Research Paper

Pharmacological evidence supporting a role for IL-1, IL-2 and serotonin in the inflammation induced by *Schistosoma mansoni* Soluble Egg Antigen (SEA) in rat paws

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THIS study intended to characterize pharmacologically the mediator(s) released in the inflammation induced by Soluble Egg Antigen (SEA), the main antigen released from eggs of *Schistosoma mansoni*, in rat hindpaws. A single intraplantar injection of 0.1–100 µg SEA at day zero induced a dose-dependent increase in the volume of rat hindpaws characterizing an oedema of quick onset (within 15 min) and 4 h-duration, which was confirmed by histopathological analysis of the paws. A second injection of SEA in the same paw (1–10 µg) 28 days later induced an increased dose-dependent oedematogenic response. The early oedematogenic response following SEA sensitization was derived from serotonin release and interleukin-1 (IL-1), since treatment with either pizotifen or an antibody against IL-1, reduced the response by 60% and 48%, respectively. The increased oedematogenic response derived from SEA-challenge (10 µg) of rat paws derived from a local rather than systemic reaction, since it was not observed if the sensitization was in the contralateral paw or the peritoneal cavity of the animals. Chronic treatment with inhibitors of IL-2 synthesis/release such as cyclosporin or dexamethasone during the sensitization phase reduced the oedematogenic response due to SEA challenge by 51% and 55%, respectively. These data suggested that SEA-challenge was immune-derived and dependent of IL-2 release. It is discussed the association between cytokine release and the resistance of rats to *S. mansoni* infection.

Key words: *Schistosoma mansoni*, Soluble Egg Antigen (SEA), Oedema, Serotonin, IL-1, IL-2, Cytokines

Introduction

Schistosomiasis, a chronic disease caused by infection with the helminths *Schistosoma mansoni*, *Schistosoma haematobium* or *Schistosoma japonicum*, presents a worldwide distribution and a varied degree of severity.[1,2] *S. mansoni* is the predominant schistosome found in the Western world, apart from being also found in the Middle East and Africa,[2] which induces a disease of moderate severity.[1]

The severity of schistosomiasis relies on the modulation of a tissue granulomatous response derived from the release of adult worm antigens in the host circulation associated with the slow release of larval-egg cross-reactive antigens and subsequently by egg-specific antigens.[3,4] Soluble Egg Antigen or SEA is the term used to collectively characterize the antigens released by the eggs deposited by *Schistosoma* worms in the tissues and is composed by nine fractions of proteins with varied profile of immunogenicity.[4] The principal source of SEA is the skin of the embryo inside the egg, which at this stage, is called miracidium, although it can also be released from the adult worm and from cercariae.[3]

The host granulomatous response to *S. mansoni* infection in sensitive species can be down-modulated by enhanced T-suppressor cell-activity and diminished CD4^{+} T-lymphocyte responsiveness expressed by reduction in cytokine release and lymphocyte proliferation.[5,6] Curiously, it has been demonstrated that rats are resistant[7] or 'non-permissible'[8] hosts to *S. mansoni* infection.

In past years, SEA has been used as a tool to study immune-mediated inflammatory reactions[9] and the development of a vaccine against schistosomiasis mansoni has progressively been appreciated.[10–13]

In the present study, we used a preparation of eggs and SEA from *S. mansoni* injected intraplantarly to evaluate the mechanism of inflammation induced in rat paws.

Materials and Methods

Animals

Holtzman female rats weighing 150–200 g were housed at 25±3°C, under a 12 h light/dark cycle (lights on 07:00 h) with water and food *ad libitum*. Male albino Swiss mice were used as a source of *S. mansoni* eggs. In all experiments, the number of animals used varied between 3 and 16.

Obtaining *S. mansoni* eggs and SEA

S. mansoni eggs were obtained from the liver of mice infected with cercariae 8 weeks before, as previously described.[14] The purified eggs were fragmented in tissue grinders using phosphate-buffered saline (PBS, 0.01 M, pH=7.4). This procedure determined miracidium release thus facilitating the obtainment of SEA (*Schistosoma mansoni* Soluble Egg Antigen). The crude material was centrifuged at 100 000 g for 2 h at 4°C. Various supernatants (SEA) were combined and evaluated for protein content by the Lowry method[15] and kept at –20°C until assayed. Only one pool of SEA has been used throughout the present studies.

Oedema measurements

Measurements of the volume of rat paws were carried out using a hydroplethysmometer Ugo Basile (model 7150) before and after an intraplantar (i.pl.) injection of various doses of a suspension of *S. mansoni* eggs (2000 eggs/ml in PBS) or SEA diluted in 0.1 ml of physiological saline at zero time. A second injection of SEA (in 0.1 ml sterile physiological saline) was also given 28 days later in the same paw whenever otherwise stated. The contralateral paw received the same volume of sterile physiological saline. Results are presented as the mean difference between volumes (ΔV, μl) obtained from the paws injected with eggs or SEA and those injected with saline ± standard error of the mean (SEM) in each group. In all the experiments the measurements were obtained at 5, 15, 30, 60, 120, 240 min and 24 h following intraplantar (i.pl.) injections (n=4–13/group).

Selection of an inhibitory dose of an antibody against interleukin 1β (IL-1β)

To assess an inhibitory dose of an antibody against human IL-1β, two dilutions (1/20–1/200) of a commercial preparation of an antibody injected by subcutaneous (s.c.) route were tested in an assay of leukocyte migration induced by intraperitoneal (i.p.) injection of human IL-1β in rats. The technique used to count leukocytes was described elsewhere.[16] In the assay, it has been shown that 1.45×10^{-10} M of human IL-1β increased the rat peritoneal leukocyte number by 29% ($P < 0.05$, Anova t-test, Fig. 1). A
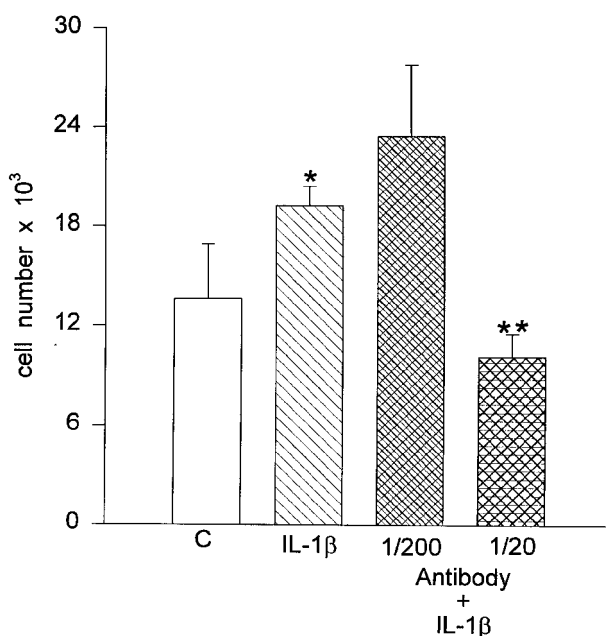

FIG. 1. Inhibition of human interleukin-1β-induced leukocyte recruitment to peritoneal cavity of rats by subcutaneous injection of an antibody against human IL-1β. Cell counts were performed 4 h after i.p. injections. Control bar (C) represents counts of leukocytes from animals injected i.p. with 1 ml of sterile physiological saline. Sterile IL-1β was injected i.p. at 1.45×10^{-10} M. Dilutions of the antibody were also made in physiological saline. *Represents $P < 0.05$ (Anova t-test) compared with control animals (n=5–7).**Represents $P < 0.01$ from comparison with IL-1β-injected animals (Anova t-test).

dilution of 1/20 of the antibody reduced the leukocyte counts increased by human IL-1β to the level of control animals (> 99% reduction, Fig. 1), whereas a 1/200 dilution of the antibody did not significantly alter human IL-1β-induced effect. The 1/20 dilution of the antibody was used in rat paws injected with SEA for further studies.

Experimental protocols

Four sets of experiments were established to evaluate the participation of known mediators release in the inflammation induced by eggs or SEA from *S. mansoni* in rat paws. All antagonists used inhibited at least 40% of the maximum oedematogenic effect of their respective agonists injected into the rat paws and tested in parallel assays. In the case of HOE 140 (bradykinin antagonist), L-NAME (nitric oxide synthase inhibitor), indomethacin and dexamethasone (non-steroidal and steroidal anti-inflammatory drugs, respectively) the agonist used was carrageenin (250 µg/site). For pizotifen, pyrilamine and SR 140333[17] a dose of 5 µg of serotonin (5-HT), 50 µg of histamine (H) and 50 µg of Substance P (SP), respectively, were used per site. In the first set, effective doses of pizotifen, pyrilamine, indomethacin or intramuscular (i.m.) dexamethasone were acutely admin-

istered 30 min before i.pl. injection of SEA (time zero), as shown in a previous work.[18] The compound SR 140333 was i.p. injected 15 min before SEA. The increase in paw volume was obtained as described above during sensitization and challenge phases. In the second set of experiments, pizotifen, dexamethasone and indomethacin were acutely administered by i.pl. route 30 min before challenge with SEA (10 μg/paw). In the third set, dexamethasone and cyclosporin A (an immunosuppressive drug) were chronically administered by i.m. route from day 0 to day 14 following SEA sensitization (10 μg/paw) and oedema measurements were obtained after SEA challenge (10 μg/paw). Finally, a 1/20 dilution of an antibody against human IL-1β was also administered by i.pl. route 15 min before SEA sensitization (10 μg/paw) and the resultant effect on SEA sensitization (10 μg/paw) was also evaluated. The dose of all drugs used were depicted in the tables or figure legends.

Histopathological studies

Rats injected intraplantarly with SEA were sacrificed with ether in different time points and the hindpaws immediately cut at the tibial-tarsic joint. A square of 1 cm was made with a sharp scalpel in the pads of each paw (control and SEA-injected) around the site of injection to permit easy fixation of the tissue which was then quickly immersed in Bouin fixative overnight. The following day Bouin solution was exchanged by ethanol 70% and the paws were left in this solution until embedded in parafin and cut in sections of 5 μm. Slides containing the sections of paw tissue stained by haematoxilin-eosin were evaluated for oedema and cell migration in various time points by optical mycroscopy (1000-fold amplification).

Drugs

Salts used in the present work and the following chemicals were purchased from Sigma: pyrilamine, indomethacin, L-NAME, dexamethasone, human interleukin 1β and anti-human IL-1β (developed in rabbit, IgG fraction). Pizotifen and cyclosporin A, SR140333 (a NK₁-receptor antagonist) and HOE 140 were kindly donated by Flávio J. R. de Aguiar (Sandoz, Brazil), Xavier Emonds-Alt (Sanofi, Montpellier) and Dr Mauro M. Teixeira (Departamento de Farmacologia, ICB, UFMG), respectively.

Statistical analysis

The increase in volume of rat paws or peritoneal cellular counts (mean ± standard error of the mean) following administration of the agonists whether or not under different treatments were compared with a control using Student's *t*-test for single comparisons
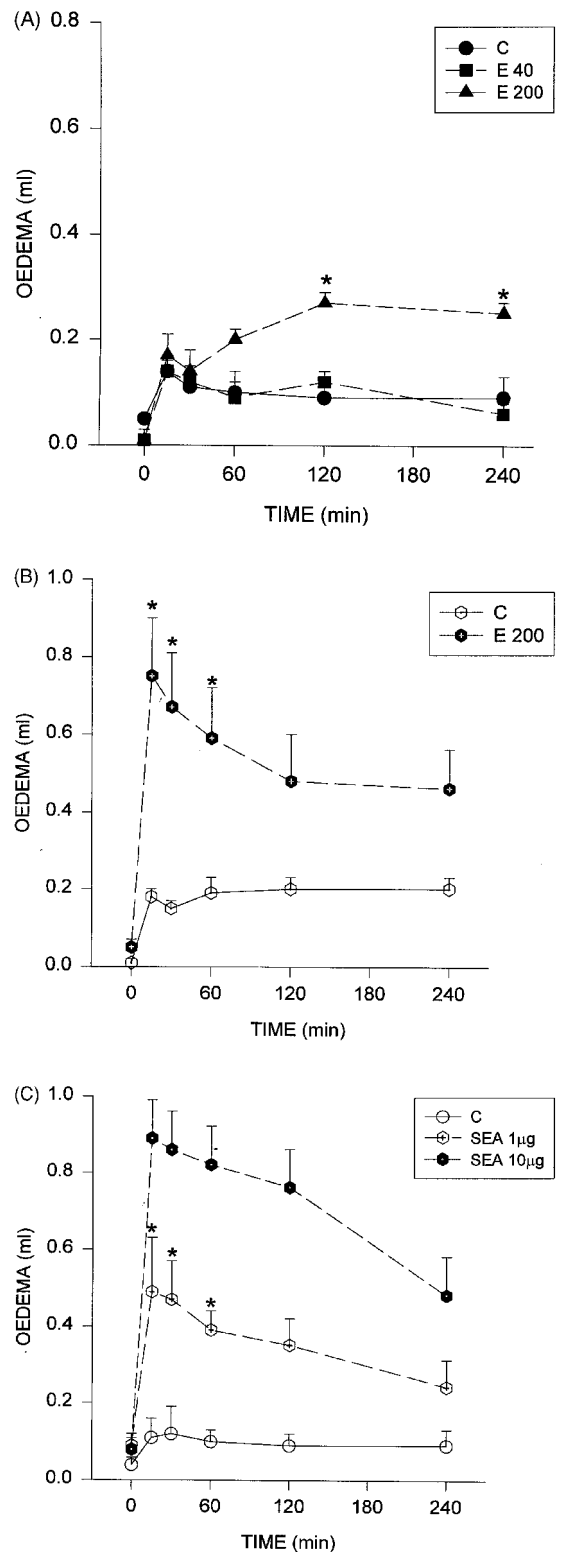

FIG. 2. Intraplantar injection of SEA (*S. mansoni* Soluble Egg Antigen) in rats reproduces the oedematogenic response induced by challenge of sensitized rats with *S. mansoni* eggs. In (A), it is shown the oedematogenic effect induced by different doses of eggs in sensitization (*n*=4–6). In (B), the oedematogenic effect induced by a second injection of eggs in rat paws is shown (*n*=4–6). In (C), 1 or 10 μg/site of SEA were injected i.pl. instead the eggs in challenge (*n*=3–6). Measurements were obtained in a hydroplethysmother (Ugo Basile) at zero, 15, 30, 60, 120 and 240 min following i.pl. administration of the 40 or 200 eggs in 0.1 ml of physiological saline/site [E].

Table 1. Anti-oedematogenic effect of known inhibitors of the syntesis of inflammatory mediators and antagonists on the oedema induced by SEA following sensitization of rat paws

| Drug (mg/kg); route	Receptor/mechanism involved	Agonist	Oedema on sensitization[a] (ml)	
			Control	Treated
Pizotifen (2); s.c.	5-HT$_2$	Serotonin	0.47 ± 0.04	0.19 ± 0.05*
Pyrilamine (2); s.c.	H$_1$	Histamine	0.30 ± 0.05	0.31 ± 0.05
SR 140333 (1); i.p.	NK$_1$	Substance P	0.37 ± 0.04	0.35 ± 0.03
Indomethacin (2); s.c.	Cyclooxygenase inhibitor	Carrageenin	0.29 ± 0.02	0.26 ± 0.06
Dexamethasone (1); i.m.	Phospholipase inhibitor	Carrageenin	0.30 ± 0.09	0.20 ± 0.07
L-Name (0.5); s.c.	Nitric oxide synthase inhibitor	Carrageenin	0.36 ± 0.06	0.32 ± 0.02

[a]Values were recorded at maximal oedematogenic effect (mean ± SEM). Significance of changes are indicated (*$P < 0.05$, Anova *t*-test).

or Anova for multiple comparisons. Analysis was developed by the SigmaStat software, accepting a value as statistical significant when $P<0.05$.

Results

Intraplantar administration of *S. mansoni* eggs (40 or 200 eggs/0.1 ml) induced a dose-dependent increase in rat paw volume thus characterizing oedema formation which was maximal at 120 min following administration (0.27 ± 0.03 ml), remained significantly elevated by 240 min (0.25 ± 0.02 ml; Fig. 2A) and subsided 24 h later (data not shown). A second injection of 200 eggs in the same paw 28 days later

induced an increased oedematogenic response at 15 min following injection (0.75 ± 0.15 ml; Fig. 2B), which was reproduced if 10 μg SEA was used instead of *S. mansoni* eggs in the challenge (0.89 ± 0.10; Fig. 2C). Sensitization of rats with SEA also induced oedema which was maximal 15–30 min following injection and lasted for 4 h, depending on the dose used (Fig. 3). No oedema was detected following i.pl. injection of SEA after 24 h (Fig. 3). However, the oedematogenic activity of SEA was progressively decreased during the development of the experiments. Such variation is illustrated in the column of control results in Table 1. The acute oedema following SEA sensitization of rats was 60% reduced by previous systemic administration of pizotifen (2 mg/kg) s.c., but it was not modified by effective anti-oedemagenic doses of pyrilamine (2 mg/kg) s.c., SR 140333 (1 mg/kg) i.p., indomethacin (2 mg/kg) s.c., L-NAME (0.5 mg/kg) s.c., or dexamethasone (1 mg/kg) i.m. (Table 1). In addition, the increased oedematogenic effect presented by the latter animals to SEA challenge was not modified 28 days later (data not shown). Challenge of animals with 1 or 10 μg SEA induced a dose-dependent increase of the oedematogenic response in animals previously sensitized with 10 μg SEA (Table 2). However, this increase was not seen if
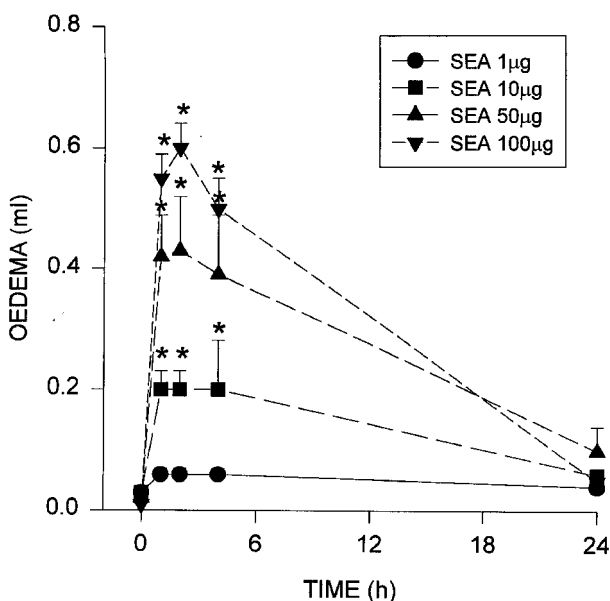
FIG. 3. Dose–response (oedema) curve induced by administration of *S. mansoni* Soluble Egg Antigen (SEA) in rat paws. Various doses (1, 10, 50 and 100 μg/site) of the antigen were diluted in 0.1 ml physiological saline and injected intraplantarly in rat hindpaws. Measurements were obtained in a hydroplethysmother (Ugo Basile) at zero, 15, 30, 60, 120, 240 min and 24 h following administration of the antigen (*n*=6–15). Statistical significance is indicated by *$P<0.05$ (Anova *t*-test).

Table 2. Increase of the oedematogenic effect induced by different doses of SEA in challenge of rat paws sensitized 28 days before

SEA dose on sensitization (μg/site)	Oedema[a] (ml)	
	1 μg	10 μg
Control	0.08 ± 0.02	0.32 ± 0.05
1	0.06 ± 0.01	0.39 ± 0.07
10	0.38 ± 0.10	0.57 ± 0.01*
50	0.40 ± 0.10	0.40 ± 0.08
100	ND	0.50 ± 0.05

[a]Values were recorded at maximal oedematogenic effect (mean ± SEM) 28 days later (challenge). Significance of changes is indicated (*$P < 0.05$, Anova *t*-test) and determined vs. control for the same dose of SEA.

Table 3. Local effect of pizotifen, indomethacin and dexamethasone on oedema induced by SEA challenge of rat paws

Drug	Receptor/mechanism involved	Agonist	Oedema in challenge[a] (ml)	
			Control	Treated
Pizotifen	5-HT$_2$	Serotonin	0.41 ± 0.03	0.18 ± 0.02*
Indomethacin	Cyclooxygenase inhibitor	Carrageenin	0.30 ± 0.03	0.32 ± 0.03
Dexamethasone	Phospholipase A$_2$ inhibitor	Carrageenin	0.32 ± 0.04	0.21 ± 0.05

[a]Values were recorded at maximal oedematogenic effect (mean ± SEM). Significance of changes is indicated (* $P < 0.05$, Anova *t*-test). Drugs (100 μg/site) were administered intraplantarly 30 min before SEA.

higher doses of SEA were used in sensitization, as shown in Table 2. This secondary oedema was also 57% reduced by acute local treatment of the paws with pizotifen (100 μg/site), but not with the same dose of indomethacin or dexamethasone (Table 3). The dose of 10 μg/paw of SEA in challenge was chosen as standard for posterior studies. Interestingly, if the sensitization with 10 μg SEA was done in the contralateral paw (clp) or intraperitoneally (i.p.) in the animals 28 days before, no increase was observed in challenge (oedema following clp: 0.30 ± 0.04; i.p. = 0.30 ± 0.04, respectively). In addition, this lack of effect on challenge to SEA in other location of the body was independent of the dose of the antigen used in sensitization (data not shown). Histopathological studies confirmed the early oedema (15 min) induced by SEA, showing vasodilation of the pad micro-vasculature, separation of collagen fibres and oedema fluid being drained by lymphatic vessels at this time point (data not shown). Recruitment of a mixed population of leukocytes (poly and mononuclear cells) was observed around 2 h of the injections, being increasingly substituted by mononuclear cells at 24 h following injection. Chronic treatment of rats with i.m. 0.1 mg/kg/day dexamethasone or 5 mg/kg/day cyclosporin from day 0 to day 14 of SEA-sensitized animals reduced by 51% and 54%, respectively, the oedematogenic response on SEA challenge, i.e. 14 days later (Table 4). Observation of the animals treated with such doses on sensitization (day 0) did not change the immediate followed oedematogenic

response (data not shown). Acute treatment of rat paws with an antibody anti-human IL-1β reduced by 48% the oedematogenic response induced by SEA on sensitization of rat paws (Fig. 4).

Discussion

Development of schistosomiasis, a parasitic disease due to the Trematoda *Schistosoma mansoni*, is highly dependent on the antigen burden derived either from the adult worms present in the circulation or from the eggs usually deposited in the liver of the host.[8] The substances extracted from the eggs of *S. mansoni* are collectively named by SEA[4] which seems to be the main inducer of a modulated response in the chronic phase (granulomatous) of the disease,[5,19] In addition, SEA has been used as a

Table 4. Effect of chronic treatment with systemic dexamethasone and cyclosporin on the oedematogenic effect of SEA on challenge of rat paws
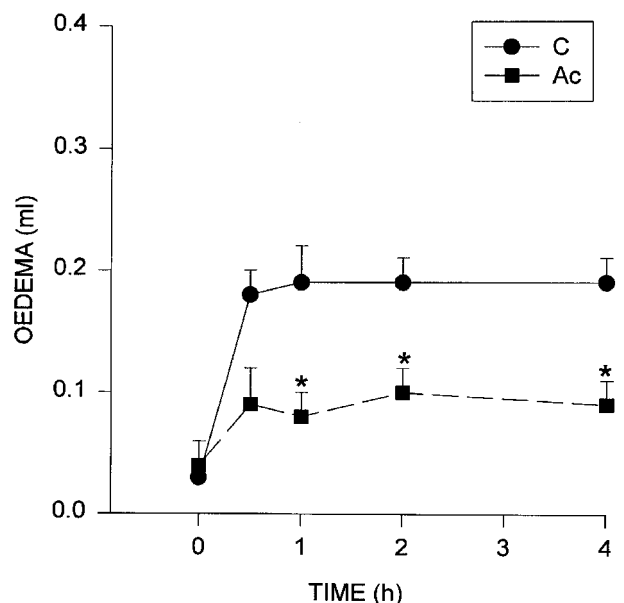
Drug (mg/kg/day)	Oedema[a] (ml)		% Inhibition
	Control	Treated	
Dexamethasone (0.1)	0.39 ± 0.03	0.20 ± 0.02*	51
Cyclosporine (5)	0.62 ± 0.10	0.34 ± 0.05*	54

[a]Values were recorded at maximal oedematogenic effect (mean ± SEM). Drugs were administered at the indicated doses from day 0 to 14 of sensitization. *$P < 0.05$ (Anova *t*-test).

FIG. 4. Reduction of oedematogenic response to SEA sensitization by an antibody anti-IL-1β. The antibody was injected s.c. 30 min before injection of the antigen. Measurements were obtained in a hydroplethysmother (Ugo Basile) at zero, 15, 30, 60, 120, 240 min and 24 h following administration of the antigen ($n = 4$–5). Statistical significance is indicated by *$P < 0.05$ (Anova *t*-test).

tool to understand the mechanisms of disease involving the immune system in experimental studies.[9] In the present work, we have shown that *S. mansoni* eggs induced inflammation in rat pads, and that SEA reproduced the oedematogenic effect of the injection of *S. mansoni* eggs, thus demonstrating that the main oedematogenic effect derived from eggs is due to SEA. We have also shown that SEA induced an increased oedematogenic response on a second administration to the animals (challenge), after a period of 28 days, suggesting the involvement of the immune system in the latter response. A surprising observation, however, was that the involvement of the immune system was local rather than systemic, since the increased effect on SEA challenge was only observed if the antigen were injected in the same location of the sensitization, i.e. into the ipsilateral paw. This result suggested that (1) SEA is a weak antigen, or conversely, rats restrain very effectively the immune response to it, and (2) SEA is very easily cleared out of the animal bodies, unless it is continuously delivered from the eggs into the circulation, as it happens during the course of the disease in sensitive species. Irrespective of the increase in polymorphonuclear and mononuclear cells (macrophages and lymphocytes) into the rat paws, as shown by histopathological studies, the cellular source of the 'local' immune response to SEA was not addressed in the present work. It was established at least 30 years ago by Parrat and West[20] that serotonin (5-HT) was the main inflammatory mediator released in rat pads. In fact, here we demonstrated that serotonin accounted for 60% and 57% of the oedematogenic response to SEA in sensitization and challenge of rat paws, respectively, since pizotifen, a 5-HT$_2$ antagonist,[21] blocked both systemically and locally the response. The release of other vasoactive substances by SEA, such as bradykinin, histamine, substance P, prostaglandins, leukotrienes, nitric oxide or platelet activating factor (PAF) was discarded, since previous acute administration of their specific antagonists or synthesis inhibitors have not modified the oedematogenic response observed following SEA sensitization. Therefore, it was reasonable to suppose that the early serotonin release (by 15 min) induced vasodilation and increased vascular permeability and together with IL-1 (see below) contributed to the arrival of circulating leukocytes to the paw tissue shown in later times by histopathological studies. As those treatments have not modified the oedematogenic response to SEA observed on challenge, we could also conclude that all the mediators above mentioned were not important in the pathophysiology of the latter response. However, to our knowledge, it is the first demonstration of an oedematogenic effect which was not acutely sensitive to the potent and efficacious steroidal antiinflammatory drug dexamethasone.

The participation of cytokines in the inflammatory reaction due to SEA has been reported elsewhere.[4,12] Our data support the notion that the oedematogenic effect induced by SEA in challenge is immune-derived and depends on the release of cytokines in early stages of the reaction. Basically, three arguments favour our hypothesis: (1) a second injection of the antigen induced a potentiated oedematogenic response; (2) drugs with immunosuppressive activity, such as dexamethasone and cyclosporin chronically administered during the early stages of the reaction greatly reduced (> 50%) the oedematogenic effect observed in challenge; and (3) an antibody specific against IL-1β was effective in inhibiting by approximately 50% the oedematogenic response to SEA. It has been exhaustively shown that dexamethasone and cyclosporin are able to inhibit the synthesis/release of various cytokines, specially interleukin-1 and 2,[22,23] the mainstay cytokines involved in the processing of immune responses.[24,25] As an antibody specific to human IL-1β significantly blocked the oedema from 1 to 4 h of SEA sensitization of rat paws by approximately 50%, it was concluded that IL-1 was an important effector of the pathophysiology of this oedema. In such case, IL-1 could effect this response through recruitment and activation of leukocytes as observed by the arrival of these cells to paw tissue from 2 h of SEA injections as confirmed by histopathological studies. As dexamethasone treatment of rat paws before SEA sensitization did not modify the early oedematogenic response, it was concluded that the detected reduction of the oedema by the antibody during sensitization was, at least partially, due to its binding to a preformed IL-1, eventually the biological/immunological analogous of IL-1β, IL-1α.[24] In addition, IL-1 could be acting as an important effector of other reactions of the immune response to SEA, such as the activation and proliferation of T-cell lines, since the potentiated oedematogenic response on SEA challenge was observed just in a narrow dose range (1–10 μg). It might be emphasized that, although we have used a human IL-1β and a antibody anti-human IL-1β in rats, the migration of leukocyte and its blockade by the antibody were very consistent (29% increase and > 99% inhibition, respectively), suggesting that the site of interaction between IL-1 and the host is relatively independent of its source and was conserved in the evolution. Our data have strongly suggested that interleukin 2 is involved in the processing of the immune response to SEA in rats, since early treatment of the sensitized animals with cyclosporin, as with dexamethasone, greatly inhibited the oedematogenic response on challenge (> 50%). Assuming that interleukin-2 is released by SEA, it is tempting to speculate that the immune response derived from SEA injection in this species might involve predominantly the differentiation of the lymphocytes into a Th1 subset, rather than a Th2
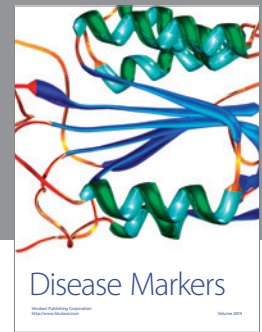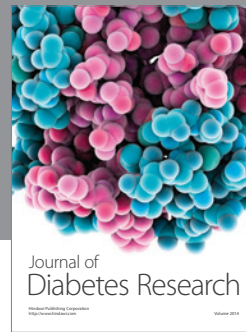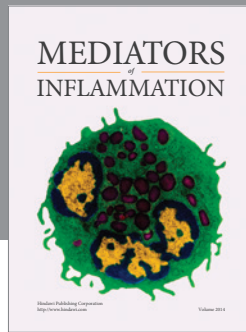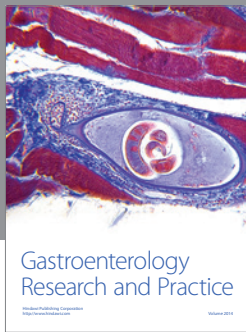
subset.[26,27] If it is so, other cytokines such as interferon gamma and interleukin 12 (IL-12) may eventually be detected in this model. Recent papers have shown that exogenous IL-12 can increase vaccine-induced immunity to *S. mansoni* in mice.[11,28] Therefore, the association of IL-12 release with a very effective immune response induced by IL-1 and IL-2, as suggested by our results, could explain at least in part, the resistance of rats to *S. mansoni* infection.

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