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Nitric Oxide-Mediated Immune Complex-Induced Prostaglandin E_2 Production by Peripheral Blood Mononuclear Cells of Humans Infected with *Schistosoma mansoni*

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Granuloma reaction around *Schistosoma mansoni* eggs is the prominent lesion in human schistosomiasis. Studies have suggested the involvement of a series of suppressive mechanisms in the control of this reaction. Using an *in vitro* model of granuloma formation, we have shown that immune complexes (IC) isolated from sera of chronic intestinal schistosomiasis patients were able to reduce granulomatous reaction developed against soluble egg antigen-conjugated polyacrylamide beads. In this system, the role of the L-arginine–nitric oxide (NO) pathway in the formation of prostaglandin E_2 (PGE_2) by human peripheral blood mononuclear cells (PBMC) of patients infected with schistosomiasis was investigated using IC. Preincubation of PBMC with IC produced a significant increase of both nitrite and PGE_2 levels in the cell supernatant. This effect was inhibited by coincubation of cells with *N*ω-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor, showing that the release of PGE_2 subsequent to IC stimulation was driven by NO. The inhibitory effect of L-NAME on PGE_2 release by IC-treated PBMC was reversed by sodium nitroprusside, a known NO donor. Our results indicate that NO could be an important second signal for the stimulation of PGE_2 production induced by IC in PBMC from human schistosomiasis patients. © 1999 Academic Press

Key Words: **prostaglandins; nitric oxide; granuloma; *Schistosoma mansoni;* modulation.**

INTRODUCTION

Schistosomiasis is a worldwide helminth-induced disease characterized by chronic granulomatous inflammation mediated by T lymphocytes and soluble factors (1–3). As the disease progresses from acute to chronic phase, regulation of the immune response takes place, characterized by a decline in soluble egg antigen (SEA)[1]-induced responses, spontaneous regression of granuloma size, and a decrease of pathology (4–7). This regulation has been associated with many factors, such as cytokines, idiotype–anti-idiotype interactions, suppressor T cells, macrophages, and immune complexes (IC) (8–12). Although most of the studies examining the role of IC in schistosomiasis investigate their participation in pathologic mechanisms (13, 14), a few have dealt with IC participation in suppressive mechanisms (15, 16). Using an *in vitro* assay of granuloma formation (17) we have demonstrated that IC isolated from sera of schistosomiasis patients regulate granulomatous hypersensitivity to polyacrylamide beads conjugated to *Schistosoma mansoni* egg antigen. In this model it appears that IC may inhibit this activity by stimulating the monocyte–macrophage lineage to release inhibitory mediators such as the prostaglandin E series (9, 10).

Nitric oxide (NO) and cyclo-oxygenase (COX) pathways share a number of similarities (18). NO is the mediator generated from the nitric oxide synthase (NOS) pathway (19). Cyclo-oxygenase converts arachidonic acid to prostaglandins (PG), prostacyclin (PGI_2), and thromboxane A_2 (TXA_2). Two major forms of NOS and COX have been identified to date. In normal circumstances, constitutive isoforms of these enzymes are found in virtually all organs. Their presence accounts for the regulation of several important physiological effects (20, 21). On the other hand, in an inflammatory setting, these enzymes are induced in a variety of cells, resulting in the production of large amounts of proinflammatory and cytotoxic NO and PG. Release of these molecules has been associated with pathological roles

[1] Abbreviations used: NO, nitric oxide; NOS, nitric oxide synthase; PGE_2, prostaglandin E_2; L-NAME, *N*ω-nitro-L-arginine methyl ester; SNP, sodium nitroprusside; COX, cyclo-oxygenase; SEA, soluble egg antigen; SWAP, soluble adult worm antigen preparation; IC, immune complexes; PBMC, peripheral blood mononuclear cells; PB, polyacrylamide bead; PEG, polyethylene glycol.

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in several disease states. An important link between NOS and COX pathways is that NO activates COX enzymes, resulting in an augmented production of PG (22, 23). Thus, the principal aim of our studies is to analyze the influence of IC on NO production, its effect on prostaglandin E_2 release, and the importance of these mediators on immunoregulation of granulomatous hypersensitivity to *S. mansoni* eggs.

MATERIALS AND METHODS

Study Population

Intestinal chronic schistosomiasis patients, not being treated with drugs, ranging in age from 18 to 30 years old, were selected based on clinical examination, the presence of *S. mansoni* eggs on stool, cellular response to parasite antigens on *in vitro* proliferation, and granuloma formation (24); patients were from the Santa Luzia district (a known endemic area) and located through a cooperative program of Federal University of Minas Gerais (UFMG), Brazilian National Health Foundation (FNS), and Santa Luzia Municipal Health Service. The patient protocols used throughout this study were approved by the human subject ethics committee of the UFMG.

Cell Preparation

PBMC were isolated from heparinized blood (20 U/ml) by Ficoll–diatriazoate density gradient centrifugation (9). These cells were suspended on RPMI 1640 culture medium (Sigma) containing 1.6% L-glutamine, 300 U/ml of penicillin, 0.3 mg/ml of streptomycin, and 10% AB^+ serum (RPMI–10% AB^+).

In Vitro Granuloma Formation Assay

This assay was performed using antigen-coated polyacrylamide beads, according to a previously described method (25). Approximately 3×10^5 PBMC from schistosomiasis patients in triplicate were stimulated with 25 μg of *S. mansoni* antigens (SEA, SWAP), 160 μg of IC, or 1 mM L-NAME (and control without any stimulus) and cocultured with 200 antigen-conjugated polyacrylamide beads (PB-SEA and PB-SWAP) or beads without antigens, as control (PB), in 200 μl of RPMI–10% AB^+, on 96-well flat-bottomed tissue culture plates (Nunc). *In vitro* reactivity was evaluated on day 5, using a phase-contrast inverted microscope, by morphological observations, examining for visual evidence of blast-transforming cells accompanied by cellular migration and adherent cell layers around the beads. A total of 300 separate determinations were made for each experimental group. A numerical score equivalent to the following classification was assigned to each *in vitro* cell/bead reaction observed: (1) no cells binding to the bead; (2) <5 cells binding to the bead; (3) >5 cells binding; (4) >5 cells binding to the bead accompanied by a circumoval mononuclear cell migration; (5) adherent cell monolayer attached to the bead accompanied by circumoval mononuclear cell migration; and (6) multiple cell layers surrounding the bead accompanied by circumoval mononuclear cell migration. The granuloma index (GI) was the resultant weighted average of these determinations. Granuloma reactivity was compared to the nonspecific binding of activated PBMC to beads without antigens (PB)

Isolation of Immune Complexes

Polyethylene glycol 6000 (PEG, Sigma) was used to precipitate soluble IC either in pooled human schistosomiasis chronic serum diluted ¼ in PBS or in manufactured IgG-SEA IC (9). These solutions were mixed in equal proportions with PEG in aqueous solution at a final concentration of 4% under sterile conditions. The mixtures were then incubated for 18 h at 4°C, followed by centrifugation at 500*g* for 30 min. PEG-induced precipitates were resuspended in PBS to the original serum volume or dilution volume, heated at 37°C for 30 min, vortexed, and stored at 4°C for use in the following assays. IC-free serum and supernatant obtained after manufactured IgG-SEA IC precipitation were used as controls (9). The quantity of protein in isolated IC was determined by the Bradford method (26).

Nitrite Determination

Nitrite concentration in culture, a measurement of NO synthesis, was assayed by a standard Griess reaction adapted to microplates (27). Supernatants of PBMC cultures for the *in vitro* granuloma reaction assay were collected on day 7. The Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 2.5% H_3PO_4 and 0.1% naphthylethylenediamide in 2.5% H_3PO_4. A volume of 50 μl of reagent was mixed with 50 μl of supernatant and incubated at room temperature for 10 min. Absorbance of the chromophore that formed was measured at 540 nm using an automated microplate reader (Multiskan MCC/340, Labsystems). Nitrite concentrations were calculated by means of a $NaNO_2$ standard curve and data were expressed as micromolars of nitrite. It is worth mentioning that IC and PEG were used as controls of nitrite contaminants (blank) in the IC preparation.

Prostaglandin E_2 Determination

Prostaglandin E_2 concentrations were determined on supernatants collected from the *in vitro* granuloma reaction assay using a commercial competitive ELISA kit (Biotrak, Amersham), according to the manufacturer's protocols. IC and PEG were also used as controls of PGE contaminants (blank) in the IC preparation.

Statistical Analysis

Data were assessed using the SAS Systems analysis of variance (ANOVA) procedure, Student *t* test, with $P < 0.05$ was taken as the lower limit of significance.

RESULTS

Effect of Immune Complexes and L-NAME on in Vitro Granuloma Formation by PBMC from Schistosomiasis Patients

The effect of IC on *in vitro* granuloma response of PBMC from 12 patients was investigated (Fig. 1A). Significant reactions to PB-SEA (GI = 4.4 ± 0.5) and PB-SWAP (GI = 3.8 ± 0.2) were observed when compared to PB (GI = 2.0 ± 0.4). As shown previously, IC from chronic schistosomiasis patients sera induced a marked suppression of granulomatous hypersensitivity to PB-SEA (GI = 2.8 ± 0.5) and PB-SWAP (GI = 1.6 ± 0.2) ($P < 0.05$) (Fig. 1 A). Addition of L-NAME reversed the suppressive effect induced by IC on granuloma reaction to PB-SEA (GI = 4.0 ± 0.5) and PB-SWAP (GI = 3.8 ± 0.1) (Fig. 1B). The L-NAME also induced granuloma enhancement against these antigens, clearly due to GI increasing in cultures without IC (Fig. 1B).

NO Production Induced by IC

The capacity of PBMC from *S. mansoni*-infected individuals induced by IC to produce NO on *in vitro* granuloma reaction was measured. Results demonstrated that addition of IC to human PBMC cultures caused an increase in NO production to PB-SEA and PB-SWAP on day 7 (Fig. 2A). L-NAME clearly inhibited NO release when PBMC were stimulated either with PB-SEA or with PB-SWAP. However, L-NAME did not interfere with the capacity of IC to induce increased NO production (Fig. 2B).

The Relationship between Prostaglandin and NO Production on in Vitro Granuloma Formation in the Presence of IC

IC was able to increase the PGE_2 released on day 7 by PBMC from *S. mansoni*- infected individuals stimulated with PB-SEA or PB-SWAP (Fig. 3). In the same experiment, we observed an equivalent degree of inhibition of PGE_2 production by the addition of L-NAME to cultures (Fig. 3). However, the addition of SNP, which releases NO directly, stimulated PGE_2 production by PBMC, even in the absence of IC (Table 1). This means that the effect of SNP on PGE_2 release was related to NO formation by PBMC from schistosomiasis patients.

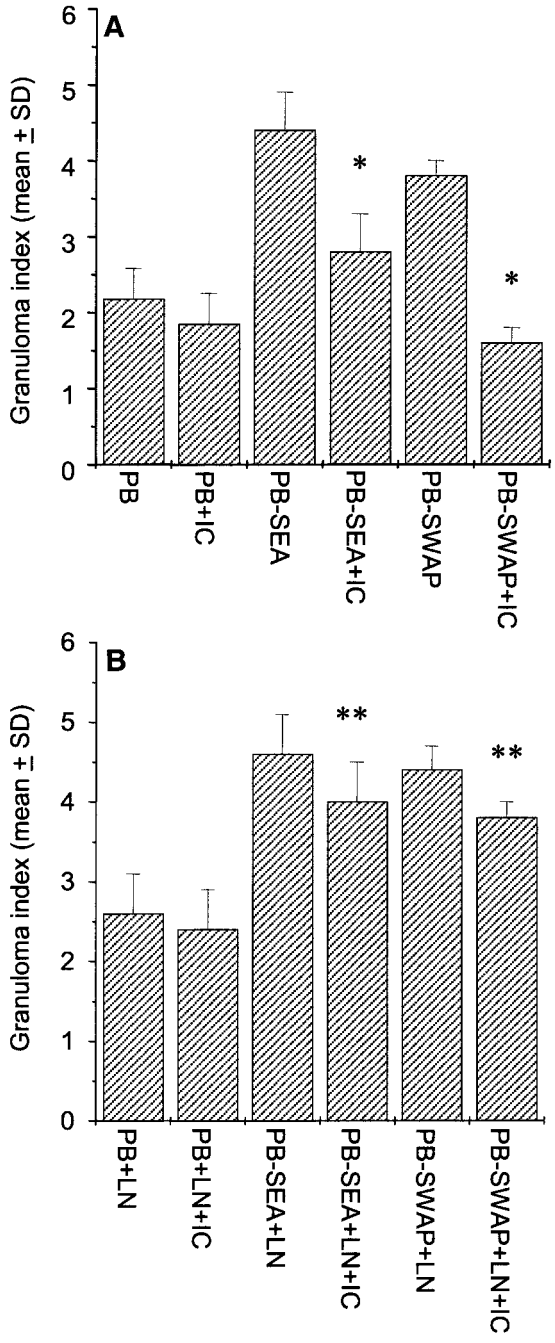

FIG. 1. Granuloma modulation induced by IC in the absence (A) or in the presence of 1 mM L-NAME (B). 3×10^5 PBMC from intestinal chronic schistosomiasis patients, with no treatment or with 160 μg/ml of IC, were cocultured for 5 days with *S. mansoni* antigens conjugated to polyacrylamide beads (PB-SEA, PB-SWAP) or beads alone (PB) in 96-well flat-bottomed plates. The granuloma index (GI) was evaluated by cellular reactivity around the beads. Data are reported as mean granuloma index ± SD for $n = 12$. *$P < 0.05$, significantly different from controls PB-SEA or PB-SWAP without IC. **$P < 0.05$, significantly different from controls PB-SEA or PB-SWAP treated with IC.

DISCUSSION

Recent studies have demonstrated that several immunoregulatory mechanisms may operate in human schistosomiasis (9, 10, 12, 28). The mechanisms associated with granuloma formation and subsequent modulation are complex, involving many types of cells (2, 29, 30) and interactive molecules (3, 12). Circulat-

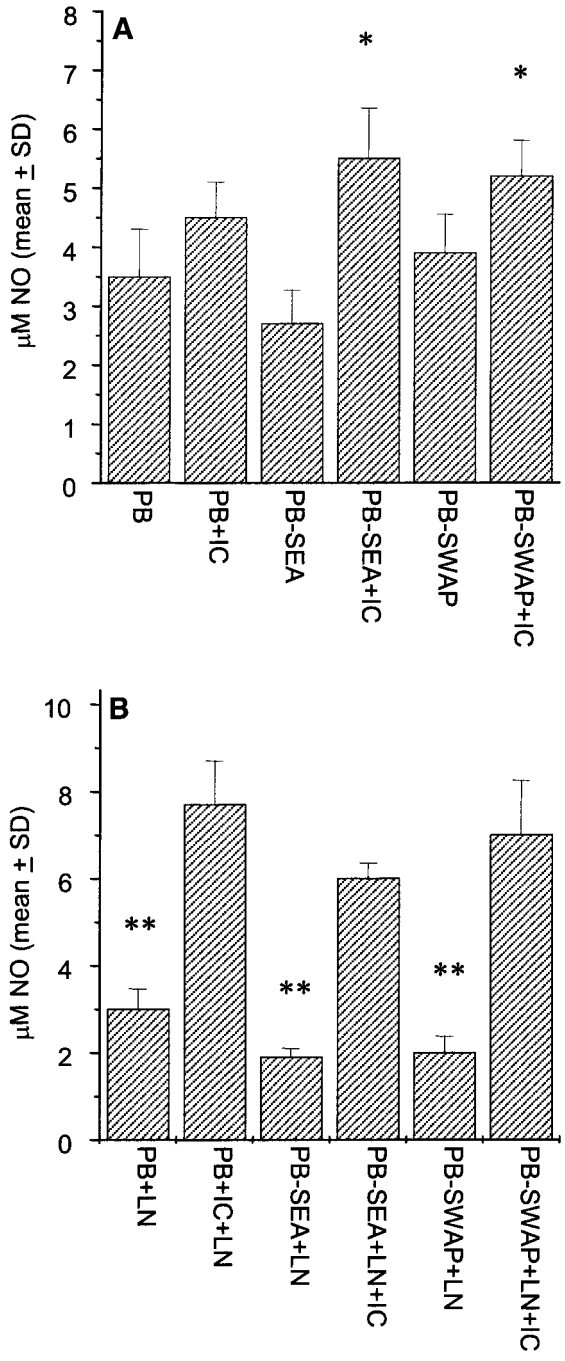

FIG. 2. Comparison of NO production in granuloma modulation induced by IC in the absence (A) or in the presence of 1 mM L-NAME (B). Nitrite levels were detected in supernatants of 3×10^5 PBMC from intestinal chronic schistosomiasis patients, with no treatment or with 160 μg/ml of IC, cocultured for 7 days with *S. mansoni* antigens conjugated to polyacrylamide beads (PB-SEA, PB-SWAP) or beads alone (PB) in 96-well flat-bottomed plates. Data are expressed as mean concentration of NO (μM) ± SD for $n = 12$. *$P < 0.05$, significantly different from controls PB-SEA or PB-SWAP. **$P < 0.05$, significantly different from controls PB-SEA or PB-SWAP treated with IC and L-NAME.

ing IC have been considered to affect profoundly host defense mechanisms against pathogens and to modulate cellular immune response. The possible interference of IC with cellular defense mechanisms against *S. mansoni* has been suggested by others (10, 15, 16). Other studies on *in vitro* granuloma reactions have shown that IC obtained from sera of intestinal chronic schistosomiasis patients are able to suppress PBMC reactivity to PB-SEA and PB-SWAP (9, 10, 12). In chronic schistosomiasis, modulation of the immune response accounts for the reduction of granuloma reactions. IC may in some way stimulate this response through the production of soluble factors. The results of the present study demonstrate that one of the mechanisms by which IC exert *in vitro* inhibitory action on granuloma formation to *S. mansoni* antigens is by stimulating PGE_2 production in the presence of NO. In this study, we showed that both exogenous and endogenous NO increased the IC-stimulated biosynthesis of prostaglandin E_2 by PBMC derived from human schistosomiasis patients in *in vitro* granuloma reactions. In fact, L-NAME, an inhibitor of NO synthase, significantly suppressed both nitrite formation and PGE_2 release in supernatants of IC-treated PBMC. Also, these responses were restored by addition of SNP, a

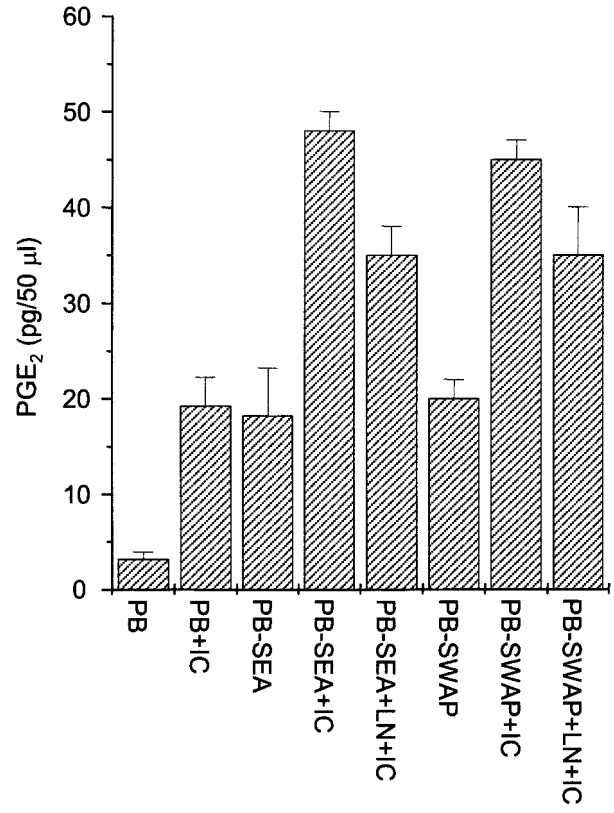

FIG. 3. Prostaglandin E_2 determination in granuloma modulation induced by IC in the absence or in the presence of 1 mM L-NAME. Prostaglandin E_2 levels were evaluated in supernatants of 3×10^5 PBMC from intestinal chronic schistosomiasis patients, with no treatment or with 160 μg/ml of IC, cocultured for 7 days with *S. mansoni* antigens conjugated to polyacrylamide beads (PB-SEA, PB-SWAP) or beads alone (PB) in 96-well flat-bottomed plates. Data are expressed as concentration of PGE_2 (pg/50 μl) (mean for $n = 4$). *$P < 0.05$, significantly different from controls PB-SEA or PB-SWAP.

TABLE 1
Nitrite and PGE_2 Release from PBMC *S. mansoni*-infected Patients

Treatment	Nitrite (μM)	PGE_2 (pg/50 μl)
SNP	62 ± 2	—
SNP + PB	198 ± 21	136 ± 15*
SNP + PB +IC	202 ± 8	137 ± 21*
SNP + PB-SEA	159 ± 30	163 ± 26*
SNP + PB-SEA + IC	105 ± 2	231 ± 62*
SNP + PB-SWAP	159 ± 3	138 ± 13*
SNP + PB-SWAP + IC	128 ± 13	285 ± 10*

Note. Nitrite and PGE_2 concentrations in the incubation medium were determined as described under Materials and Methods. Values are the mean ± SD of four independent experiments. Controls: PGE_2, PB (3.16 ± 0.5 pg/50 μl); PB-SEA (48 ± 13 pg/50 μl); PB-SWAP (20 ± 5 pg/50 μl); no PGE_2 was detected in the IC preparation or in the PEG solution. NO, PB (1.6 ± 0.3 μM/50 μl); PB-SEA (3.5 ± 1.2 μM/50 μl); PB-SWAP (4.2 ± 0.5 μM/50 μl); no nitrite contaminants were detected in the IC preparation or in PEG solution.

* $P < 0.05$, significantly different from control.

known NO donor. Inhibition of NOSi by L-NAME (3) or PGE_2 synthesis (9, 10) resulted in an increase of granuloma reaction. The interaction between the NO and the COX pathways should be relevant in granulomatous hypersensitivity that appears to be modulated by NO, since in this type of process the degree of reaction was reduced by IC and exacerbated by NOS and PGE_2 inhibitors (10).

Results presented by Mollace *et al.* (31) demonstrated that *N*-methyl-D-aspartate (NMDA)-induced release of PGE_2 by human cultured T 67 astrocytoma cell line is mediated by NO. In fact, L-NAME, an inhibitor of NO synthase, signficantly inhibited both nitrite formation and PGE_2 levels in supernatants of NMDA-treated astrocytoma cells. PGE_2 release was restored by addition of arachidonic acid, an effect suppressed by indomethacin, thus demonstrating that inhibition of NO-generating machinery specifically interferes with cyclo-oxygenase enzymes in astrocytoma cells. Our results are in agreement with these and other data suggesting that both endogenous NO and exogenous NO increase cyclo-oxygenase activity in activated human cells (32) and mouse macrophages (20).

The discovery that NO regulates cyclo-oxygenase activity was originally made using cellular systems and purified enzymes (21). However, the molecular mechanism by which this activation takes place remains to be identified (33). A few possibilities can be put forward. An antioxidant effect is generated during COX activation and has been postulated to be involved in the autoinactivation of the COX enzyme (34). NO nitrosylating cysteine residues in the catalytic domain of COX enzymes can lead to the formation of nitrosothiols, which can produce changes in enzyme structure, resulting in increased catalytic efficiency (35). NO and O_2^- can interact to form the cytotoxic molecule peroxynitrite, which in turn can be decomposed to give rise to OH (36, 37).

On the other hand, we observed in our experiments that the interaction between IC and PBMC induced an increase in NO levels even in the presence of L-NAME. We believe that the mechanisms by which IC stimulated high concentrations of NO in the presence of L-NAME do not involve the IC purification method. On the other hand, we cannot attribute the failure of L-NAME to inhibit NO production in the presence of IC to PEG contaminants. Our explanation for IC insensitivity to L-NAME is that activation of the adenylate cyclase system by IC and L-NAME in a synergistic fashion subsequently increased cyclic AMP (cAMP) levels. Increases in intracellular cAMP have been suggested as mediating the stimulatory effect of PG on the NOS pathway (39). We suggest that PBMC from schistosomiasis patients when stimulated *in vitro* by IC/IC–L-NAME–cAMP increase iNOS activity with subsequent high levels of NO. Recently, experiments done by Inoue *et al.* (40), using rat vascular smooth muscle cells stimulated with L-Arg (30 mM), showed that there was an increase in NO production when L-Arg was added with IL-1 and L-NAME to the cultures. Stimulation of rat peritoneal macrophages with IC induced NO production and the expression of inducible iNOS protein (41). These findings corroborate our results involving PB-SEA or PB-SWAP + IC + L-NAME.

Since it is known that IC induces PGE_2 release, these findings support the hypothesis of PGE_2-mediated overproduction of NO. A role for PG as enhancers of NO production has been reported in other systems (20, 38, 42).

Ongoing analysis of *in vitro* granuloma formation indicates that IC modulation of granulomatous hypersensitivity against *S. mansoni* antigens is driven by NO release, which plays a crucial role in the biosynthesis of prostaglandin E_2.

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