifuged at 1500 r.p.m. for 10 min and the cell pellet resuspended in sterile RPMI containing 10% heat-inactivated foetal calf serum, 2 μg/ml amphotericin B and 10 μg/ml gentamycin. A 5-μl sample of lavage 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 subsamples of lavage cells at 400 r.p.m. for 5 min at 4°C in a Beckman TJ-6 benchtop centrifuge prior to differential staining. Four hundred to 500 cells were identified microscopically at ×1000 magnification and the relative percentage of lymphocytes, monocytes/macrophages, eosinophils, neutrophils, basophils and mast cells determined.

Nitric oxide production by lavage cells

The levels of nitrite in culture supernatants of lavage cells of Merino and Indonesian Thin Tail sheep and rats, following stimulation with cytokines and/or LPS, were used as an indicator of nitric oxide production (27). Lavage cells were incubated at 37°C in 96-well, flat-bottom ELISA plates, in a humidified incubator in the presence of 95% air and 5% CO_2. Aliquots of $0{\cdot}5–2 \times 10^5$ viable cells were cultured for 0–10 days in 200 μl RPMI containing 10% heat-inactivated foetal calf serum, 2 μg/ml fungizone, 10 μg/ml gentamycin, with or without 0·5–2·0 μg/ml LPS, 50–1000 U/ml rBoIFN-γ, 50–1000 U/ml rOvTNF-α, or combinations of these stimulants. In addition, 0·5 mM L-NMMA was included in some incubations as a competitive inhibitor of nitric oxide synthase (28). Nitrite concentration in the culture media, at the end of the incubation period, was assayed by a standard Greiss reaction (29). Briefly, 100 μl of media was added to 50 μl of 1%

sulphanilamide in 2·5% H_3PO_4 and 50 μl of 0·1% naphthylenediamine dihydrochloride. After 15 min, the absorbance was read at 540 nm using a Titertek Multiskan MCC spectrophotometer. Nitrite concentration was determined with reference to a standard curve generated using concentrations from 1 μM to 280 μM sodium nitrite in culture media. Under these conditions, the detection limit was 1 μM nitrite.

Superoxide production by lavage cells

Lavage cell monolayers (2×10^6 cells) in 24-well plates were incubated at 37°C, in 2 ml RPMI and 10% heat-inactivated foetal calf serum, 2 μg/ml fungizone and 10 μg/ml gentamycin for 2 h. Immediately prior to assaying superoxide generation, the media was gently removed and replaced with substrate. Superoxide production was measured by the cytochrome c reduction method. 75 μM cytochrome c, 40 μg/ml catalase and with or without 50 NEJ liver fluke or 0·5 μg/ml LPS were added to triplicate wells and the final volume made up to 1 ml with RPMI media. Wells used as blanks contained 40 μg/ml superoxide dismutase. After incubation for 45 min at 37°C, the absorbance of the supernatant at 550 nm was determined using the Shimadzu UV-160 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

Incubation of NEJ liver fluke with lavage cells

Incubations in 96-well ELISA plates contained a single NEJ liver fluke in each of 10 replicate wells whereas for 24-well tissue culture plates, up to 50 NEJ liver fluke per well were used. Plates were incubated for 2–5 days in 200 μl (96-well plates) or 2 ml (24-well plates) 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% rat or sheep serum, 0·5 μg/ml LPS, 0·5 mM L-NMMA, 10 μg/ml SOD or combinations of these reagents, and lavage cells at an effector:target ratio of 2×10^5 cells : 1 NEJ liver fluke. Mean nitrite values were obtained by assaying the nitrite levels in 100 μl of culture supernatant of lavage cells at the end of the incubation period. Subsequently, NEJ liver fluke were incubated for 4 h in a solution of 2 mg/ml MTT and killing of NEJ liver fluke was then determined microscopically as loss of motility, an opaque appearance and an inability to reduce the tetrazolium, MTT (30).

Origin of sera

Sheep were each infected with 500 metacercariae of *F. hepatica* by intraruminal injection and blood collected 8–10 weeks postinfection by jugular venipuncture. The blood was allowed to clot at room temperature for 1 h and centrifuged in a Beckman CS-6R centrifuge at 3000 ***g*** for 20 min. The serum was then removed and complement activity inactivated by heating at 56°C for 30 min prior to storage at − 20°C. Blood was collected from *F. hepatica*-naive rats and from rats 11 weeks postinfection with 50 metacercariae by cardiac puncture after euthanasia. Serum was obtained as described above.

RESULTS

Free radical generation by peritoneal lavage cells of *F. hepatica*-naive rats in response to stimulation by NEJ liver fluke or LPS

The PLCs of *F. hepatica*-naïve rats consisted of 70–90% monocytes/macrophages, 5–15% lymphocytes and the percentage of eosinophils, neutrophils and mast cells did not exceed 10% for any of these individual cell types in our assays (Figure 1). NEJ liver fluke were able to stimulate significant levels of two major types of free radicals from rat PLCs, namely, reactive oxygen and reactive nitrogen species, when compared to control incubations without NEJ liver fluke (Figure 2). Under our assay conditions, LPS stimulated even greater levels of reactive oxygen/nitrogen species. Therefore, to attain maximal free radical generation, LPS was added in subsequent experiments with incubations of rat PLCs and NEJ liver fluke. Nitrite levels

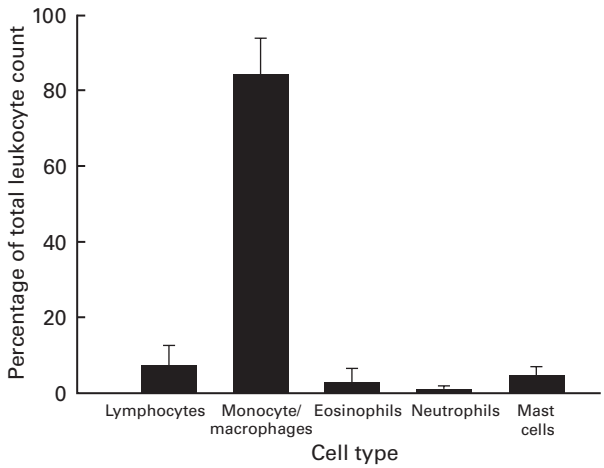

Figure 1 Differential cell counts of peritoneal lavage cell samples from *F. hepatica*-naïve rats. Cytospin preparations were stained using the Diff Quik® system and each cell type identified and expressed as a percentage of the total number of leucocytes counted. Values represent the mean ± SD from five rats.

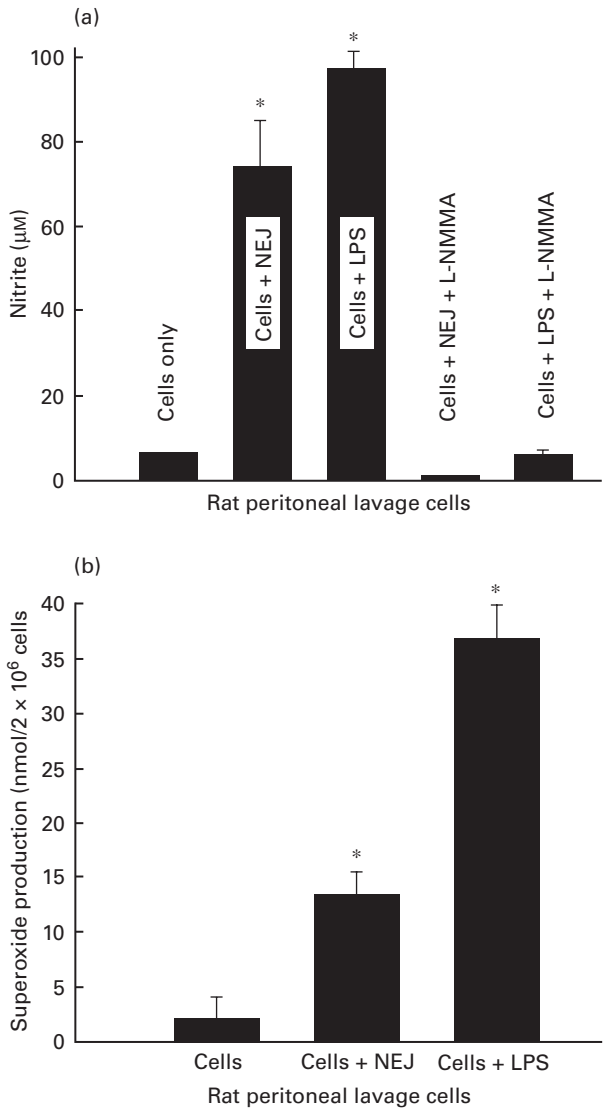

Figure 2 (a) Nitrite levels and (b) superoxide levels in culture supernatants of rat peritoneal lavage cells stimulated with either NEJ liver fluke or lipopolysaccaride. For (a), each of 10 replicate wells containing 2×10^5 cells were incubated for 48 h in 96-well plates containing ± a NEJ liver fluke or ± L-NMMA or ± LPS in 200 μl of media and the nitrite level in 100 μl of the supernatant then determined. Values represent the mean ± SD of five experiments. For (b), each of three replicate wells containing 2×10^6 cells were plated into 24-well plates and ± 50 NEJ liver fluke or ± LPS in 1 ml of media and the total amount of superoxide generated in a 45-min period was calculated. Values represent the mean ± SD of three experiments. *Represents means that were significantly different, by the Dunnett's multiple comparison test at $P < 0{\cdot}05$, to control incubations (cells only) within each experiment.

in culture supernatants of rat PLCs were not detected until at least 4 h post stimulation with LPS and peak levels of nitrite in culture supernatants were detected 48 h after LPS stimulation (data not shown).

The effect of sera from *F. hepatica*-infected rats on the killing of NEJ liver fluke incubated with LPS-stimulated rat peritoneal lavage cells

Incubation of NEJ liver fluke with sera from *F. hepatica*-infected rats resulted in the formation of a precipitate surrounding the NEJ liver fluke tegument within 5 h of culture. Precipitate was not observed on NEJ liver fluke incubated with media alone or with sera from *F. hepatica*-naive animals. The precipitate, which was presumably antigen-antibody complexes on the parasite surface (14,31), was continuously shed throughout the 5-day incubation period and large amounts accumulated within the incubation wells. In the presence of homologous infection sera, large numbers of rat lavage cells adhered to a high proportion of NEJ liver fluke (> 80%) via the NEJ liver fluke tegument and this prevented an accurate count of the total number of attached cells (Table 1). Incubation without sera or with sera from *F. hepatica*-naive animals resulted in adherence of lavage cells only to a small number of NEJ liver fluke (0–20%, Table 1).

Incubation of NEJ liver fluke with LPS and rat PLCs, in the absence of sera from *F. hepatica*-infected rats, resulted in a mean killing of only 18% of NEJ liver fluke despite the high levels of nitrite (73 μM) in culture supernatants (Table 2). The inclusion of sera from *F. hepatica*-naive rats also resulted in high levels of nitrite (60 μM) in culture supernatants but only a mean killing of 2% of NEJ liver fluke by LPS-stimulated rat PLCs was observed (Table 2). However, when NEJ liver fluke were incubated with LPS-stimulated rat PLCs, in the presence of sera from *F. hepatica*-infected rats, 61% of NEJ liver fluke were killed even though nitrite levels in culture supernatants (73 μM) were similar to incubations without sera (73 μM) or sera from *F. hepatica*-naïve rats (60 μM). Dead NEJ liver fluke were characterized by extensive cell attachment, an opaque appearance due to loss of internal structures, immotility, inability to reduce the tetrazolium salt MTT and general loss of parasite shape (30). Although peak nitrite levels in culture supernatants occurred 48 h post LPS stimulation, a 5-day incubation was required to detect irreversible damage of NEJ liver fluke. The addition of the nitric oxide synthase inhibitor, L-NMMA, reversed the cytotoxic effects of LPS-stimulated rat PLCs (9% killing) and the level of nitrite in culture supernatants (6 μM). In contrast, addition of the reactive oxygen species inhibitor superoxide dismutase (SOD) did not reverse the cytotoxic effects of LPS-stimulated rat PLCs (93% killing) or the level of nitrite in culture supernatants (85 μM, Table 2). The level of killing of NEJ liver fluke by LPS-stimulated rat PLCs in the presence of sera from *F. hepatica*-infected rats was highly correlated ($r^2 = 0{\cdot}77$) to the mean level of

Table 1 Adherence of rat peritoneal lavage cells to NEJ liver fluke of *F. hepatica* incubated in the presence or absence of sera obtained from *F. hepatica*-naïve or -infected rats

Incubation	% of NEJ liver fluke with 20 or more cells adherent to the outer tegument						
	0·1 h	8 h	16 h	24 h	48 h	72 h	120 h
NEJ + LPS + rat PLCs	0	10	0	10	0	10	0
NEJ + LPS + rat PLCs + naïve sera	0	0	0	10	10	20	0
NEJ + LPS + rat PLCs + immune sera	0	70	70	90	90	80	80

Each of 10 replicate wells containing 2×10^5 cells and a single NEJ liver fluke in 200 μl of media were incubated for 5 days in the presence or absence of sera from *F. hepatica*-naïve or -infected rats. Results presented here represent typical values obtained in four other experiments. Values are the percentage of NEJ with 20 or more cells adherent to the outer tegument.

nitrite detected in culture supernatants after 5 days culture with $>50\%$ killing observed above 60 μM nitrite (Figure 3).

Killing of NEJ liver fluke by LPS-stimulated rat peritoneal lavage cells is dependent on contact between NEJ liver fluke and cells

The killing of the NEJ liver fluke was dependent on the presence of sera from *F. hepatica*-infected rats which was necessary to promote high levels of cell attachment to the NEJ tegument, since little attachment of cells occurred in incubations with no sera or sera from *F. hepatica*-naive rats (Table 1). This suggested that the effector cells required close contact with the NEJ liver fluke tegument to mediate cytotoxicity. In order to investigate this possibility, we performed incubations in the presence of sera from *F. hepatica*-infected rats in wells with tissue culture inserts in which the PLCs were separated from the NEJ by a 0·45-μm filter to inhibit direct contact between NEJ liver fluke and PLCs. Under these conditions, a mean of only 10% NEJ liver fluke were killed compared with a mean killing of 60% NEJ liver fluke in the absence of tissue culture inserts, even though the mean levels of nitrite produced by the rat PLCs were equivalent between the incubations (Figure 4).

Comparison of nitric oxide production and killing of NEJ liver fluke by lung and peritoneal lavage cells from *F. hepatica*-naïve sheep

In contrast to rats, sheep do not aquire resistance to *F. hepatica* infections and we investigated whether sheep cells rich in monocyte/macrophages lacked the reactive nitrogen species-dependent cytotoxic mechanism exhibited by rat PLCs. We used two populations of cells rich in monocyte/macrophages from *F. hepatica*-naïve sheep:

Table 2 Effect of incubation of rat peritoneal lavage cells with sera from *F. hepatica*-naïve and -infected rats on the killing of NEJ liver fluke of *F. hepatica*

Incubation	Rat PLCs	
	% dead NEJ	Nitrite (μM)
NEJ + LPS	1 ± 3	< 1
NEJ + LPS + naïve sera	5 ± 5	< 1
NEJ + LPS + immune sera	1 ± 3	< 1
NEJ + LPS + cells	18 ± 11	73 ± 10
NEJ + LPS + cells + naïve sera	2 ± 2	60 ± 4
NEJ + LPS + cells + immune sera	61 ± 17*	73 ± 13
NEJ + LPS + cells + immune sera + L-NMMA	9 ± 10	6 ± 2
NEJ + LPS + cells + immune sera + SOD	93 ± 9*	85 ± 30

Each of 10 replicate wells containing 2×10^5 cells and a single NEJ liver fluke in 200 μl of media were incubated for 5 days and the nitrite level in 100 μl of the supernatant determined. Viability of the NEJ liver fluke in the remaining 100 μl of media was then determined. Values represent the mean ± SD of 15 experiments. *Represents means that were significantly different by the Dunnett's multiple comparison test at $P < 0{\cdot}05$, to control incubations (NEJ + LPS + cells).

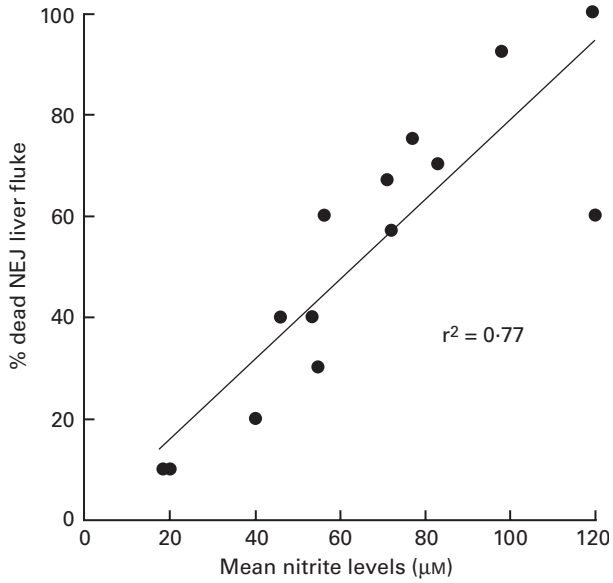

Figure 3 Relationship between mean nitrite levels in supernatants of cultures of naive rat peritoneal lavage cells and the percentage of dead NEJ liver fluke of *F. hepatica*. Each of 10 replicate wells containing up to 2×10^5 cells, LPS and sera from *F. hepatica*-infected rats and a single NEJ liver fluke in 200 μl of media were incubated for 5 days and the nitrite level in 100 μl of the supernatant then determined. Viability of the NEJ liver fluke in the remaining 100 μl of media was then determined. Each value represents the mean nitrite level and percentage of dead NEJ liver fluke for each experiment.

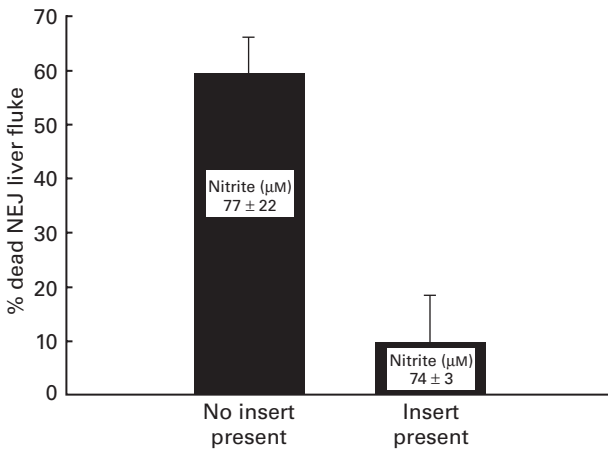

Figure 4 Effect of physical separation of NEJ liver fluke from rat PLCs on the killing of NEJ liver fluke. 25–50 NEJ were placed in 24-well tissue culture plates with sera from *F. hepatica*-infected rats. Inserts containing rat PLCs at an E : T ratio of 2×10^5 cells : 1 NEJ liver fluke were placed in the 24-well tissue culture wells containing the NEJ liver fluke; the rat PLCs were separated from the NEJ liver fluke by a 0·45-μm membrane. Following incubation for 5 days, the nitrite level in 100 μl of the supernatant was assayed and viability of the NEJ liver fluke was then determined. Values represent the mean ± SD of three experiments.

PLCs, as a direct comparison with rat PLCs and lung lavage cells (LLCs) as a source of macrophages. Lung and peritoneal lavage washings of naïve sheep consisted of greater than 85% monocytes/macrophages, with less than 10% lymphocytes, eosinophils, neutrophils and basophils (Figure 5). No detectable nitrite (< 1 μM) was found in culture supernatants of LPS-stimulated Merino sheep LLCs or PLCs after 48 h incubation (Table 3). In some systems, production of nitric oxide requires costimulation with cytokines such as IFN-γ and TNF-α. To determine whether this applies in sheep, LLCs and PLCs of Merino sheep were incubated with a combination of LPS, recombinant bovine interferon gamma (rBoIFN-γ) and recombinant tumour necrosis factor alpha (rOvTNF-α) to determine whether the addition of these stimulants could induce increased nitric oxide production. Nitrite was not detected in culture supernatants of Merino sheep LLCs and PLCs when incubated with LPS, rBoIFN-γ, rOvTNF-α or combinations of these stimulants for 48 h (Table 3) or after prolonged culture for up to 10 days. However, LPS, rBoIFN-γ, and NEJ were all able to stimulate superoxide production from Merino LLCs, showing that sheep LLCs did respond to the stimulants (data not shown). It was considered important to establish whether the inability of lavage cells of Merino sheep to produce detectable nitric oxide was specific to this breed. Consequently, LLCs were harvested from Indonesian Thin Tail sheep, a breed of non-European origin (32). Incubation of LLCs of Indonesian Thin Tail sheep with LPS, rBoIFN-γ, rOvTNF-α or combinations of these stimulants also failed to produce detectable levels of nitrite

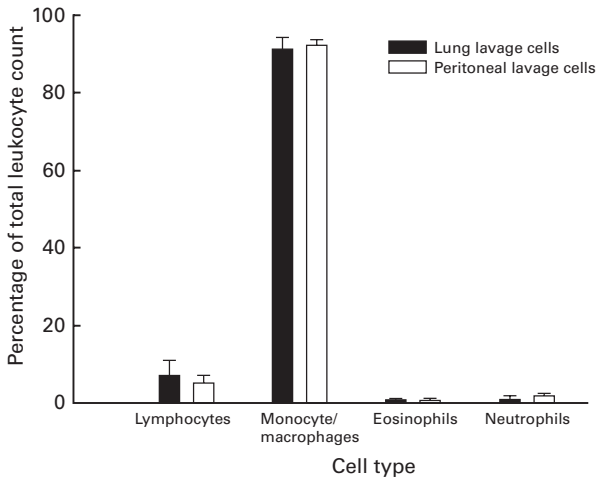

Figure 5 Differential cell counts of lung and peritoneal lavage cell samples from *F. hepatica*-naïve Merino sheep. Cytospin preparations were stained using the Diff Quik® system and each cell type identified and expressed as a percentage of the total number of leucocytes counted. Values represent the mean ± SD from three sheep.

Table 3 Nitrite levels in culture supernatants of lung and peritoneal lavage cells from Merino and Indonesian thin tail (ITT) sheep in response to LPS and cytokine stimulation

	Merino or ITT sheep nitrite level (μM)	
Incubation	LLCs	PLCs
Cells	< 1	< 1
Cells + LPS (0·5, 1 or 2 μg/ml)	< 1	< 1
Cells + rBoIFN-γ (50, 100, 500 or 1000 U/ml)	< 1	< 1
Cells + rOvTNF-α (50, 100, 500 or 1000 U/ml)	< 1	< 1
Cells + rBoIFN-γ (100 U/ml) + rOvTNF-α (50, 100 or 500 U/ml)	< 1	< 1
Cells + rBoIFN-γ (100 U/ml) + LPS (0·5, 1 or 2 μg/ml)	< 1	< 1

Each of four replicate wells containing 2×10^5 cells in 200 μl of media and with or without stimulants were incubated for 48 h and the nitrite level in 100 μl of the supernatant then determined. Values represent the mean ± SD of three experiments.

Table 4 Effect of incubation of sheep lung lavage cells with sera from *F. hepatica*-infected sheep on the killing of NEJ liver fluke of *Fasciola hepatica*

	Merino sheep LLCs	
Incubation	% dead NEJ	Nitrite (μM)
NEJ + LPS	0	< 1
NEJ + LPS + immune sera	0	< 1
NEJ + LPS + cells	3 ± 5	< 1
NEJ + LPS + cells + immune sera	7 ± 9	< 1

Each of 10 replicate wells containing 2×10^5 cells and a single NEJ liver fluke in 200 μl of media were incubated for 5 days and the nitrite level in 100 μl of the supernatant determined. Viability of the NEJ liver fluke in the remaining 100 μl of media was then determined. Values represent the mean ± SD of three experiments.

in culture supernatants after 48 h (Table 3). Incubation of NEJ liver fluke for 5 days with LPS-stimulated Merino LLCs in the presence of sera from *F. hepatica*-infected sheep resulted in only a mean 7% killing of NEJ liver fluke and no detectable levels of nitrite in culture supernatants (Table 4), despite attachment of high numbers of cells to the NEJ tegument.

To determine whether the inability of sheep LLCs to kill NEJ liver fluke by the nitrite dependent cytotoxic mechanism of rats was due solely to the cell source and not the source of sera, NEJ liver fluke were incubated with LPS-stimulated rat PLCs and sera from *F. hepatica*-infected sheep or from *F. hepatica*-infected rats. Incubation of NEJ liver fluke with LPS-stimulated rat PLCs in the presence of sera from *F. hepatica*-infected sheep or sera from *F. hepatica*-infected rats resulted in similar levels of killing of NEJ liver fluke and nitrite levels in culture supernatants (Table 5). Killing of NEJ by rat PLCs in the presence of either serum source was inhibited by L-NMMA (Table 5). Conversely, incubation of NEJ liver fluke with LPS-stimulated sheep LLCs in the presence of sera from *F. hepatica*-infected rats resulted in no killing of NEJ liver fluke and undetectable nitrite levels in culture supernatants (data not shown).

Table 5 Effect of source of host serum on the killing of NEJ liver fluke of *F. hepatica*

Incubation	% dead NEJ	Nitrite (μM)
NEJ + LPS + rat immune sera	0	< 1
NEJ + LPS + sheep immune sera	0	< 1
NEJ + LPS + rat PLCs + rat immune sera	69 ± 8	111 ± 20
NEJ + LPS + rat PLCs + sheep immune sera	60 ± 8	118 ± 13
NEJ + LPS + rat PLCs + rat immune sera + L-NMMA	15 ± 5	2 ± 1
NEJ + LPS + rat PLCs + sheep immune sera + L-NMMA	5 ± 5	7 ± 3

25–50 NEJ were placed in 2 ml of media in 24-well tissue culture plates containing rat PLCs at an E : T ratio of 2×10^5 cells : 1 NEJ liver fluke, with either sera from *F. hepatica*-infected rats or sheep and incubated for 5 days, and the nitrite level in 100 μl of the supernatant then determined. Viability of the NEJ liver fluke in the remaining media was then determined. Values represent the mean ± SD of four experiments.

DISCUSSION

This study demonstrates that rat PLCs, but not sheep LLCs, are able to kill NEJ liver fluke by a mechanism dependent on antibody and the production of reactive nitrogen species. Furthermore, this study shows differences in the ability of rat and sheep lavage cell populations, rich in monocytes/macrophages, to generate nitric oxide in response to LPS and cytokines. We suggest that the ability of PLCs from *F. hepatica*-naive rats to produce high levels of nitric oxide could be a mechanism by which migrating juvenile flukes are killed within the peritoneal cavity of rats.

In this first report demonstrating a mechanism of cytotoxicity against NEJ liver fluke, we have identified a host cell killing mechanism dependent on nitric oxide production by LPS-stimulated rat PLCs and the presence of sera from *F. hepatica*-infected animals. Evidence that cytotoxicity was dependent on the production of reactive nitrogen species and not reactive oxygen species was three-fold. First, the level of killing of NEJ liver fluke was directly proportional to the level in culture supernatants of one of the stable end products of nitric oxide catabolism, nitrite. Second, the killing of NEJ liver fluke was inhibited by the addition of the nitric oxide synthase inhibitor L-NMMA as seen with killing of schistosomula of *Schistosoma mansoni* (33). Finally, the killing of NEJ liver fluke was not inhibited by the addition of superoxide dismutase, which prevents the formation of the reactive oxygen species, superoxide radicals.

The presence of sera from *F. hepatica*-infected animals was also essential for high levels of killing of NEJ liver fluke. The addition of sera from *F. hepatica*-infected animals to LPS-stimulated rat PLCs and NEJ liver fluke resulted in large numbers of cells attaching to the NEJ liver fluke tegument, high levels of nitrite in culture supernatants and killing of NEJ liver fluke. Sera from infected animals was not directly toxic to NEJ liver fluke. Conversely, when NEJ liver fluke were incubated with LPS-stimulated rat PLCs and sera from *F. hepatica*-naive rats, or without sera, low levels of cells attached to the NEJ liver fluke tegument and less than 18% of NEJ liver fluke were killed, despite similar levels of nitrite within the culture supernatants. Furthermore, when we performed incubations in the presence of sera from *F. hepatica*-infected rats in wells with tissue culture inserts in which the PLCs were separated from the NEJ, to inhibit direct contact between NEJ liver fluke and PLCs, killing of NEJ liver fluke was also inhibited. These findings therefore suggest killing of NEJ liver fluke by LPS-stimulated rat PLCs requires both attachment of effector cells to the NEJ liver fluke tegument and the production of high levels of nitric oxide at the parasite surface. This suggestion is consistent with our previous work demonstrating that antibody-independent cell cytotoxicity is ineffective in mediating death to NEJ liver fluke (30) and the view that, due to the short half-life of nitric oxide, susceptible parasites are required to be in close contact with the effector cells producing the nitric oxide (19).

The ability of lavage washings, rich in monocytes/macrophages, to produce nitric oxide in response to stimulants differed between sheep and rats. LPS-stimulation of sheep lung and peritoneal lavage cells resulted in undetectable levels of nitrite in culture supernatants. In contrast, levels of nitrite within supernatants of LPS-stimulated rat peritoneal lavage cells were consistently high. LPS alone, however, does not stimulate significant nitric oxide production by several types of mammalian cells; for example, production of nitric oxide by resident mouse monocytes/macrophages requires stimulation with recombinant murine IFN-γ alone or in combination with recombinant murine TNF-α or LPS (34,35). Incubation of sheep LLCs and PLCs with rBoIFN-γ, rOvTNF-α and LPS under a wide range of combinations and concentrations did not result in detectable levels of nitrite in culture supernatants. Thus, exposure to a range of stimulants, which are potent inducers of nitric oxide production by rodent macrophages, apparently fails to induce the production of elevated levels of nitric oxide by sheep lavage cells. Similarly, under a variety of conditions, only low levels of nitrite were detected in culture supernatants of sheep alveolar macrophages and sheep, goat and cattle peripheral blood mononuclear cells (36–38).

The inability of Merino sheep LLCs and PLCs to produce detectable nitric oxide prompted us to analyse nitric oxide production in a sheep breed of non-European origin to determine whether nitric oxide production was breed specific. Indonesian Thin Tail sheep are a breed of sheep derived originally from Asia (32). Nitrite was not detected in culture supernatants of lavage cells of Indonesian Thin Tail sheep following incubation with LPS, rBoIFN-γ or rOvTNF-α or by combinations of these stimulants, indicating that the inability of sheep lavage cells to generate a detectable level of nitric oxide was not specific to the Merino breed.

It has been suggested that several mammalian hosts potentially recognize protective fluke antigens but only rats possess the necessary cellular effector mechanisms for killing recently excysted juvenile liver fluke (39). Interestingly, the level of free radical production of PLCs in challenged rats in response to crude adult antigens of *F. hepatica* is 30-fold greater than that of the susceptible mouse host (40). Thus, the ability of different hosts to kill NEJ liver fluke may be due to differences in the magnitude of the free radical response. Large numbers of

LPS-stimulated monocytes/macrophages of sheep were unable to mediate NEJ liver fluke cytotoxicity despite the addition of sera from *F. hepatica*-infected animals. In contrast, a mean killing of 61–69% of NEJ liver fluke resulted when rat cell populations containing large numbers of monocytes/macrophages were used, regardless of whether homologous or heterologous sera from *F. hepatica*-infected animals were used. Killing of NEJ liver fluke therefore appears dependent on the origin of the host cells, not the origin of the host sera. Therefore, the inability of lavage cells of sheep to kill NEJ liver fluke appeared to be a result of the lack of nitric oxide generation by these ruminant cells. Thus, if the level of nitric oxide produced by resident host lavage cells is essential in the killing of migrating NEJ liver fluke, then the use of the rat to determine immunological mechanisms of resistance that may apply in ruminants is highly questionable.

The protection of naive rats by intraperitoneal injection of sera from *F. hepatica*-infected sheep, cattle or rats suggests a killing mechanism at that site dependent on the presence of parasite-specific antibody (20–25). The protective response in these studies required challenge infection on the day of transfer with killing of NEJ liver fluke occurring within 48 h, prior to entry into the liver (20–23) and therefore is likely to involve resident PLCs. Our study has identified a mechanism capable of killing NEJ liver fluke that is not dependent on homologous sera from *F. hepatica*-infected animals and is mediated by reactive nitrogen species produced by resident PLCs of naive rats. We therefore suggest that the production of reactive nitrogen species by resident PLCs of naive rats could be the unidentified component responsible for the mechanism of killing of NEJ liver fluke in these earlier studies.

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