# Nitric oxide interaction with IL-10, MIP-1α, MCP-1 and RANTES over the *in vitro* granuloma formation against different *Schistosoma mansoni* antigenic preparations on human schistosomiasis

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SUMMARY

Nitric oxide (NO) produced by cytokine-activated macrophages is reported to be cytotoxic against the helminth *Schistosoma mansoni*, although this is a controversial issue. Previous work in our laboratory identified a fraction of *S. mansoni* soluble adult worm antigenic preparation (SWAP), named PIII, able to elicit significant *in vitro* cell proliferation and at the same time lower *in vitro* and *in vivo* granuloma formation when compared either to soluble egg antigen (SEA) or to SWAP. Here we report that, in comparison to other *S. mansoni* antigenic preparations (SEA and SWAP), supernatants of PBMC cultivated with PIII possess higher concentrations of interleukin-10 (IL-10) and macrophage inflammatory protein (MIP-1α), concomitantly with lower concentrations of monocyte chemoattractant protein (MCP-1) and regulated on activation, normal T expressed and secreted (RANTES). In the particular case of NO inhibition, supernatants of PBMC cultivated with PIII present decreased IL-10 levels. Altogether, these results indicate that IL-10, MIP-1α, MCP-1 and RANTES are distinctively important elements in the PIII modulating role, while NO seems to be pivotal in the regulation of granulomatous responses.

Key words: nitric oxide, IL-10, MIP-1α, MCP-1, RANTES, *in vitro* granuloma, schistosomiasis.

## INTRODUCTION

Previous work by our laboratory (Hirsch & Goes, 1996) identified a fraction of *S. mansoni* soluble adult worm antigenic preparation (SWAP), named PIII, able to elicit significant *in vitro* cell proliferation and, at the same time, lower *in vitro* (Hirsch & Goes, 1996) and *in vivo* (Hirsch *et al.* 1997) granuloma formation when compared either to SEA (soluble egg antigen) or to SWAP. Using this model, we previously investigated some biological activities, such as nitric oxide (NO) production and cytokine profile, of different *S. mansoni* antigenic preparations, particularly of PIII (Oliveira *et al.* 1999). NO has been identified as a cytotoxic factor and as an important and versatile messenger in the immune system (Khatsenko *et al.* 1993). However, its precise role in inflammation remains unclear, since anti- and/or pro-inflammatory properties have been widely and conversely attributed to NO (Hibbs *et al.* 1989; Xie *et al.* 1992). Recently, our laboratory looked into NO correlation with *in vitro* granuloma (IVG) reaction using *S. mansoni* antigens (SEA and SWAP) and demonstrated a possible regulatory role of NO during granulomatous reaction (Oliveira *et al.* 1998, 1999).

Here we describe that, in comparison to SEA and SWAP, supernatants of PBMC cultivated with PIII possess higher concentrations of interleukin-10 (IL-10) and macrophage inflammatory protein (MIP-1α), concomitantly with lower concentrations of monocyte chemoattractant protein (MCP-1) and regulated on activation, normal T expressed and secreted (RANTES). In the particular case of NO inhibition, supernatants of PBMC cultivated with PIII present decreased IL-10 levels. Altogether, these results on IVG reaction might indicate that IL-10, MIP-1α, MCP-1 and RANTES are important elements in the PIII modulating role, while

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NO seems to be pivotal in the regulation of granulomatous responses in human schistosomiasis.

## MATERIALS AND METHODS

### *Reagents*

$N^{\omega}$-Nitro-L-arginine methyl ester (L-NAME) was purchased from Sigma Chemical Co. (St Louis, MO, USA).

### *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, parasitological stool examinations (Katz, Chaves & Pellegrino, 1972), cellular response to IVG formation. A total of 20 schistosomiasis mansoni patients, from the Santa Luzia district (a known local endemic area), were located through a cooperation programme among 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 in Brazil and also conform to the guidelines of the Declaration of Helsinki. Two healthy, uninfected individuals (normal controls) were selected among volunteers.

### *Cell preparations*

Human peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood (20 U/ml heparin) of patients with chronic intestinal schistosomiasis mansoni, or uninfected controls, by Ficoll diatriazoate density-gradient centrifugation (LSM, Organon Teknica, Charleston, SC, USA) (Thorsby & Bratlier, 1970). PBMC were suspended with culture medium RPMI 1640 (Sigma, St Louis, MO, USA) containing 1·6% L-glutamine, 300 U/ml penicillin, 0·3 mg/ml streptomycin and 10% heat-inactivated human $AB^+$ serum, and then counted in a Neubauer chamber.

### S. mansoni *antigens conjugated to polyacrylamide beads* (*PB*)

PB, 40–60 $\mu$m in diameter (Bio-Gel P-4, Bio-Rad, Richmond, CA, USA), were washed in 0·5 M bicarbonate buffer, as previously described (Doughty *et al*. 1984). The beads were mixed with 20 mg of *S. mansoni* antigens obtained from eggs (SEA) or adult worms (SWAP and PIII), the latter as a fraction of the former, according to previously described techniques (Doughty *et al*. 1987; Hirsch & Goes, 1996). The protein content was measured according to the microassay of Bradford (1976) and the same amount of protein of each antigenic preparation was provided to couple to the same number of polyacrylamide beads. To obtain PIII, SWAP was fractionated by anion-exchange chromatography on FPLC (Fast Protein Liquid Chromatography), as previously described by Hirsch & Goes (1996). Briefly, proteins were eluted in a multistep increasing gradient and flow-through fractions were concentrated by lyophilization. The concentrated material was dialysed, sterilized by filtration and stored at −70 °C. The fraction was called PIII and was used in different immunological assays. Purified protein derivative (PPD) from *Mycobacterium tuberculosis* (Connaught Laboratories, Canada) was used as a positive control for the IVG reaction, since the Brazilian population is BCG-vaccinated and reacts to PPD, whereas PB alone (not conjugated to any antigen) was the negative control specific for the IVG reaction.

### In vitro *granuloma* (*IVG*) *reaction*

The reaction was determined utilizing antigen-coated beads as previously described by Doughty *et al*. (1987). Briefly, 200 polyacrylamide beads, not coated to antigen (PB), or conjugated to *S. mansoni* antigens (PB-SEA, PB-SWAP and PB-PIII) or to *M. tuberculosis* antigen (PB-PPD), were added to the bottom of 96-well tissue culture plates together with $3 \times 10^5$ cells/well. The PBMC were cultivated in a final volume of 200 $\mu$l of RPMI containing (or not) L-NAME in a concentration of 1 mM, and with 10% heat-inactivated fetal calf serum (instead of $AB^+$ human serum because it could possibly interfere with chemokine detection). Each experimental (infected individuals) and control (uninfected individuals) group was set in triplicate occupying as many individual wells as needed for all trials in different days of analysis. Culture plates were maintained at 37 °C in a 5% $CO_2$ incubator. Cellular reactivity was determined by morphological observations, using a phase-contrast inverted tissue culture microscope (Nikon TMS), of visual evidence of cellular migration and adherent cell layers surrounding the beads. Two hundred separated determinations of cellular reactivity 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) absence of cells binding to the bead; (2) $< 5$ cells binding to the bead; (3) $\geqslant 5$ 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; (6) multiple cell layers surrounding the bead accompanied by mononuclear cell migration. The total score was then summed and the resultant

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weighted average expressed as the granuloma index (GI) (Doughty *et al*. 1987). Granulomatous reactivity to PB-SEA, PB-SWAP, PB-PIII and to PB-PPD was compared to the non-specific binding of activated macrophages against polyacrylamide beads not conjugated to antigen (PB), the negative control specific for the IVG reaction.

### *Nitrite determination*

Nitrite concentration in cell culture supernatant, an indirect measurement of NO synthesis, was assayed by a standard Griess reaction adapted to microplates (Green *et al*. 1982). Concentrations were calculated by means of a $NaNO_2$ standard curve and data were expressed as $\mu$M nitrite (Xiong *et al*. 1996). Supernatants from PBMC on the IVG reaction were analysed at the 7th day of IVG cultures for nitrite determination.

### *Cytokine detection*

Using a quantitative enzyme immunoassay technique with commercially available kits (Amersham Life Science, Bucks, UK), IL-2, INF$\gamma$, TNF$\alpha$ and IL-10 concentrations were detected in supernatants of PBMC cultivated with PB, PB-SEA, PB-SWAP and PB-PIII on the IVG reaction (groups with and without L-NAME) at 72 h (3rd day) of cell culture. Results were determined in pg/ml and all are representative of at least 4 experiments in duplicate.

### *Chemokine detection*

Using a quantitative enzyme immunoassay technique with commercially available kits (Quantikine, R & D Systems, Minneapolis, MN, USA), MIP-1$\alpha$ (macrophage inflammatory protein-1$\alpha$), MCP-1 (monocyte chemoattractant protein-1) and RANTES (regulated on activation, normal T expressed and secreted) concentrations were detected in supernatants of PBMC cultivated with PB, PB-SEA, PB-SWAP and PB-PIII on the IVG reaction (groups with and without L-NAME) at the 3rd day of cell culture. Samples of supernatants were diluted $\times 10$ for MIP-1$\alpha$ and $\times 4$ for MCP-1 and RANTES quantitative determinations, following the manufacturer's recommendations. Results were determined in pg/ml and all are representative of at least 4 experiments in duplicate.

### *Data analysis*

Statistical analysis was assessed using the Student *t*-test, analysis of variance (ANOVA) procedure (SAS Systems, 1990). A $P < 0{\cdot}05$ was taken as the lower limit of significance.

## RESULTS

### In vitro *granuloma* (*IVG*) *reaction and NO production induced by PIII*

PIII antigenic fraction conjugated to polyacrylamide beads (PB-PIII) significantly lowered GI values when compared either to PB-SEA or to PB-SWAP in IVG cultures of PBMC from schistosomiasis patients (Table 1). PBMC obtained from control (not infected) individuals usually do not react on IVG (data not shown). Addition of L-NAME (1 mM) to cultures of PBMC generally increased the GI, which was evaluated at the 5th day of cell culture. This L-NAME-induced enhancement of IVG reaction occurred regardless of the conjugated antigen (Table 1).

Comparison among tested *S. mansoni* antigens conjugated to polyacrylamide beads (PB-SEA, PB-SWAP and PB-PIII) showed that PB-PIII induced the highest nitrite levels on IVG reaction (Table 1). Culture supernatants were analysed for nitrite determination at the 7th day of IVG culture. A general time-dependent increase in NO production was also observed (data not shown). Using the SAS systems (ANOVA procedure), we were able to testify that PIII-induced nitrite levels were statistically different from SEA and SWAP. Addition of L-NAME (1 mM) was able to partly block nitrite production measured at the 7th day of the IVG cell culture.

### *Cytokine profile induced by PIII on IVG reaction*

Release of IL-2, TNF$\alpha$ and IFN$\gamma$, measured by our methods, did not present substantial differences when comparing the three *S. mansoni* antigenic *in vitro* stimulations (SEA, SWAP and PIII) and, also, it seemed not to be affected by inhibition of NO. Levels of these cytokines measured in supernatants from PBMC cultivated with polyacrylamide beads conjugated (or not) to *S. mansoni* and *M. tuberculosis* antigens are about the same independently of L-NAME being added or not to the culture (data not shown). This fact apparently reveals a lack of influence of NO synthesis over the release of these cytokines in the IVG reaction, at least under our experimental conditions.

On the other hand, regarding IL-10, statistical comparison among tested *S. mansoni* antigenic stimulations (PB-SEA, PB-SWAP and PB-PIII) showed that PB-PIII induced the highest significant levels on IVG reaction (Fig. 1). Moreover, inhibition of NO synthesis generally decreased IL-10 production, as seen in Fig. 1. The use of L-NAME changed the IL-10 releasing pattern, which became significantly lower for both PB-SEA and PB-SWAP supernatants, but not so much for PB-PIII, as could be statistically confirmed by ANOVA procedure, SAS Systems. This comparatively greater PIII-

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Table 1. Comparison between *in vitro* granuloma reaction and NO production

| Antigenic stimulation | Granuloma index (GI) | | Nitrite concentration ($\mu$M) | |
|---|---|---|---|---|
| | w/o | +L-NAME | w/o | +L-NAME |
| PB | 2·3±0·14 | 3·8±0·12 | 8·0±0·35 | 1·3±0·32 |
| PB-SEA | 4·2±0·13 | 5·6±0·18 | 7·3±0·43 | 1·2±0·38 |
| PB-SWAP | 4·3±0·15 | 5·8±0·17 | 8·1±0·47 | 1·3±0·26 |
| PB-PIII | 3·2±0·13 | 5·1±0·18 | 9·5±0·28 | 1·5±0·42 |
| PB-PPD | 4·0±0·14 | 5·2±0·19 | 7·1±0·39 | 1·4±0·52 |

(Data are expressed as means±standard deviation for $n = 20$. Nitrite concentrations ($\mu$M) were detected in supernatants of PBMC from *Schistosoma mansoni*-infected individuals on the 7th day of *in vitro* granuloma (IVG) culture. Addition of L-NAME (1 mM) is shown in the right-hand column; w/o stands for absence of L-NAME. Statistical comparison (ANOVA procedure, SAS Systems) of NO production by PBMC stimulated with PB-PIII to that with PB-SEA and PB-SWAP demonstrated that PB-PIII induced significantly higher nitrite levels.)

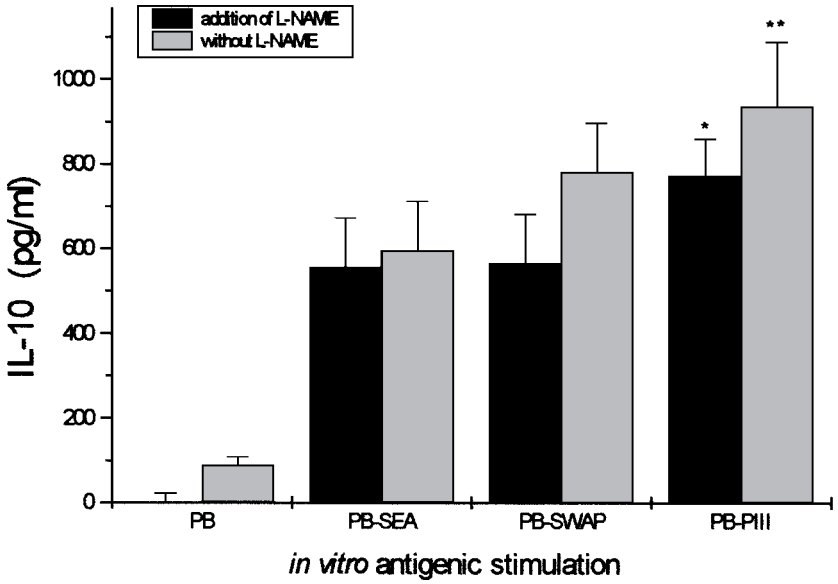


Fig. 1. IL-10 production by PBMC from schistosomiasis patients on the *in vitro* granuloma (IVG) reaction. Cells ($3 \times 10^5$/well) were cultured with polyacrylamide beads (PB) coated with *Schistosoma mansoni* antigens (PB-SEA, PB-SWAP, PB-PIII) or not (PB). Supernatants were collected on the 3rd day of IVG culture and assayed for IL-10 concentrations by ELISA. The results are expressed as pg/ml and represent the mean±S.E. of 4 experiments in duplicate. Statistical comparison (by ANOVA procedure, SAS Systems) among PB-SEA, PB-SWAP and PB-PIII confirmed that PB-PIII induced significantly higher IL-10 release (*). Effect of NO inhibition over IL-10 release was analysed by the addition of L-NAME (1 mM), which was equally performed to all *in vitro* antigenic stimulation. A $P < 0{\cdot}05$ was taken as the lower limit of significance. Levels of IL-10 are significantly lower in all L-NAME-added treatments when compared to those concentrations in the absence of L-NAME (**).

induced IL-10 release associated with the L-NAME effect of decreasing IL-10 production seems to reveal an interesting influence of NO on its release.

### *Chemokine profile induced by PIII on IVG reaction*

Comparison among tested *S. mansoni* antigenic stimulation showed that supernatants of PBMC cultivated with PIII had statistically significant higher MIP-1$\alpha$ concentrations than PB-SEA or PB-SWAP on IVG reaction (Fig. 2). Release of MIP-1$\alpha$ was, to a large extent (over 20%), increased when NO production was blocked with L-NAME for both PB-SEA and PB-SWAP, but not so much referring to PB-PIII, as shown in Fig. 2.

Referring to MCP-1 and RANTES levels, supernatants of PBMC cultivated with PIII showed the lowest levels when compared to PB-SEA and PB-SWAP on IVG reaction (Figs 3 and 4), as statistically confirmed by ANOVA procedure, SAS systems. Measurement of MCP-1 and RANTES did not show significant changes that could be detected by our methods when L-NAME was used (data not shown).

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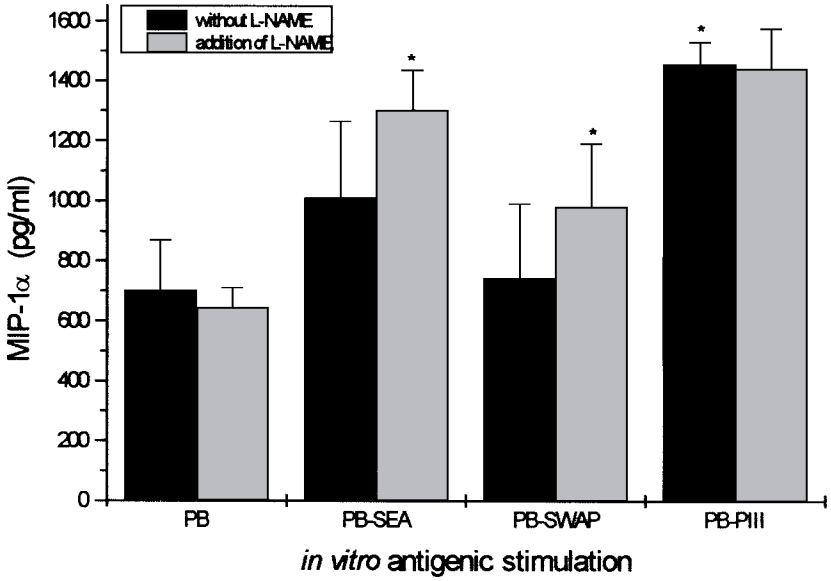


Fig. 2. MIP-1α production by PBMC from schistosomiasis patients on the *in vitro* granuloma (IVG) reaction. Cells ($3 \times 10^5$/well) were cultured with polyacrylamide beads (PB) coated with *Schistosoma mansoni* antigens (PB-SEA, PB-SWAP, PB-PIII) or not (PB). Supernatants were collected on the 3rd day of IVG culture and assayed for MIP-1α concentrations by ELISA. The results are expressed as pg/ml and represent the mean ± S.E. of 4 experiments in duplicate. Statistical comparison (by ANOVA procedure, SAS Systems) among PB-SEA, PB-SWAP and PB-PIII confirmed that PB-PIII induced significantly higher MIP-1α production (*). Obs: Supernatants for MIP-1α quantitative determination were diluted ×10, as recommended by the manufacturer of the ELISA kit (R & D Systems). A $P < 0{\cdot}05$ was taken as the lower limit of significance. Effect of NO inhibition over MIP-1α release was analysed by the addition of L-NAME (1 mM) which was equally performed to all *in vitro* antigenic stimulation and, for SEA and SWAP, it was responsible for significantly higher MIP-1α release when compared to those concentrations in the absence of L-NAME.

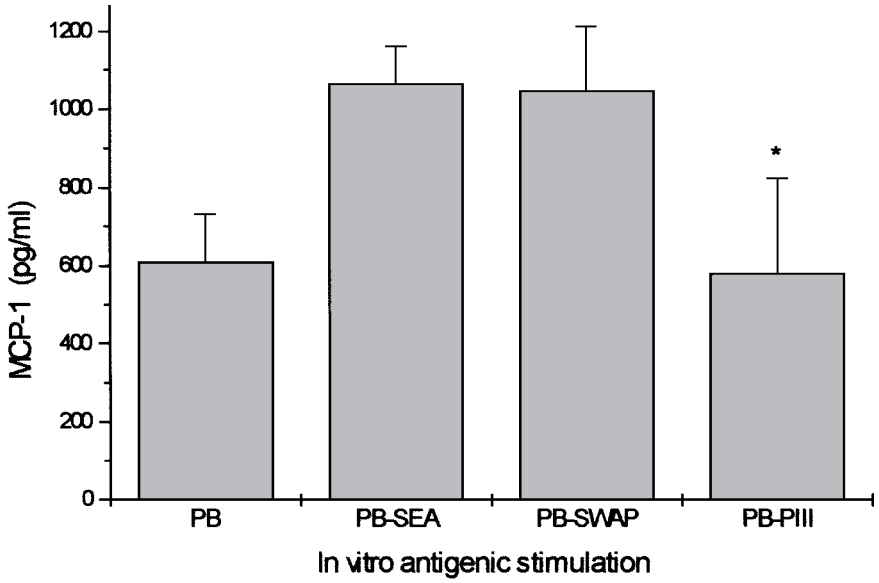


Fig. 3. MCP-1 production by PBMC from schistosomiasis patients on the *in vitro* granuloma (IVG) reaction. Cells ($3 \times 10^5$/well) were cultured with polyacrylamide beads (PB) coated with *Schistosoma mansoni* antigens (PB-SEA, PB-SWAP, PB-PIII) or not (PB). Supernatants were collected on the 3rd day of IVG culture and assayed for MCP-1 concentrations by ELISA. The results are expressed as pg/ml and represent the mean ± S.D. of 4 experiments in duplicate. Statistical comparison (by ANOVA procedure, SAS Systems) among PB-SEA, PB-SWAP and PB-PIII validated that PB-PIII induced significantly lower MCP-1 production (*). Obs: Supernatants for MCP-1 quantitative determination were diluted 4×, as recommended by the manufacturer of the ELISA kit (R & D Systems). A $P < 0{\cdot}05$ was taken as the lower limit of significance.

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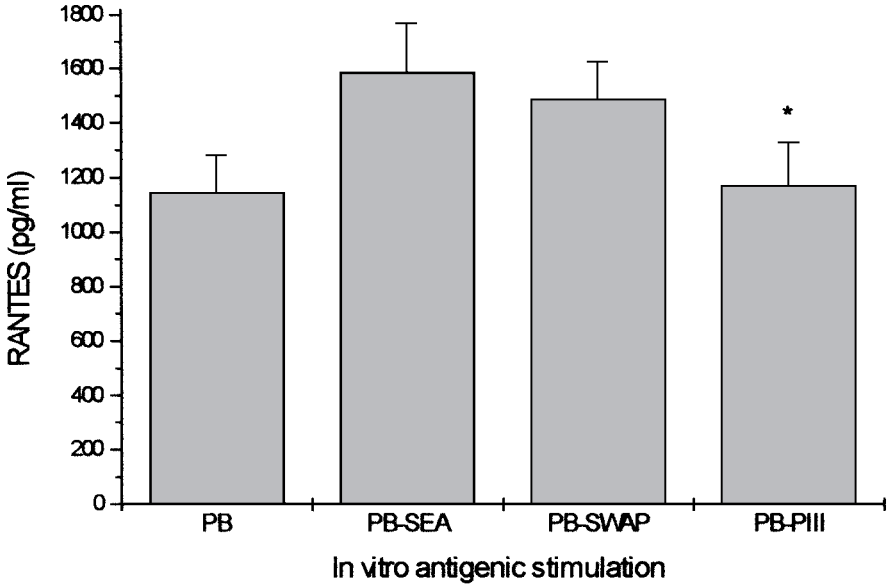


Fig. 4. RANTES production by PBMC from schistosomiasis patients on *in vitro* granuloma (IVG) reaction. RANTES concentrations (pg/ml) were detected in supernatants of PBMC from *Schistosoma mansoni*-infected individuals on the 3rd day of IVG culture. Cells ($3 \times 10^5$/well) were cultivated with polyacrylamide beads (PB) conjugated to *S. mansoni* antigens (SEA, SWAP and PIII) or not (PB alone). Data are expressed as mean and S.D. for $n = 4$ and all experiments were set in duplicate. Statistical comparison (by ANOVA procedure, SAS Systems) among PB-SEA, PB-SWAP and PB-PIII validated that PB-PIII induced significantly lower RANTES production (*). Obs: Supernatants for RANTES quantitative determination were diluted $\times 4$, as recommended by the manufacturer of the ELISA kit (R & D Systems). A $P < 0{\cdot}05$ was taken as the lower limit of significance.

## DISCUSSION

In order to better understand the immunoregulation of granulomatous response and the role of PIII in the induction of regulatory events, we searched for putative anti-inflammatory or modulatory elements associated with PIII. Previously, we showed that *in vitro* granuloma reactivity to *S. mansoni* antigens, SEA and SWAP, produced NO in an inverse relationship, that is, the higher the GI the lower the NO release and vice-versa (Oliveira *et al*. 1998). Since PIII was able to reduce GI, acting as a modulator of the IVG reaction (Hirsch & Goes, 1996), we decided to search for its capacity to induce NO production and for evidence of a possible NO connection with the IVG modulation. A regulatory role for NO on schistosomiasis using human cells in an *in vitro* response to SEA and SWAP has been described (Oliveira *et al*. 1998) and, concerning PIII antigenic stimulation, we reported induction of higher NO and IL-10 production *in vitro* by human mononuclear cells during a granulomatous response (Oliveira *et al*. 1999).

IL-10 is now known to be a major immunoregulatory and anti-inflammatory cytokine with multiple biological properties (Mosmann, 1994), including potent inhibitory effects on monocyte/macrophage function. Considering that these cells are capable of releasing MIP-1$\alpha$, which is a potent chemoattractant for activated T cells, it is interesting to comment on experiments in which IL-10 appears as an inhibitor of the induced transcription of MIP-1$\alpha$ mRNA and of the MIP-1$\alpha$ protein release. Some authors suggest that IL-10 may indirectly regulate effects on activated T lymphocytes partly through the inhibition of MIP-1$\alpha$ expression from monocytes and macrophages (Berkman *et al*. 1995). Furthermore, IL-10 is a potent macrophage deactivator which suppress TNF (Fiorentino *et al*. 1991), known to be crucial for NO synthesis (Drapier, Wietzerbin & Hibbs, 1988), and IL-10 itself downregulates or inhibits NO production (Gazzinelli *et al*. 1992; Fiorentino *et al*. 1991). It is apparent that there are pivotal interactions among IL-10, MIP-1$\alpha$ and NO, especially when we evaluate the results obtained on IVG reaction to PIII antigenic stimulation.

Some studies corroborate with ours in that they have shown MIP-1$\alpha$ and MCP-1 increased levels in L-NAME-treated mice and the authors suggest that these increases were likely responsible for the increased cellularity of granulomatous lesions and for the altered cytokine profile in L-NAME-treated mice (Hogaboam *et al*. 1997). In our findings, NO inhibition seems to act as a kind of granulomatogenic condition, since it increases IVG formation and MIP-1$\alpha$ release while decreasing IL-10 concentrations. However, other researchers have reported a different effect of NO inhibition in which L-NMMA ($N^G$-monomethyl-L-arginine) reduces LPS-stimulated expression of MIP-1$\alpha$ in human adherent PBMC (Muhl & Dinarello, 1997).

Since growing evidence suggests that chemokines play a regulatory role in Th cell response, NO may also exert effects on the cytokine profile through its

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direct modulation of chemokine production. It has been reported that MCP-1 participates in Th2 cell-mediated responses and may be stimulated by IL-4 (Chensue *et al*. 1996), while MCP-1 and RANTES are reported to have chemoattractant properties for mononuclear cells, therefore displaying potential relevance to granuloma formation (Taub & Oppenheim, 1994; Chensue *et al*. 1996). Type 1 and 2 responses show differential chemokine expression and regulation that may dictate the nature and extent of the granulomatous response (Chensue *et al*. 1997). In our experiments, supernatants of PBMC cultivated with PIII do possess higher concentrations of IL-10 and MIP-1$\alpha$ concomitantly with lower concentrations of MCP-1 and RANTES. Furthermore, in the particular case of NO inhibition, they present decreased IL-10 levels. Altogether, these results indicate that IL-10, MIP-1$\alpha$, MCP-1 and RANTES are important elements on PIII modulating role and also that NO might be pivotal in the interactions that occur during granulomatous responses. Although aware of the restrictions in assumptions like the present one, we favour a possibility that the PIII biological activities we have been investigating will contribute to provide support for potential future studies in the development of vaccines and/or treatments against the granulomatous immunopathogenesis of the disease.

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