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Research Article

Arginase expression in peritoneal macrophages and increase in circulating polyamine levels in mice infected with *Schistosoma mansoni*

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Abstract. We investigated the nitric oxide (NO) synthase and arginase pathways in resident peritoneal macrophages of mice infected with the tropical parasite *Schistosoma mansoni*. The two enzymes may have opposite effects, insofar as NO may be involved in the killing of the parasite whereas arginase may stimulate parasite growth via polyamine synthesis. We determined the effects of the infection on the expression and activity of the two enzymes in macrophages, before and after cytokine activation. Cells from infected mice expressed the hepatic type I arginase, whereas in control cells, the enzyme was expressed only after cytokine activation, as were NO synthase II and type II arginase in both groups of cells. Moreover, we found that in infected mice, arginase expression in macrophages was associated with a ten fold increase in the concentration of circulating ornithine-derived polyamines. This may be of pathological importance, since parasitic helminths are though to be dependent on their hosts for the uptake and interconversion of polyamines.

Key words. Arginase; NO synthase; polyamine; infectious disease; schistosomiasis.

There is striking parallelism between nitric oxide (NO) synthase (NOS) and arginase. Both enzymes have constitutive and inducible isoforms and they share the same substrate, arginine. They are co-induced at inflammatory sites [1, 2], and in macrophages activated by lipopolysaccharide [3, 4]. NO production by NOSII in inflamed tissues is involved in the killing of intracellular pathogens [5], including *Trypanosoma* [6], *Leishmania* [7] and *mycobacteria* [8]. NO also has implications for tissue injury and hemodynamics. Arginase catalyzes the conversion of arginine to ornithine and urea. Type I arginase (AI) is expressed constitutively in the liver as part of the urea cycle. Its role in cells without a complete urea cycle is not clearly understood. In addition, AI and NOSII interact in many aspects. Arginase activity modulates NO production by reducing the availability of L-arginine to NOS [9], thereby limiting the potential pathological effects of high NO throughput [10]. Furthermore, arginase produces ornithine, the precursor of polyamine synthesis via ornithine decarboxylase (ODC) activity, which is modulated by NO [11]. Polyamines are essential for the proliferation of normal and neoplastic cells [12].

Thus, according to the review by Boucher et al. [13], the pathway used by macrophages to metabolize arginine may influence the type of host immune response against pathogens, parasites, and tumors. In particular, *Leishmania* growth is dependent on macrophage AI induction [14]. Parasites of relatively large size with no intracellular stage, such as *Schistosana mansoni*, raise however specific questions. The worms settle in the portal vein

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and the eggs lodge in the liver, inducing chronic liver inflammation with consecutive periportal fibrosis and portal hypertension. NO may exert a microbicidal effect at the lung stage of larval development [15]; however, NO is not sufficient to eliminate the parasite [16]. We showed earlier that the deposition of parasite eggs triggers NOSII expression in the liver. In this case, the overall effect of NO may be deleterious insofar as the NOS inhibitor monomethyl L-arginine (L-NAME) reduced liver injury [17].

In the present report, we examine the involvement of arginase AI in the pathology of the infection. Interest in this enzyme is reinforced by the fact that the AI gene maps to human chromosome 6q22–q23. The locus, denoted SM2, encodes a factor predisposing for severe disease in Sudanese populations at risk of schistosomiasis [18]. As AI is constitutively expressed in the liver, we investigated its induction in peritoneal macrophages following infection. Even though it is not in direct contact with the parasite, the peritoneal cavity is a secondary site of *S. mansoni*-induced inflammation [19]. Thus, we found that there is a fivefold increase in the number of macrophages harvested after a peritoneal wash (unpublished data). We also investigated the expression and activity of NOSII and the extrahepatic (AII) arginase isoform in the macrophages. We compared enzyme expression and activity in 'infected' and control cells (for the sake of brevity, we refer to peritoneal cells harvested from infected mice as 'infected' cells, even though they do not contain parasites). Studies were performed with or without activation with a combination of interferon (IFN)-γ and tumor necrosis factor (TNF)-α, since the two cytokines act together to activate macrophages to produce NO [20]. Moreover, mRNA transcripts of both cytokines are up-regulated in the liver tissue as a result of infection with *S. mansoni* [21].

We found that the infection triggered AI but not NOSII expression and activity in peritoneal macrophages. The event was associated with a ten fold increase in the circulating concentration of the polyamines of the ornithine pathway, putrescine, spermidine, and spermine. Moreover, polyamine levels correlated positively with the number of parasite eggs deposited in the liver.

Materials and methods

Materials

Culture medium (RPMI 1640) and superscript II Rnase H reverse transcriptase were obtained from Gibco-BRL (Grand Island, N.Y.); murine rIFN-γ and rTNF-α were from Boehringer Mannheim (Mannheim, Germany); rabbit anti-mouse NOSII and anti-mouse AI were from Transductions Laboratories (Lexington, Ky.); peroxidase-labelled anti-rabbit IgG was from Jackson Laboratories (West Grove, USA); [^{3}H]-L-arginine was from Dupont-NEN (Boston, Mass.); N$^{\omega}$-hydroxy-L-arginine (NOHA) was from Calbiochem-Novabiochem (Läufelfingen, Switzerland); L-NAME was from Calbiochem (La Jolla, Calif.); Taq DNA polymerase was from Appligen (Illkirch, France); prestained molecular- weight standards were from Biorad (Hercules, Calif.); nitrocellulose membrane and ECL were from Amersham (UK). All other reagents were purchased from Sigma (St. Louis, Mo.).

Animals and parasites

Eight-week-old female CBA/J mice (IFFA CREDO, France) were used. They had free access to water and a complete rodent diet. Animals were infected percutaneously with 150 cercariae of the Puerto Rican strain of *S. mansoni*, which is maintained in our laboratory by passage through *Biomphalaria glabrata* snails. Parasites circulate in the vascular system as schistosomula larvae. They reach sexual maturity 5 weeks post-infection (p.i.). Control mice received a saline injection and were raised under the same conditions as their littermates. All animals received humane care, according to the rules of 'Décret no.87-848 du 19/10/87, Paris.'

Cell culture

Non-elicited peritoneal exudate cells were collected from the experimental and control groups by peritoneal wash, after 8 weeks of infection. The exuded cells were seeded at 5×10^5 or 5×10^6 cells/well in 96- or 24-well culture plates, respectively, in RPMI 1640 medium supplemented with 2 mM glutamine, 1 mM HEPES, 50 µg/ml of gentamycin and 10% heat-inactivated fetal calf serum. They were incubated at 37 °C under 5% CO_2 in air. The culture medium contained 1 mM L-arginine. After a 2-h incubation, non-adherent cells were removed. The number of remaining, adherent, cells was determined by differential counting. Part of the cultures was activated with 10 U/ml rIFN-γ and 30 U/ml of rTNF-α.

Reverse transcription-polymerase chair reaction

Attached cells were harvested to determine NOSII, AI, and AII mRNA expression. The constitutively expressed HPRT gene was used as a control to verify equal loading of RNA and cDNA in the reverse transcription (RT) and polymerase chain reaction (PCR) reactions. Total RNAs were prepared from 5×10^6 cells using the guanidinium thiocyanate/phenol/chloroform single-step method [22]. Activated cells were harvested 48 h after activation; control cells were collected at the same time. Two-microgram aliquots of each RNA sample were reverse-transcribed using the superscript II Rnase H reverse transcriptase with random hexamers to prime the reverse transcriptase, following the manufacturer's instructions. PCR amplification of the cDNAs was conducted with

2.5 U of Taq DNA polymerase, 1.5 mM $MgCl_2$ and 200 ng of primers in a PTC 200 apparatus (Watertown, Mass). The amplification procedure consisted of (i) denaturation at 94 °C for 30 s, (ii) primer annealing for 30 s, and (iii) extension at 72 °C for 1 min. The following primer sets were used:

for NOSII: forward primer 5′-GTG-AGG-ATC-AAA-AAC-TGG-GG-3′; reverse primer 5′- ACC-TGC-AGG-TTG-GAC-CAC-TG-3′ (nt 419–438 and 781–798 in Genbank no M84373);

arginase I: forward primer 5′ GTC-CAG-AAG-AAT-GGA-AGA-GTC-AG-3′; reverse primer 5′-GTT-CCC-CAG-GGT-CTA-CGT-CTC-G-3′ (nt 400–418 and 679–700 in Genbank no. U51805);

arginase II: forward primer 5′-GGC-TAC-AGC-TGT-GTC-ACC-ATG-G-3′; reverse primer 5′-CAG-GAG-GCT-CCA-CAT-CTC-TCA-G-3′ (nt 382–403 and 646–667 in Genbank no. U90886);

HPRT: forward primer 5′-GTT-GGA-TAC-AGG-CCA-GAC-TTT-GTT-G-3′; reverse primer 5′-GAG-GGT-AGG-CTG-GCC-TAT-AGG-CT-3′ (nt 601–625 and 930–952 in Genbank no. J00423).

The temperature, number of cycles, and amplified fragment size were as follows. NOSII: 63 °C, 32 cycles, 380 bp; AI: 58 °C, 30 cycles, 300 bp; AII: 58 °C, 35 cycles, 280 bp; HPRT: 60 °C, 35 cycles, 350 bp. The amplified products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Western blot analysis

Cells were lysed in boiling lysis buffer (50 mM Tris-HCl pH 6.8, 1% SDS, 100 mM DTT, 10% glycerol, and 0.01 % bromophenol blue). Proteins (30 µg per lane) were separated on an SDS-polyacrylamide gel (7% for NOSII and 10% for AI) in the presence of prestained molecular-weight standards, and then transferred to nitrocellulose membranes. The membranes were incubated with a polyclonal anti-mouse NOSII or an anti-mouse AI diluted 1:2500. The membranes were subsequently incubated with horseradish peroxidase-labelled anti-rabbit IgG diluted 1:2500, then washed with Tris-buffered saline containing 0.1% Tween 20, and finally developed by an enhanced chemiluminescence technique (ECl; Amersham).

Nitrite assay

Nitrite in culture supernatants was measured using the Griess reagent; optical density was measured at 548 nm. Concentrations were determined by a standard curve using sodium nitrite.

Intracellular arginase activity

Intracellular arginase activity was measured in cell lysates as previously described [23]. Briefly, 5×10^5 cells were lysed in the presence of a cocktail of protease inhibitors. The extracts were centrifuged, then $MnCl_2$ (10 mM) and L-arginine (0.5 M) were successively added to the supernatants. After the reaction had been stopped by acidification of the medium, the urea formed was revealed by α-isonitrosopropiophenone. The colored product formed was quantified by absorption at 540 nm. Activity is expressed as $mU/10^6$ cells.

Conversion of arginine to ornithine and citrulline

Conversion of arginine to ornithine (arginase activity) and to citrulline (NOSII activity) were measured in intact cells using a method modified from Zhao et al. [24]. Briefly, after 48 h incubation in the presence of [^{3}H]-L-arginine, aliquots of supernatants were collected. Arginine, ornithine and citrulline were separated by thin layer chromatography, according to Iyengar et al. [25]. The plate was cut according to location of the standards and radioactivity was measured by scintillation counting.

Polyamine assay

Blood samples (200 µl) were collected and the red blood cells (RBCs) were separated by centrifugation. Cell and plasma compartments were assayed separately. Samples were homogenized in ice-cold $HClO_4$. The acid added was adjusted to a final volume of 200 µl and a final concentration of 0.4 N. After centrifugation, extracts and standards were dansylated according to the procedure described previously [26]. Briefly, 40-µl extracts were dansylated and prepurified on a Waters Sep-Pak C18 cartridge. Separation and quantification of polyamines were then performed by reverse phase-HPLC, using a Waters system with a Merk F 1050 fluorescence spectrophotometer (350 nm excitation and 495 nm emission). Polyamines were identified by their retention times. Peak areas were measured automatically by the integrator and evaluated according to the calibration method. The absolute limit of detection was 1 pmol for spermidine and spermine, and 7 pmol for putrescine.

Eggs deposited in the liver were counted after lysis of the organ in 4% KOH at 37 °C overnight.

Statistical analysis

All experiments were done in triplicate and were repeated at least three times. For mRNA and protein expression, the figures show typical images obtained. For polyamine assays, the number of animal used is mentioned in the table. The infected group was compared to the control group using the Student t test.

Results

All PCR reactions resulted in the amplification of a single product of the predicted size (fig. 1). Macrophages harvested from control mice expressed neither NOSII nor arginase before cytokine activation. The mRNAs were

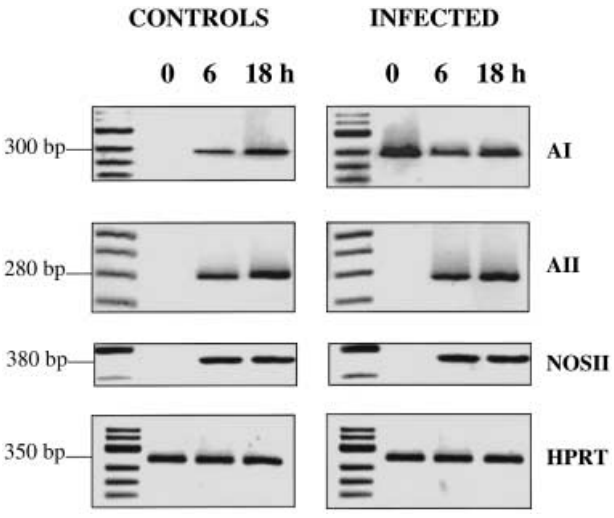

Figure 1. RT-PCR analysis of arginase AI, arginase AII, NOSII, and HPRT mRNAs in resident peritoneal macrophages, before and after in vitro activation with a combination of IFN-γ and TNF-α (6 and 18 h after activation). HPRT mRNA was used as a quantitative reference. Cells were harvested from mice infected with *S. mansoni* (INFECTED) and from their uninfected littermates (CONTROLS). Macrophages from control and infected mice behaved similarly, with the exception of arginase AI, which was expressed in cells from infected animals, even without cytokine activation.

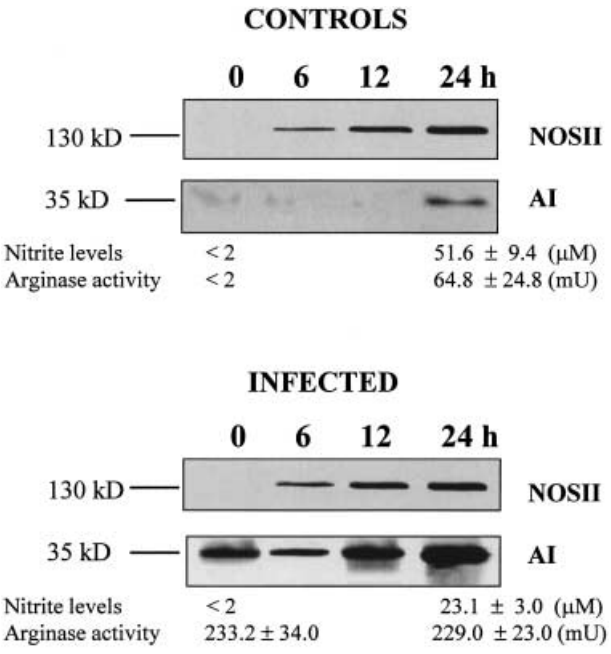

Figure 2. Western blot analysis of NOSII and arginase AI in peritoneal macrophages, before and after in vitro activation by cytokines (6, 12, and 24 h after activation). Arginase AI protein was present in cells harvested from infected animals, even without activation. In control cells, the enzyme appeared as a tiny band only 24 h after activation, whereas NOSII was detected as early as 6 h after activation by cytokines. Nitrite levels in the culture supernatants and intracellular arginase activity were determined either before or 48 h after cytokine activation. Arginase activity is expressed as mU per 10^6 cells.

present 6 h after activation. AI and AII expression were slightly delayed compared to NOSII, since a plateau was observed at 6 h for NOSII and later for the arginases. Beyond 18 h, mRNA concentrations remained constant for at least 48 h (data not shown). In macrophages harvested from infected animals, after 8 weeks of infection, the pattern of mRNA expression was similar to that of the controls for NOS and AII, with, however, the striking difference that AI mRNA was present even in non-activated cells.

The patterns of immunoreactive bands for NOSII and AI were in agreement with the mRNA expression described above (fig. 2). In controls, the NOSII immunoreactive band appeared as early as 6 h after the addition of cytokines and then increased with time. The appearance of arginase, however, was delayed compared to NOSII and it appeared only as a tiny immunoreactive band 24 h after activation. In cells harvested from infested animals, a 35-kDa band corresponding to the arginase AI protein was already present in non-activated cells. Arginase expression decreased slightly 6 h after cytokine stimulation and then increased. The time course of NOSII expression was comparable to that observed in controls.

Nitrite production and arginase activity were determined in the same cultures and the values are indicated below the protein lines in figure 2. Non-activated macrophages from both groups did not express NOSII and, accordingly, did not produce significant amounts of nitrite. Cytokine activation triggered enzyme expression and nitrite production in the two groups of cells. However, nitrite levels reached about 50 μM in the medium of control cells, and only half this value in cells from infected animals. This reduced nitrite production reflects arginine depletion by arginase, since the addition of arginine to the culture medium restored normal NO production (data not shown). In control macrophages, NOSII activity was not limited by arginase activity. The NOS inhibitor L-NAME completely inhibited nitrite production in all cells, confirming that nitrite formation was by the NOS pathway (data not shown). Intracellular arginase activity was undetectable in naive control macrophages whereas it reached about 230 mU/10^6 cells in 'infected' cells. Cytokine activation induced arginase activity in controls; however, the activity remained low (about 70 mU/10^6 cells) compared to the experimental group (230 mU/10^6 cells).

^{3}[H]-Arginine conversion confirmed the high activity of the parasite-induced arginase: almost all the arginine present in the culture medium of non-activated 'infected' macrophages was converted to ornithine (fig. 3). By contrast, no arginine conversion was observed in non-activated control cell cultures. Activation by cytokines, which induced the expression of NOSII (in both types of cell) and of arginase (in control cells) was followed by cit-

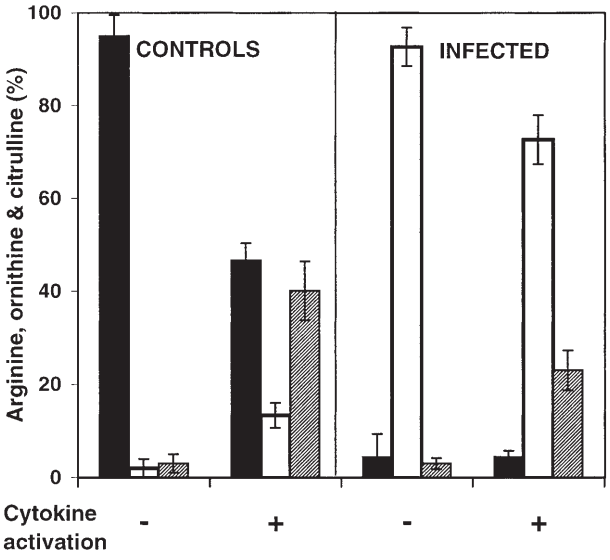

Figure 3. Conversion of [^{3}H]arginine to ornithine (arginase activity) and to citrulline (NOSII activity) by naive and cytokine-activated macrophages. Concentrations of arginine, ornithine, and citrulline were determined after 48 h of culture. Black columns, residual arginine; open columns, ornithine; hatched columns, citrulline.

rulline and ornithine production, accordingly. The reduced rate of arginine to ornithine conversion by 'infected' macrophages after cytokine treatment confirmed the reduction of arginase activity by IFN-γ.

Arginase induction in peritoneal macrophages was closely associated with parasite egg deposition in the liver, since AI mRNA was undetectable during the period of parasite larval development, i.e., during 5 weeks p.i. The mRNA became apparent after the onset of parasite egg laying, i.e., at 6 weeks p.i., and mRNA levels then increased (fig. 4). Arginase induction, combined with an increase in the number of resident peritoneal macrophages, was accompanied by a drastic increase in putrescine, spermidine, and spermine levels in the blood. The levels reached a ten fold increase at 8 weeks p.i., as compared to the controls (table 1). In the case of cadaverine, circulating levels were at the limit of detection in control mice and were undetectable in infected ones. About 80% of the blood putrescine was in the RBCs; for spermidine and spermine, the percentage was above 90%. Therefore, polyamine levels are expressed as nmol per 10^9 RBCs. Blood polyamine levels increased as soon as parasite eggs appeared in the liver (fig. 5). In mice sacrificed

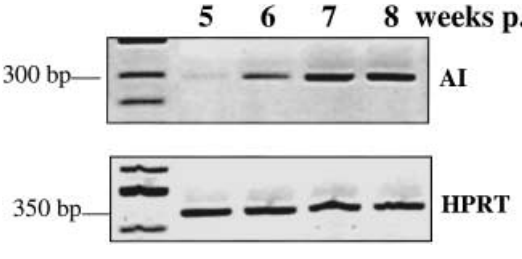

Figure 4. Time course of arginase AI mRNA expression in peritoneal macrophages following infection. Arginase expression was triggered 6 weeks after infection, i.e., when parasite eggs appeared in the liver.

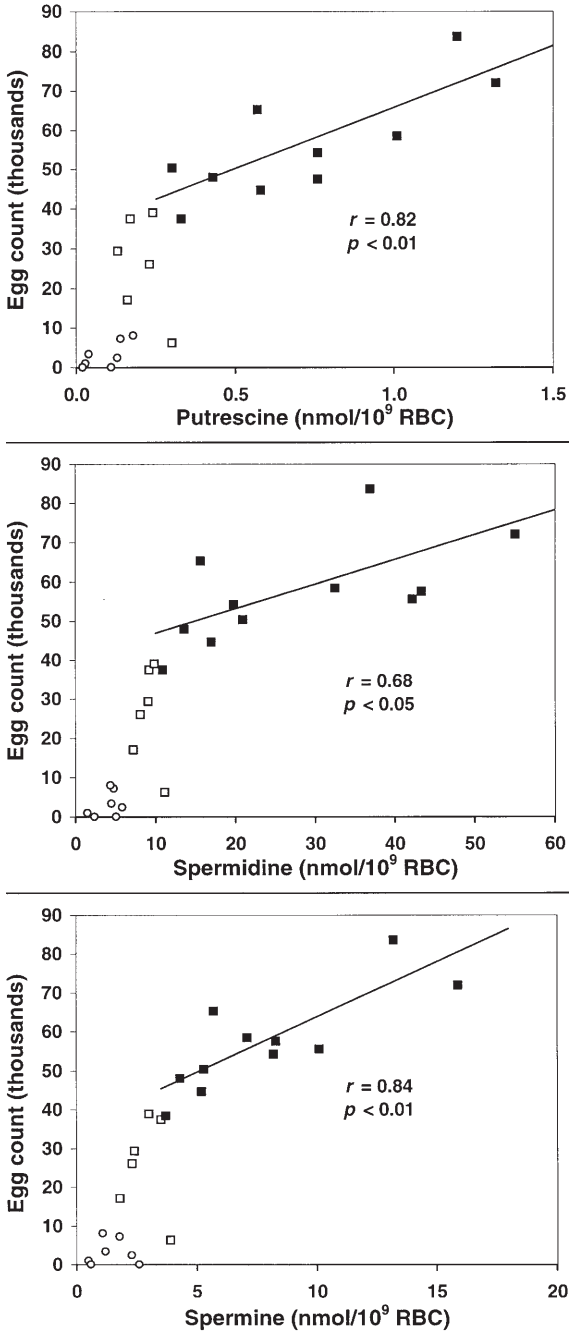

Figure 5. Number of eggs (thousands) deposited in the liver as a function of blood polyamine levels. Egg number and polyamine levels were determined at 6, 7, and 8 weeks p.i. (open circles, open squares and solid squares, respectively). The regression line for the third group (8 weeks p.i.) is displayed; r, p, correlation coefficient and the associated probability, respectively.

Table 1. Blood polyamine levels in infected mice and their control littermates.

Weeks post-infection	Infected			Controls 8 (n = 8)
	6 (n = 7)	7 (n = 6)	8 (n = 10)	
Putrescine	0.10 ± 0.06	0.21 ± 0.06	0.73 ± 0.38[a]	0 .07 ± 0.01
Spermidine	4.07 ± 1.43	9.09 ± 1.25	23.6 ± 5.35[a]	2.69 ± 1.19
Spermine	1.46 ± 0.77	2.83 ± 0.71	5.7 ± 2.48[a]	0.51 ± 0.23
Cadaverine	< DL	< DL	< DL	0.04 ± 0.05[b]

Polyamine levels are expressed as nmol/10^9 RBCs. Values are means ± SD; n, number of animals used.
[a] Significantly different ($p < 0.01$) from the cognate control as calculated by Student's t test.
[b] Value at the limit of detection of the method; < DL, value below the limit of detection.

8 weeks p.i., the number of deposited eggs correlated positively with putrescine, spermidine, and spermine levels.

Discussion

We found that hepatic arginase AI, but not NOSII expression and activity were triggered in peritoneal macrophages in response to infection with the parasite *S. mansoni*. AI expression started after the onset of egg laying and the levels of the enzyme paralleled the severity of hepatic injury. Accordingly, macrophages from infected animals displayed a high rate of arginine to ornithine conversion in vitro. The infection triggers NOSII expression only in tissue in direct contact with the eggs i.e., in the normal conditions of pathology, the liver [17]. In cultivated macrophages, the differential induction of NOSII, AI, and AII depends on the stimuli used to activate the cells [27]. Under our experimental conditions, treatment with IFN-γ and TNF-α induced the expression of the three enzymes in cells from uninfected animals. AI protein, however, appeared only as a tiny band with a 24-h delay as compared to NOSII. These findings are in agreement with those of Munder et al. [28] who found that IFN-γ in combination with TNF-α strongly induce NOSII but induce arginase only weakly. In macrophages from infected animals, the cytokine treatment induced NOSII and AII expression as it did in controls. Therefore, we can assume that, in vivo, the absence of NOSII activity in peritoneal macrophages of infected mice was not due to alteration of the NOSII pathway. As a logical consequence of the above statements, we observed a limited in vitro competition of the two enzymes in control macrophages. In contrast, in macrophages from *S. mansoni*-infected mice, arginase overexpression severely limited NO production. This finding is in agreement with the respective Km values of the two enzymes, which are in the millimolar range for arginase and the micromolar range for NOSII: a relatively high level of arginase activity is required to observe a marked reduction in NO production [27, 29].

Arginase AI expression in peritoneal macrophages confirms that the peritoneal cavity is another site of *S. mansoni*-associated inflammation, even though it is not in direct contact with parasites. Our finding is in agreement with quite old data showing an increase in arginase activity in serum and the liver of *S. mansoni*-infected mice [30]. As a result, there should be an increase in the synthesis of polyamines, as shown in activated RAW 264.7 macrophage cells [27]. We indeed found a tenfold increase in the ornithine-derived putrescine, spermidine, and spermine levels in the blood following infection with *S. mansoni.* By contrast, cadaverine, which is derived from lysine, was undetectable.

Another effect of the infection is to inhibit carbamoyl phosphate synthetase and carbamoyltransferase activities in mouse liver [19, 30]. Both enzymes contribute to ornithine consumption for citrulline synthesis; therefore, the inhibition reinforces the effect of arginase activity, the final result being an increase in ornithine stores available for polyamine synthesis.

Circulating polyamines were found almost entirely in RBCs, in agreement with studies in mice grafted with tumors, and the levels were comparable: about 30 nmol and 5 nmol per 10^9 RBCs for spermidine and spermine, respectively [31]. Polyamines are crucial for cell differentiation and proliferation [32]. Accordingly, the products of the arginase pathway, as well as exogenous putrescine, reduce macrophage cytotoxicity toward tumor cells and enhance tumor cell proliferation [33]. Moreover, tumor rejection is accompanied by an upshift in NOS activity and a decrease in arginase activity, whereas tumor proliferation is associated with a decline in the production of citrulline and a marked increase in ornithine and polyamine synthesis [34]. Polyamines are also required for the growth of parasites such as *Trypanosoma* and *Leishmania*, therefore providing a potential target for chemotherapy [35, 36]. No data are available regarding the occurrence and role of polyamines in *S. mansoni*. Enzymes of polyamine biosynthesis were found to be present in very low to negligible amounts in the helminth species examined. This suggests the dependence of these parasites on their hosts for the uptake and interconversion of polyamines [37]. Therefore, the high levels of circulating polyamine found in *S. mansoni*-infected mice might benefit the parasite. This assumption is strengthened by the fact that *S. mansoni* feeds on RBCs, where polyamines are stored. Moreover, we showed that the number of eggs deposited by the parasites correlates positively with circulating polyamine levels. However, a cause and effect relationship between the two events remains to be established. Polyamines might also stimulate liver fibrosis, since they are known to produce an environment fa-

vorable for fibroblast replication and collagen deposition [38]. The relationship between the arginase pathway and fibrosis is illustrated by the fact that interleukin 13, which up-regulates arginase expression [39], is a profibrotic agent [40]. Therefore, hepatic arginase induction following infection and the associated elevated polyamine levels in the blood might be relevant physiopathological parameters in human schistosomiasis.

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