Early and transient cytotoxic response of peritoneal cells from Fasciola hepatica-infected rats

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Vet. Res. 35 (2004) 573–584

DOI: 10.1051/vetres:2004033

Original article

Early and transient cytotoxic response of peritoneal cells from *Fasciola hepatica*-infected rats

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(Received 2 December 2003; accepted 24 March 2004)

Abstract – Experimental infection by *F. hepatica* was performed on rats. Early recruitment of the peritoneal cell population was observed and revealed transient parasite-killing activity, preceded and followed by a state of total unresponsiveness. The activation peaked at seven days post-infection (dpi) and was characterised by a massive peritoneal cell recruitment, a strong superoxide anion and nitric oxide (NO) production, that were coincident with the fasciolicide activity of these cells, as monitored by an in vitro decrease of juvenile fluke viability in a conditioned medium. The addition of L-NG-monomethyl arginine (LNMMA) to cell cultures abrogated both fasciolicide activity and NO production. Parasites started to die when NO production exceeded 25 μM and all juvenile flukes were killed by a 90 μM NO exposition (Lethal Dose 50 between 45.8 and 50.3 μM, 95% fiducial limits). However, when rat peritoneal cells were cultured in the presence of either infected or control rat serum, juvenile flukes were much more resistant to the oxidative burst, despite a massive attachment of rat peritoneal cells to the parasite tegument. These data suggest that a transient control of fasciolosis may take place in the peritoneum following the parasite intrusion but that the parasite efficiently scavenges the host cellular response to avoid destruction.

***F. hepatica* / rat peritoneal cells / cytotoxicity / nitric oxide**

1. INTRODUCTION

The liver fluke, *Fasciola hepatica*, has a worldwide distribution and causes a liver disease which is one of the major liver parasitic infections in humans and domestic ruminants. In animals, adverse effects of fasciolosis include both decreased meat and milk production, decreased female fertility and increased veterinary expenses [6, 31]. Chemotherapy has been proven to be efficient, primarily against the adult stage. However, treatments are expensive and are responsible for residues in milk and meat. Moreover, resistance to flukicides has been observed [22]. Thus, it is necessary to develop new means for the control of fasciolosis, such as vaccination. Since conventional approaches are not effective enough for the design of a commercial vaccine [27], and since an efficient vaccine should protect against every invasive juvenile fluke, a better understanding of the host-parasite relationship is needed in order to improve the natural resistance developed by the host.

The host's reaction to *F. hepatica* and, more generally, to worm infections is quite difficult to assess. Many data concerning *F. hepatica*

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demonstrate the resistance or susceptibility of the host, depending on the species or the parameters considered [9, 13, 14, 19, 20]. In the case of rodents, resistance to infection or re-infection has been studied. It seems to be particularly efficient in the liver or at the mucosal level [32, 33]. The subsequent steps of the host-parasite interactions have been less characterised but research has clearly established that: (i) peritoneal cells may be recruited rapidly, and (ii) peritoneal cells and eosinophils, in particular, have been shown to attach to damaged juvenile flukes in the presence of immune serum [7, 12]. However, Doy and Hughes [8] showed that rats are unable to kill juvenile flukes when they receive an intraperitoneal (ip) injection of both parasites and peritoneal cells obtained from a chronically infected rat. Moreover, juvenile *F. hepatica* has been described as being resistant to in vitro killing by free radicals as compared with *Schistosoma mansoni* [24]. However, more recent data suggested that peritoneal cells (PC) could destroy juvenile flukes by an antibody-dependent, nitrogen intermediate-dependent mechanism [25]. But these studies focused on the interactions between juvenile flukes and peritoneal cells from naive rats. Many modifications occurring both in the liver [28, 30] and peritoneum in the early stages following an *F. hepatica* infection have been observed. In this work, we report an increase of cellular recruitment as well as macrophage oxidative burst, associated with a transient susceptibility of juvenile *F. hepatica* that were killed by the PC of rats infected 4 to 7 days before sacrifice. We established that the killing of the juvenile flukes is highly dependent on the production of reactive nitrogen intermediates although nitric oxide (NO) alone is unable to induce juvenile killing, as previously observed by Piedrafita et al. [24], and according to our in vitro assays involving chemical NO donors. Moreover, this in vitro killing only occurs when rats have been infected within a week, suggesting that the liver fluke parasites do rapidly and efficiently evade the host response.

2. MATERIALS AND METHODS

2.1. Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma (St. Louis, MO, USA). Cell culture products were obtained from Life Technologies.

2.2. Animals

For all experiments, we used male and female inbred Wistar Furth rats, 13–16 weeks old. The animals were reared locally in our laboratory at 20 °C, with 12 h of light per day. They were fed a pelleted diet (UAR Alimentation, Villemoisson, France) and had free access to drinking water. The metacercariae of *Fasciola hepatica* (bovine origin) were produced in our laboratory under standard conditions from *Lymnaea truncatula* [2, 3].

2.3. In vitro excystation of juvenile flukes: viability estimation

In vitro excystation of *F. hepatica* was performed as previously described [26]. Briefly, metacercariae were incubated for 2 h at 37 °C in a CO_2-saturated sodium dithionite solution (20 mM). Excystation was then performed in RPMI containing 0.1% bile salts (VWR, Fontenay-sous-Bois, France). After removal of the metacercarial walls, juveniles were washed three times in RPMI supplemented with penicillin and streptomycin and distributed into microwell plates (10/well). A final wash was performed in the plate and the volume was adjusted to 100 µL. Juvenile viability was monitored every 2 days up to 11 days after culture initiation by distinguishing the live flukes from the dead ones under an inverted microscope. Structural disorganisation and immobility were both required to ascertain the death of the parasite. Viability was expressed as the percentage of live flukes compared to the initial fluke population. S-nitroso-N-acetyl-penicillamin (SNAP) dissolved in dimethylsufoxide and stored at –20 °C (100 mM aliquots) was used as the chemical NO donor molecule.

2.4. Experimental procedures and cell culture

Groups of 4 to 6 animals were orally administered 30 metacercariae in a gelatine capsule at remote dates. All control and infected animals (0, 2, 4, 7, 14, 28, or 42 days post-infection (dpi)) were killed on the same days.

The animals were killed by chloroform inhalation. PC were obtained following the injection of 50 mL ice-cold phosphate buffered saline (PBS) containing 0.1% EDTA. After an abdominal massage, the cell suspension was collected and centrifuged for 10 min at 500 *g*. Contaminated red blood cells were removed by flash lysis in 2 mL H_2O and subsequent washing in cold PBS-EDTA. The cells were dried on glass slides and stained for histological characterisation (May-Grünwald-Giemsa staining).

Standard culture medium was used for the in vitro studies. It consisted of RPMI-1640 medium supplemented with L-glutamine, Hepes, Na pyruvate, penicillin/streptomycin (all purchased from In Vitrogen Life Technologies, Carlsbad, CA, USA). Ten percent decomplemented Fetal Calf Serum (FCS) was finally added to the culture medium. The cells were cultured either alone or with juvenile flukes (10 juveniles/10^5 cells) or 250 µM of a specific NO-synthase inhibitor (L-NG-monomethyl arginine, LNMMA, Calbiochem, La Jolla, USA). In some experiments, 10% rat serum was further added to the culture medium. The rat serum was obtained from the animals slaughtered during the experiment, pooled and filter-sterilised before use. A pool of 14 dpi-infected rat sera and a control pool from naive animals were constituted. The final volume was adjusted to 250 µL. All cell cultures were performed in triplicate.

2.5. Measurement of NO and O_2^- production

After four days of culture, macrophage NO production was assessed using the NO_2-titering Griess reagent [1]: sulfanilamide (1% w/v in HCl 1.2 N) and naphtylethylene diamine (0.3% v/w in H_20) were mixed in equal amounts and 100 µL of this solution was added to 50 µL of the cell supernatant. Optical Density (OD) was read at 550 nm. A linear standard curve was obtained with serial dilutions of $NaNO_2$ ranging from 2 to 500 µM.

O_2^- production was determined after 18 h cultivation using the oxidation of ferricytochrome C, as previously described [23]. Briefly, the plates were washed with phenol red-free Hanks balanced salt solution (HBSS) and incubated for one hour in a CO_2 incubator with 75 µL HBSS, 25 µL ferricytochrome C (2.7 mg/mL) and 10 µL phorbol myristate acetate (10 µg/mL) per well. A control plate was similarly treated, except that 25 µL superoxide dismutase (SOD, 1 mg/mL) was added to each well. After a reading at 550 nm, total ferricytochrome C was reduced by 10 µL sodium dithionite (1 mg/mL). The plates were then read again at 550 nm. O_2^- production was deduced by the following calculation: nmoles O_2^- = (OD – ODSOD)/(ODdithionite – ODSOD) × 10.9, where 10.9 represents the nmoles of added ferricytochrome C.

2.6. Statistical analysis

All data were subjected to non-parametrical Kruskall–Wallis statistical analysis (Simstat Software). Differences were considered significant when $P < 0.05$.

The analysis of the correlation between NO production and juvenile viability was performed using the SAS software Probit procedure.

3. RESULTS

3.1. Evolution of peritoneal cell populations during infection

A series of six rats per group was orally infected with 30 metacercariae of *F. hepatica* at various times. The number of recovered peritoneal cells increased drastically at

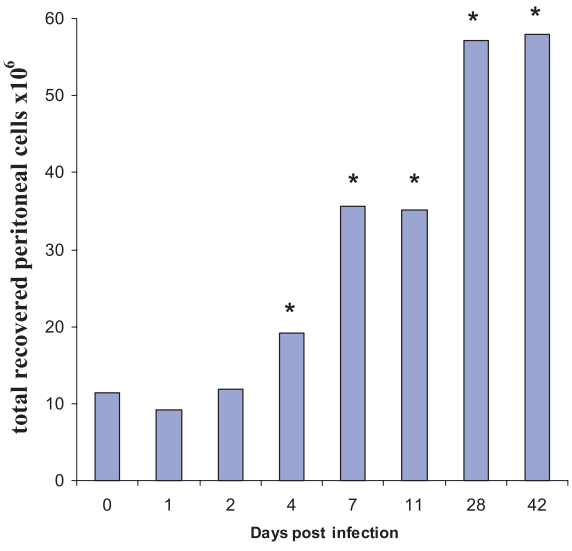

Figure 1. Influence of infection on peritoneal cell recruitment. Peritoneal cells were recovered in ice-cold PBS-EDTA, washed and counted. Viability was assessed by trypan-blue exclusion. * Indicates significant variations between infected and control rats ($P < 0.05$, Kruskall and Wallis non-parametrical test). The data are representative of five independent experiments.

4 dpi, starting from eight million cells/rat (control rats) to 35 million cells/rat at 7 dpi (Fig. 1). At the end of the experiment, the total recovered peritoneal cells reached 50 million cells per animal. Lymphocyte and mastocyte populations were drastically proportionally reduced, while eosinophil cell counts rose from near zero to more than 62% of the total population (Fig. 2). Recruitment of the monocyte/macrophage population was only slightly increased at one WPI (not statistically significant). Thereafter, their relative abundance was significantly lower at three WPI when compared to one WPI ($P < 0.05$).

3.2. In vitro viability of juvenile flukes

Peritoneal cells of infected and control rats were co-cultured with newly excysted juveniles (NEJ) and live parasites were counted every two days. The juvenile fluke organisation quickly appeared to be highly disturbed (within five days, see Fig. 3) in the wells that contained peritoneal cells from 7 dpi infected rats. However, up to ten days of culture were required to observe parasite death while the control flukes were still moving in the culture well. A significant decrease in the viability of the juvenile flukes was observed when cultured with the peritoneal cells from four- and seven-day infected rats (Fig. 4). Conversely, juvenile flukes cultured alone or in the presence of either uninfected or late-infected (i.e. > 14 dpi) rat PC were not affected. Similar results were obtained in three other studies, with slight variations of the optimum period (7–11 days, data not shown). When cells and juvenile flukes were cultured in the presence of 250 µM LNMMA, in vitro killing of juvenile flukes by the PC was totally abrogated, suggesting that NO was involved in the cytotoxic mechanism. Thus, a narrow window (4–11 dpi) was found during which peritoneal cells from infected rats were able to kill NEJ. This phenomenon seemed to be highly dependent on NO production since

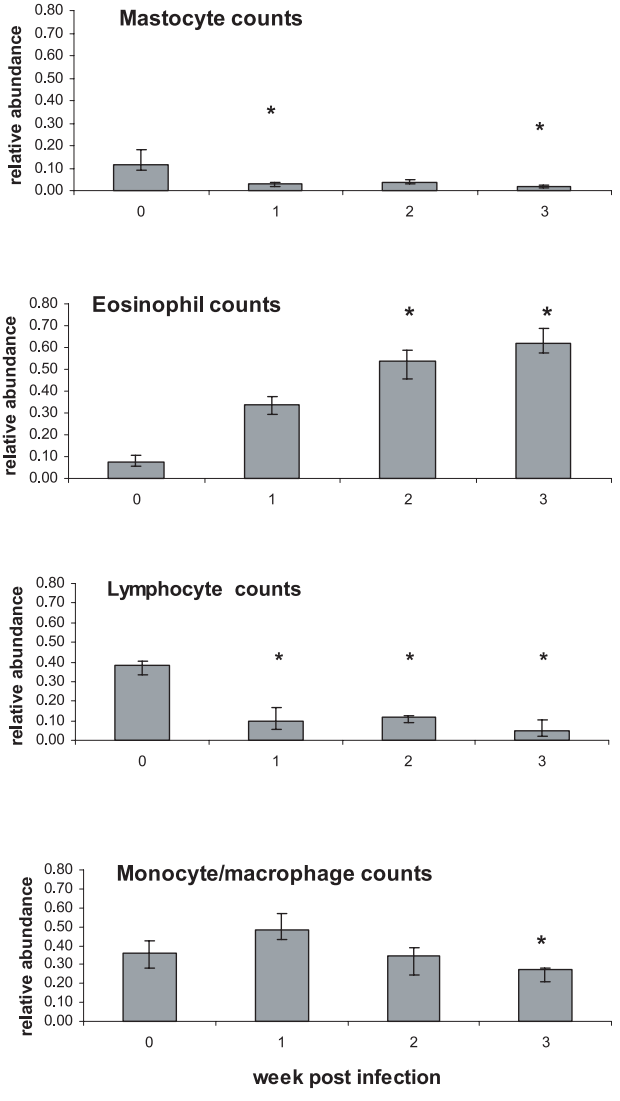

Figure 2. Variations in the recovered peritoneal cell population during the first three weeks post infection. Aliquots of the peritoneal cells were dried on glass slides before staining with May-Grünwald-Giemsa staining and histological identification. * Significant variations ($P < 0.05$, Kruskall and Wallis non-parametrical test).

specific inducible NO synthase (iNOS) inhibitors totally inhibit parasite killing. Curiously, this cytotoxic phenomenon completely vanished when the cells and the juveniles were co-incubated in the presence of either 14 dpi-infected or control rat serum (Fig. 5).

3.3. Juvenile mortality and reactive intermediate (NO, O_2^-) production

NO production by peritoneal cells after four days of in vitro culture was monitored with the Griess reagent (Fig. 6). The SAS

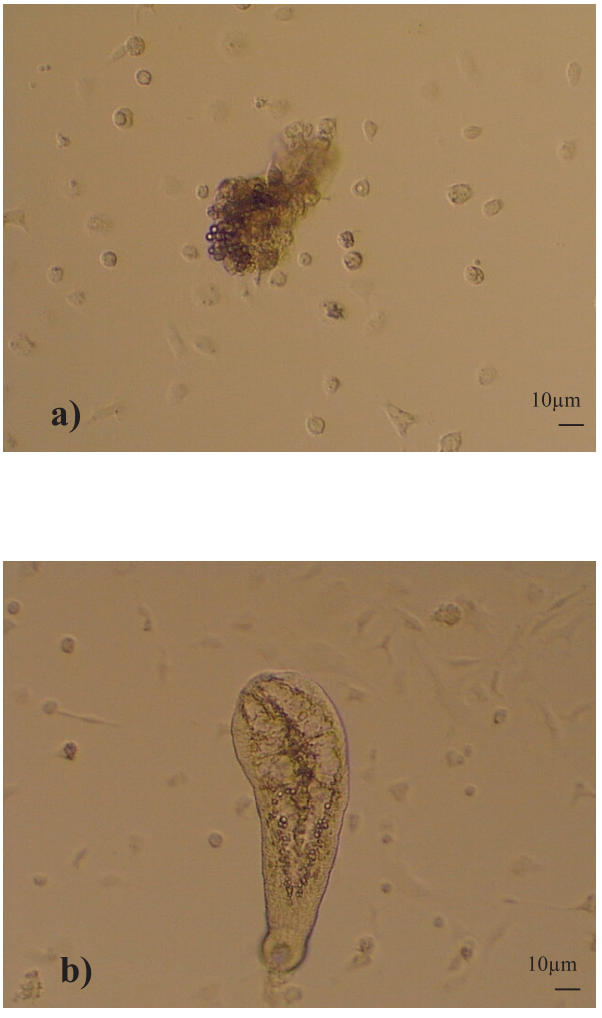

Figure 3. Microscopic aspect of juvenile flukes exposed to macrophage populations purified from seven-day infected rats. (a) shows a lysed juvenile fluke cultured with peritoneal cells of one-week infected rats, while (b) shows a juvenile fluke incubated with the same peritoneal cells in the presence of 250 µM L-NG-monomethyl arginine.

Probit procedure performed with a logistic model revealed a very high correlation (log likelihood –237.26) between the recorded nitrite level and juvenile mortality. This resulted in a highly significant sigmoïd dose-mortality curve with the following parameters (95% fiducial limits, see Fig. 7): less than 10% mortality with 28–33 µM NO_2^-; 50% mortality with 45–50 µM NO_2^-; 95% mortality with 78–96 µM NO_2^-.

O_2^- production by peritoneal cells was assayed after 18 h of in vitro culture. O_2^- production was increased in PC obtained from infected rats, but with a slight delay in peak

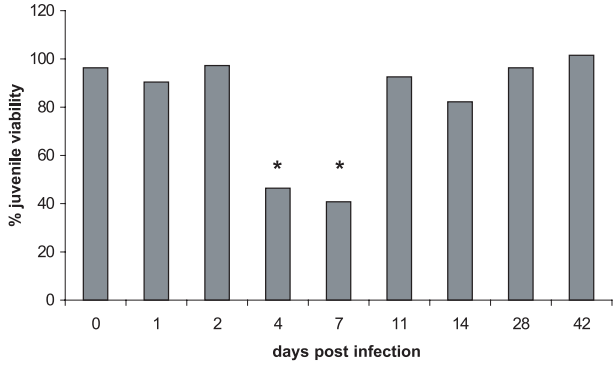

Figure 4. Viability of newly excysted juveniles cultured with peritoneal cells obtained at various times from *F. hepatica*-infected rats. Viability was assessed 11 days post culture initiation. * Represents a significant decrease of viability ($P < 0.05$, Kruskall and Wallis non-parametrical test).

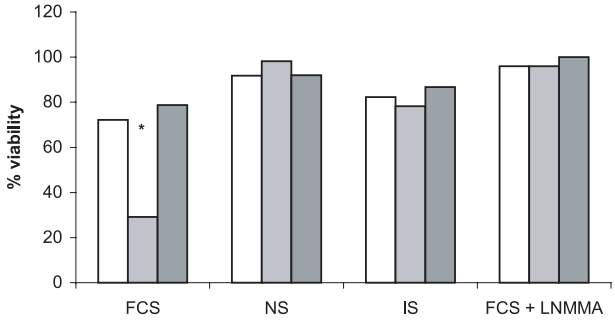

Figure 5. Cytotoxic activity of peritoneal cells in the presence of autologous serum. Peritoneal cells of control or infected rats were incubated in the presence of 10% decomplemented fetal calf serum (FCS) and in either 14 dpi infected or control naïve rat serum (IS and NS, respectively). L-NG-monomethyl arginine (LNMMA) (250 μM) was also added to some culture wells to prevent the production of nitric oxide. □ Represents peritoneal cells from control rats, ■ represents peritoneal cells from seven-dpi infected rats, ■ represents peritoneal cells from fourteen-dpi infected rats. * Indicates a significant difference when compared to uninfected rats ($P < 0.15$, Kruskall and Wallis non-parametrical test).

production as compared to NO production (7 and 11 dpi as compared to 4 and 7 dpi, respectively) (Fig. 8). At 4 and 28 dpi, macrophage $O_{2^-}^-$ production was reduced as compared to the uninfected rats. In contrast to NO production, the addition of SOD did not influence the viability of the juvenile flukes (data not shown) and no correlation could be established between the O_2^- production level and in vitro juvenile viability.

3.4. Peritoneal cell toxicity in relation with juvenile ES products and the presence of flukes

Whether or not somatic or secreted antigens were responsible for this peritoneal cell activation could not be established within the framework of the previous experiment. To discriminate between both hypotheses, we injected rats intraperitoneally with either

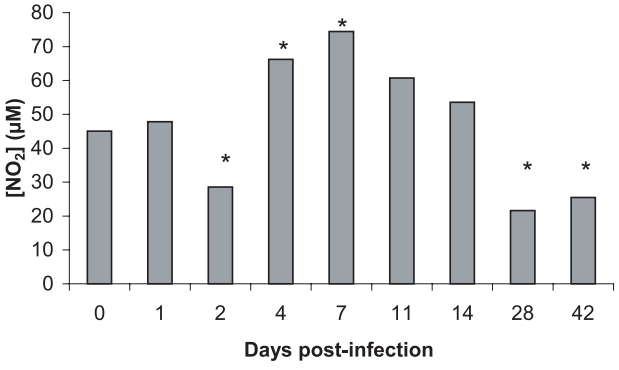

Figure 6. NO production by peritoneal cells from infected rats collected at various times after infection in the presence of juvenile flukes. NO_2^- was titrated after a four day period. * Indicates a significant difference when compared to uninfected rats ($P < 0.05$, Kruskall and Wallis non-parametrical test).

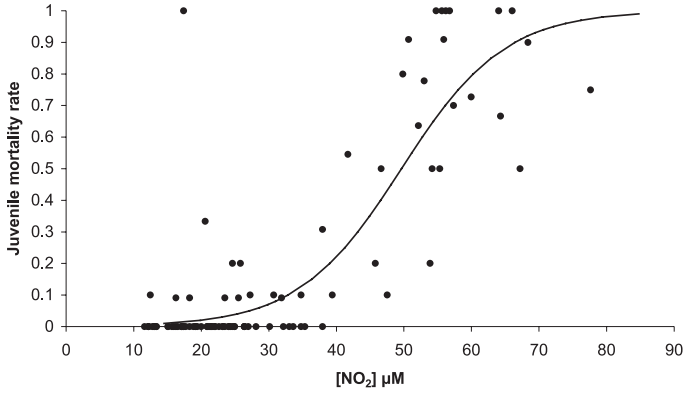

Figure 7. Juvenile toxicity represented as a function of NO concentration. Each plot represents an individual fluke. Statistical regression was performed with the SAS Probit procedure. Lethal Dose (LD)-50 and LD-95 were estimated at 45–50 and 78–96 µM, respectively.

PBS, fixed juvenile parasites (30 NEJs) or jES (24 h-culture excretion/secretion products of 30 NEJ in 1 mL RPMI medium). The rats were killed at 0, 7 or 14 days after injection. Peritoneal cells were cultured with juvenile flukes and parasite mortality was monitored every day. Mortality was recorded (after the fourth day of culture) only with parasites cultured with peritoneal cells from rats that had been injected with fixed juvenile *F. hepatica,* 7 and 14 days earlier (mortality reached 85 and 50% within 11 days of culture, respectively, Fig. 9). Individual analysis revealed that PC from half of the rats that were injected with parasites 14 days before sacrifice were able to kill parasites, while the cells from the remaining 50% were unable to do so. Significant killing was thus only observed with PC from rats exposed to fixed parasites, seven days before sacrifice. The other treatments induced no noticeable effect. Once

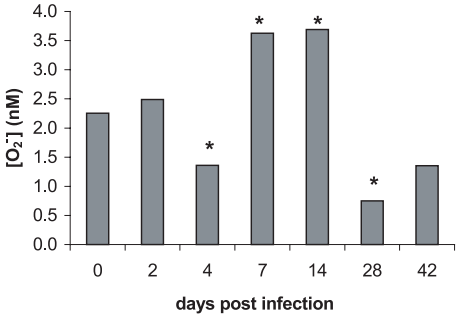

Figure 8. O_2^- production after 18 h culture of rat peritoneal cells at various post-infection times. Cell culture was performed without the addition of rat serum. * Represents a significant difference between uninfected- and infected-untreated rats ($P < 0.05$, Kruskall and Wallis non-parametrical test).

again, addition of LNMMA to the cultures totally abrogated the toxic effects.

To further characterise the role of RNI in juvenile toxicity, SNAP (S-nitroso-N-acetylpenicillamine) was used as a chemical NO donor. SNAP was selected for the high stability of the compound in aqueous solutions, and preliminary experiments established that 140 and 211 µM SNAP generated NO_2 concentrations corresponding to the lethal dose (LD) 50 (45 µM NO_2) and LD-95 (90 µM NO_2), respectively. An experimental procedure consisting of various treatments (dose and timing) revealed that NO alone was unable to induce juvenile death in a way comparable to that observed in cell culture (data not shown). These data suggest that NO alone is not toxic in itself to juvenile flukes in peritoneal cell cultures.

4. DISCUSSION

This was the first study revealing that juvenile flukes are transiently susceptible to the peritoneal cells of sensitised rats (infected or ip-injected with dead parasites). Rats develop a cytotoxic response against juvenile flukes after their penetration into the peritoneum. The following observations were

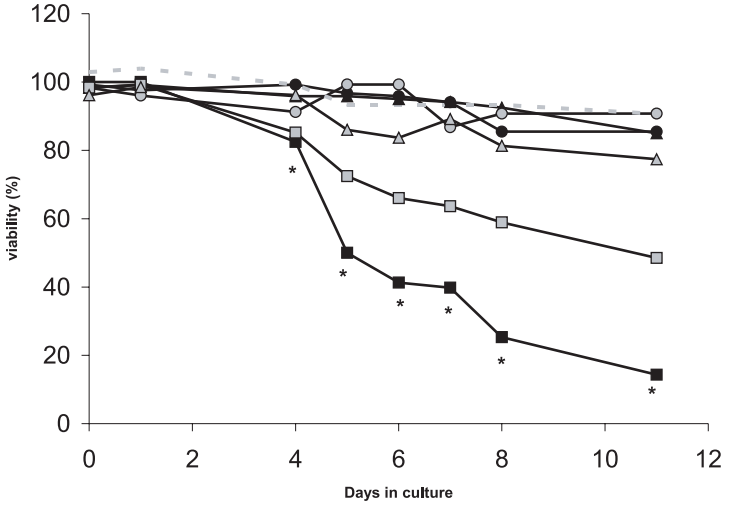

Figure 9. In vitro viability of juvenile *F. hepatica* exposed to peritoneal cells from rats that received ip injection of PBS (▲, ▲), jES (●, ●) or fixed juvenile flukes (■, ■) either seven or 14 days before sacrifice (black and grey symbols, respectively). Addition of L-NG-monomethyl arginine is represented by a dotted grey line. * Represents significant differences from the control juvenile culture ($P < 0.05$, Kruskall and Wallis non-parametrical test). Assays were conducted in the presence of 10% fetal calf serum.

made: (i) an early and sustained recruitment of peritoneal cells (from 8 to 50×10^6 collected peritoneal cells per rat); (ii) a rapid modification of the cellular composition. At the quantitative level, an increase in the eosinophil population and a decrease in both lymphocyte and mastocyte cells account for the most important phenomena. The increase in the macrophage population at 7 dpi is rather modest but coincides with the following: (i) a transient activation of these cells (O_2^- and NO production) and (ii) toxicity against NEJ at 7 dpi. This activation was independent of a previous non-specific stimulation since the juvenile mortality rate could not be achieved when rats were treated with a foetal calf serum or thioglycolate broth ip injection (personal unpublished data), although some reduction of the parasitic burden (40%) could be obtained with stronger peritoneal inflammation [3]. Conversely, one could still induce the PC cytotoxic response when dead parasites were injected ip. A previous study established that PC of four-week infected rats does not affect the survival of juvenile flukes cultured for 24 h and subsequently transferred to the peritoneal cavity of normal rats [8]. Conversely, Piedrafita et al. showed in a recent publication that naive rat PC are able to kill NEJ in vitro when cultured in the presence of immune serum of either rat or sheep origin, and that the cytotoxic mechanism largely involves NO production [25]. The discrepancies between these results and ours could be the consequence of different experimental procedures: the effector/Target (E/T) ratio in Piedrafita's study was 1 NEJ/2×10^5 PC, while the E/T ratio in the present study was 6–20 times lower (3 to 10 NEJ/10^5 PC). In both studies, the addition of naive or immune sera increased the cell attachment to juvenile flukes in vitro, but in our laboratory, this was not sufficient to promote juvenile death. This could indicate the presence of an inhibitory factor in the serum. This hypothesis is currently under investigation.

As noted for schistosomosis, *F. hepatica* infection appeared to lead to transient activation of PC since the killing of parasites in vitro was only observed with PC recovered from rats infected seven days before or injected with fixed juveniles seven days before. We hypothesize that *F. hepatica* exerts an active immunosuppression since PC recruitment still increased while the specific activity was being depressed (decreased cytotoxic response and NO production). This was in agreement with our observations showing a delayed hepatic immune response [29, 30].

The cytotoxic response by PC appeared to be mainly caused by reactive nitrogen intermediates since a strong and reproducible correlation was demonstrated between nitrite levels in the culture medium and juvenile mortality. Nitrite oxide has been widely implicated in the resistance to pathogenic agents such as bacteria [21], as well as parasites such as *Plasmodium* spp. [15] and *Trypanosoma* [11]. Juvenile *Schistosoma mansoni* schistosomules have been described as being highly sensitive to nitric oxide [16] in a developmentally stage-specific manner, the variations being attributed to changes in the larval metabolism. It is of interest to note that the killing of 95% of the juvenile flukes corresponds to nitrite concentrations three to four times greater than those required for the killing of schistosomules or plasmodium (90, 20 and 25 μM, respectively) [15, 16]. This suggests that in spite of its real ability to mediate toxicity, very high doses of NO are required in order to be effective against *F. hepatica*. Moreover, we have to consider that the effective death of the flukes required more than ten days of in vitro culture in our laboratory, although modifications in the comportment and structure of dying flukes could already be detected after 4 to 5 days of culture (consistent with Piedrafita's observations). The time of exposure to NO combined with the high doses of NO required may constitute a drastic limiting factor for an effective response to occur. In addition, we were unable to reproduce massive juvenile mortality using only SNAP as a chemical NO donor (80% mortality achieved with over 1 000 μM SNAP,

data not shown). The kinetics of NO liberation by SNAP and macrophage activation may differ but other chemicals such as DETA NONOate and sodium nitroprusside that display different dissociation kinetics in aqueous solutions yielded the same results (data not shown).

Our study shows that *F. hepatica* infection induces a significant increase and a reduction of O_2^- production by PC at 7 and 14 dpi, respectively. Curiously, this increase was slightly delayed as compared to the peak of NO production. Several studies have also demonstrated a major role played by reactive oxygen intermediates in the response of PC to invading agents. El-Ghaysh et al. reported that *F. gigantica* somatic and ES products could suppress the release of toxic oxygen intermediates by sheep neutrophils [10]. Cervi et al. showed that ES induces an O_2^- dependent decrease in normal rat splenocyte response to mitogens since the addition of SOD reverses the effects of ES antigens [4]. In our case, however, SOD in the culture medium did not affect the action of peritoneal cells against *F. hepatica* juveniles (data not shown). This result confirms what was observed by Piedrafita et al. [25].

In this experiment, jES alone was not able to modulate the cytotoxic response of peritoneal cells. Since we were unable to quantify the protein material released by the juvenile flukes (RPMI medium contains interfering free amino acids), it is possible that there was insufficient ES material to observe any biological effect or that this material was degraded between the time of collection and cell treatment. However, it is reasonable to assume that jES, just like adult ES, is able to modulate cellular responses [17, 18]. As shown by Cervi et al. [5], adult ES inhibited the proliferative response of splenocytes as well as NO production by macrophages. Modification of PC activity has also been demonstrated by these authors who showed an absence of a delayed hypersensitivity response in infected rats [5]. Similar results could also be obtained when adult ES was injected into rats one week before sacrifice. We are currently investigating the triggering and down-regulatory components released by the parasite during early *F. hepatica* infection.

To conclude, we show that the cytotoxic behaviour of PC obtained from infected rats in relation to *F. hepatica* is transient and highly correlated to NO production, although NO alone is not sufficient to mediate NEJ killing in vitro. Several complementary studies remain to be done to elucidate the particular role of jES products as immunomodulators of the early inflammatory response. In addition, we have to identify the serological factors responsible for the strong inhibition of the cellular cytotoxic response against NEJ. However, our results shed new light on the nature of host inflammatory responses to *F. hepatica*.

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