

Dexamethasone, a Drug for Attenuation of *Schistosoma mansoni* Infection Morbidity

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Received 11 February 2002/Returned for modification 9 May 2002/Accepted 26 June 2002

To investigate the possible use of immunomodulators as adjuvants in the treatment of chronic schistosomiasis, the study described in the present report evaluated the effects of dexamethasone on several parameters which reflect disease severity and morbidity. Parasitological, immunological, and histological parameters were analyzed in animals treated from the first day of infection or after 35 days of infection. In both situations, dexamethasone had no effect on the parasite burden but altered the egg distribution in tissue, indicating that under the schedule used it did not interfere with the development of adult worms or oviposition. Treated mice showed a decrease in the number of eggs in hepatic tissue, reduced granuloma sizes, reduced levels of granuloma maturation, and reduced collagen contents. Dexamethasone-treated mice also had decreased gamma interferon, interleukin-12 (IL-12), and IL-4 levels in serum and increased IL-10 levels in serum. Taken together, these data suggested a decrease in the severity of murine schistosomiasis and point to dexamethasone as a convenient and promising adjuvant agent in the therapy of this infection.

The main cause of mortality and morbidity in human schistosomiasis is hepatic fibrosis, which essentially involves portal spaces, without severe lesions of the hepatic parenchyma. In humans, the hepatic portal fibrosis is only partially related to the presence of granulomas, while in murine schistosomiasis, liver fibrosis is essentially dependent on granulomas (5, 3, 73). Granulomatous inflammation in schistosomiasis is a cell-mediated hypersensitivity to parasite egg antigens that are lodged in hepatic tissue (74). As granulomas evolve, collagen fibers deposit around the eggs, a process that leads to fibrosis (48). In the chronic phase of infection the high number of granulomas and their confluence can eventually lead to portal hypertension, gastrointestinal bleeding, and, ultimately, death (15).

The periovular granulomatous process results from immunological stimuli mediated by different cell types, especially CD4⁺ T cells (52). The granuloma size reaches its maximum during the acute phase of infection, decreasing during the chronic phase (7). It is postulated that this process, known as downmodulation, involves several mechanisms including alterations in the patterns of cytokines produced by Th1 and Th2 cells (30, 53). Cytokines are also involved in the modulation of granuloma size and fibrosis. Thus, granuloma size in interleukin-10 (IL-10)-deficient mice increases significantly during the acute phase of infection (78). Treatment with anti-IL-4 or exogenous gamma interferon (IFN- γ) led to a reduction in granuloma size and to a decrease in the amount of fibrosis (18,

80, 25), while administration of exogenous IL-4 caused an increase in the amount of periovular fibrosis (80). In addition to its effects on granuloma formation and possibly on morbidity, it has been suggested that the balance of Th1 and Th2 cytokines is very important for a mild evolution of *Schistosoma mansoni* infection (9, 51). Moreover, transforming growth factor β (TGF- β) modulates Th1 and Th2 cytokine production (59), including enhancement of the level of IL-10 production by macrophages, which has been described as a key mechanism in the control of Th1 and Th2 polarization (6, 40). Indeed, the role of TGF- β in experimental schistosomiasis is still confusing (59), presenting a profibrotic effect in baboons (29) or interfering with IFN- γ production within the murine schistosome granuloma (63, 62).

The effects of glucocorticoids on the course of several infectious diseases including schistosomiasis have been reported. When glucocorticoids are administered in early phases of experimental schistosome infection, they caused a reduction in the worm burden (17, 76, 37). However, previous studies on the effects of dexamethasone against murine schistosomiasis showed variations according to the dose, the type of corticoid, and the schedule of treatment used (34, 54, 37). It was proposed that a decrease in the parasite burden was due to impairment of the initial phase of parasite penetration into host tissues (36). Glucocorticoids also decreased the level of collagen synthesis and the levels of posttranslational enzymes associated with collagen synthesis (56, 43). In fact, dexamethasone was shown to inhibit the transcription of collagens in murine schistosomiasis (75).

The importance of the granulomatous process and fibrosis on the course of *S. mansoni* infection (28) and the role of cytokines in the modulation of infection are well known (18,

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14). Despite the known anti-inflammatory and antifibrotic properties of glucocorticoids, most studies that have investigated its effects on schistosomiasis were done during the acute phase of the infection. The study described in this paper addressed the effect of dexamethasone on C57BL/6 mice acute and chronically infected with *S. mansoni*. The results showed that chronic administration of this glucocorticoid starting either concomitantly with the infection or 35 days postinfection did not significantly affect the parasite burden but reduced the number of hepatic eggs without interfering with oviposition. Dexamethasone treatment also delayed granuloma maturation, decreased the granuloma size, and reduced the amount of fibrosis. Moreover, treated animals produced increased IL-10 levels in serum and decreased IFN- γ and IL-4 levels in serum.

(A. S. Pyrrho performed this work in partial fulfillment of the Ph.D. degree at the Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.)

MATERIALS AND METHODS

Animals, drug, and infection. Adult C57BL/6J female mice (age, 7 to 8 weeks) were infected with 45 cercariae of the BH strain of *S. mansoni* by the cutaneous route. The mice were maintained for at least 1 week in the animal facilities before drug administration or infection. Dexamethasone disodium phosphate (Decadron, Prodome, Brazil) was administered by the intramuscular route at 1 mg/kg of body weight⁻¹ three times a week until the end of the experiment (55 or 120 days postinfection).

Controls animals (N) and infected animals (I) were divided into four groups: nontreated animals (groups N and I), animals treated with saline (groups N + S and I + S), animals treated with dexamethasone from the beginning of the experiment (groups N + Dex0 and I + Dex0), and animals treated with dexamethasone starting 35 days after the beginning of the experiment (groups N + Dex35 and I + Dex35). Animals from all groups (at least 10 animals per group) were killed while they were under anesthesia at 55 or 120 days after the beginning of the experiment, and during that time, they were maintained under controlled temperature and light conditions and fed a balanced diet and given sterile water ad libitum.

Parasitological parameters. Hepatic, intestinal, and lung tissues were digested as described by Cheever (11). Briefly, tissues were maintained in 4% KOH at room temperature for approximately 12 h, followed by 1 h of incubation at 37°C. Results were expressed as the number of eggs per parasite pair per gram of tissue. Counts were done in triplicate. Perfusion of mesenteric vessels was performed as described by Duvall and Dewitt (24). After anesthesia, the abdomen and chest were opened, the portal vein was dissected, and a catheter was introduced in the thoracic aorta or the left heart ventricle. The numbers and sexes of the adult worms were determined.

Histopathological analysis. Transversal sections of all liver lobes were collected, fixed in 4% buffered formaldehyde solution, and embedded in paraffin. Sections of 5 μ m were stained with hematoxylin-eosin (H-E) and phosphomolibdic acid-picro-sirius red (PMA-PSR) (23) and read by bright-field microscopy.

The area and level of fibrosis of the hepatic granulomas were determined by histological analysis by using 20 to 30 granulomas per animal. The centers of the granulomas contained viable eggs, which were randomly chosen. The granuloma area was manually delimited in images of H-E-stained sections, in which the images were captured with a charge-coupled device camera by bright-field microscopy and automatically processed with calibrated Scion Image software (version 3b; Scioncorp). For evaluation of the areas of fibrosis, PMA-PSR-stained images obtained by laser scanning confocal microscopy (LSM-410; Zeiss) were assessed with the same image software by application of a digital segmentation procedure. All evaluations were performed by two different blinded observers.

To evaluate the granuloma stage, a simplified classification of Lenzi et al. (47) was applied. Granulomas stained with PMA-PSR were divided into four different stages of maturation according to the intensity and the arrangement of the collagen deposition (see Fig. 4a). G1 represents the exudative stage, with the central egg characterized by small, weak, and disarranged collagen deposition; G2 represents the exudative-productive stage, with a moderate and disorganized arrangement of collagen; G3 represents the advanced exudative-productive or productive stage, with accentuated and more organized collagen deposition; and

G4 represents the productive-involucional stage, with a high density of collagen, a concentric collagen arrangement, and an area with reduced amounts collagen. The results are presented as the percentage of each stage per animal group.

For microphotography, digital images were captured with a color-chilled 3-charge-coupled device camera (model C-5810; Hamamatsu), stored in the tagged-image file format (TIF), and printed on a Codonics NP-1600 photographic dye-sublimation printer.

AST levels. The levels of aspartate aminotransferase (AST), a marker of hepatocellular damage, in serum were determined at 55 and 120 days postinfection by a colorimetric assay by using a commercial kit from CHIRON Diagnostics Corporation (East Walpole, Mass.).

Cytokine assay. Serum IFN- γ , IL-12, IL-4, and IL-10 levels were measured at 120 days postinfection by a sandwich enzyme-linked immunosorbent assay technique with capture and detection antibodies according to the instructions of the manufacturer (PharMingen, San Diego, Calif.). Recombinant cytokines were used as standards. Briefly, Immunosorb plates (Nunc, Roskilde, Denmark) were coated with capture antibodies and covered with pooled serum (from five to eight animals per group) or recombinant cytokine. Following addition of the biotinylated detection antibody and streptavidin-alkaline phosphatase conjugate, the reaction was developed with *para*-nitrophenyl phosphate (Sigma) and the absorbance at 405 nm was measured with a Benchmark reader (Bio-Rad Laboratories Inc., Hercules, Calif.). Assays were performed in duplicate or triplicate. The cytokine concentration was obtained from a regression curve prepared with the help of Microplate Manager software (Bio-Rad).

Statistical analysis. Statistical analysis was performed with SigmaPlot for Windows software (version 5.0; SPSS Inc.). Comparison between groups was done by the nonpaired Student *t* test. *P* values <0.05 were considered significant.

RESULTS

Parasitological parameters. The effects of glucocorticoids on worm burden have been described previously (17, 37). As it was proposed that the reduction in parasite burden by early administration of dexamethasone was due to interference with parasite maturation (38), we evaluated the effect of a chronic dexamethasone treatment on the development and/or survival of *S. mansoni* parasites on the host. For this, groups of infected mice were perfused, and the sex and maturity of the parasites were analyzed. At 120 days postinfection, no differences were observed in the number of worm pairs between nontreated animals (3.61 ± 0.14), animals treated with saline (4.17 ± 0.14), and dexamethasone-treated groups, independent of whether treatment was started on day 0 (3.43 ± 0.45) or day 35 postinfection (3.67 ± 0.47). Thus, at the concentration and with the schedule of administration used, dexamethasone did not influence larval development or the survival of the adults.

Since pathological alterations in hepatic tissue induced by *S. mansoni* infection resulted from a granulomatous reaction in response to eggs (74), interference with oviposition could modulate the course of disease. To analyze if dexamethasone affected the oviposition capacity, we compared the amounts of eggs retained in tissues (liver, intestine, and lungs) of infected nontreated animals (groups I and I + S) and treated animals (groups I + Dex0 and I + Dex35) at 120 days postinfection.

The results in Fig. 1 show that the reduction in the number of eggs retained in the hepatic tissues of animals treated with dexamethasone was small and not significant ($P = 0.06$). Although this reduction was not significant, it could indicate either a decrease in the level of oviposition or an increase in the level of migration of eggs to other tissues. In an attempt to verify these hypotheses, we counted the eggs retained in intestinal and lung tissues. An insignificant amount of eggs was found in the lungs, but a mild increase in the number of eggs was observed in intestinal tissue (Fig. 1). However, although dexamethasone caused an alteration in the egg distribution,

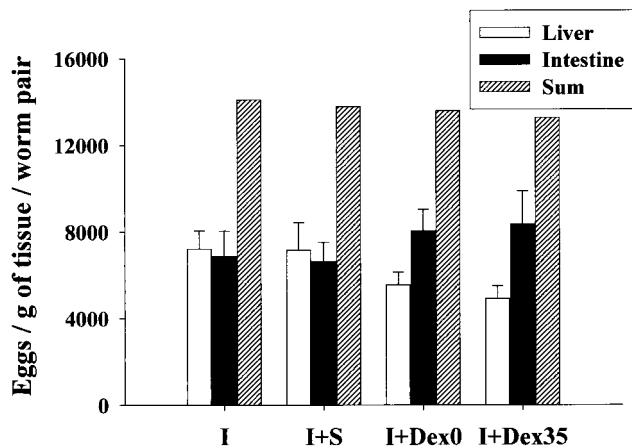


FIG. 1. Effect of dexamethasone on egg tissue distribution. Infected animals that were not treated (group I), treated with saline (group I + S), and treated with dexamethasone (groups I + Dex0 and I + Dex35) were killed at 120 days of infection. Tissues were digested as described by Cheever (11). The results represent the means \pm standard deviations for eggs from the intestine or liver or for all eggs found in these tissues.

the total number of eggs retained in tissues did not vary much between groups, indicating that it did not affect oviposition.

Histopathological parameters. According to Cheever et al. (12), a reduction in the amount of eggs in hepatic tissue may result in mild injury due to decreased granuloma formation and consequent extracellular matrix deposition (fibrosis). With the aim of evaluating the effects of dexamethasone on disease development, we used H-E- and PSR-PMA-stained histological sections to measure the area of the granulomas, the amount of collagen deposited, and the level of maturation of the granulomas.

As expected, the granuloma sizes in chronically infected animals either treated or not treated with dexamethasone showed downmodulations (Fig. 2). Moreover, significant decreases ($P < 0.001$) in the granuloma area were observed in the treated groups (groups I + Dex0 and I + Dex35) compared with those in the nontreated group (group I) at both 55 and 120 days postinfection (Fig. 2).

The area of collagen deposition was determined by confocal laser microscopy of PSR-PMA-stained sections (Fig. 3a). In general, as the infection evolves the area of collagen deposition increases (Fig. 3b). A significant reduction in the area of collagen deposition ($P < 0.02$) was observed in the I + Dex0 group in the acute phase, while in chronic phase, the granulomas of both treated groups (groups I + Dex0 and I + Dex35) presented reductions in collagen contents ($P < 0.004$ and $P < 0.0009$, respectively) (Fig. 3b).

To better understand the role of dexamethasone on the development of *S. mansoni* infection, we analyzed its effects on granuloma maturation. Since analysis of granuloma development involves several parameters, as described in Materials and Methods, granuloma development was classified into four distinct histological patterns: G1, G2, G3, and G4 (Fig. 4a). At 120 days postinfection, we observed a significant reduction in the percentage of G4 stages and a significant enhancement in

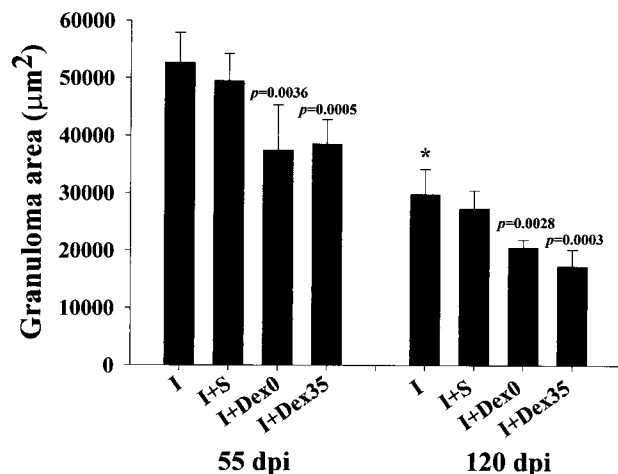


FIG. 2. Dexamethasone reduces granuloma area. The sizes of granulomas from groups of infected animals that were not treated (groups I and I + S) and that were treated with dexamethasone (groups I + Dex0 and I + Dex35) on H-E-stained slides were evaluated as described in Materials and Methods. The area of granuloma from 20 to 25 granulomas per animal was determined by using at least five animals per group. A statistical difference between acutely infected and chronically infected animals is indicated by an asterisk, while the differences between nontreated animals (group I) and treated animals (groups I + Dex0 and I + Dex35) are indicated by P values. dpi, day postinfection.

the percentage of G1 stages (Fig. 4b), showing that dexamethasone could lead to a delay in granuloma maturation.

AST activity. Apart from its role on the pathology of schistosomiasis, it has also been pointed out that the granulomatous reaction is a beneficial and protective process due to its ability to minimize the hepatotoxic effects of egg antigens (2). To evaluate whether the action of dexamethasone on granuloma size and evolution would be associated with increased hepatotoxicity, we measured the AST activity. Compared to the AST levels in the respective control group, independent of treatment schedule, the AST levels were significantly higher in all acutely infected animals, while no significant variation was observed in the chronically infected animals (data not shown).

Cytokines. Cytokines are believed to modulate the amount of fibrosis and granuloma size and, therefore, to play a fundamental role in the pathology of schistosomal infection (18, 13, 78). In order to investigate if the modulatory effects of dexamethasone on granulomas were mediated through alteration of cytokine production, the levels of these mediators in serum were measured. Dexamethasone induced decreases in the levels of IL-4, IFN- γ , and IL-12 that were more pronounced when the drug was administered from the beginning of the infection (Fig. 5). On the other hand, significant increases in serum IL-10 levels were detected in both treatment groups (groups I + Dex0 and I + Dex35; $P < 0.04$ and $P < 0.006$, respectively) (Fig. 5).

DISCUSSION

In the study described here we used a murine model to investigate the possible use of dexamethasone as a coadjuvant in the treatment of chronic schistosomiasis. Our results showed

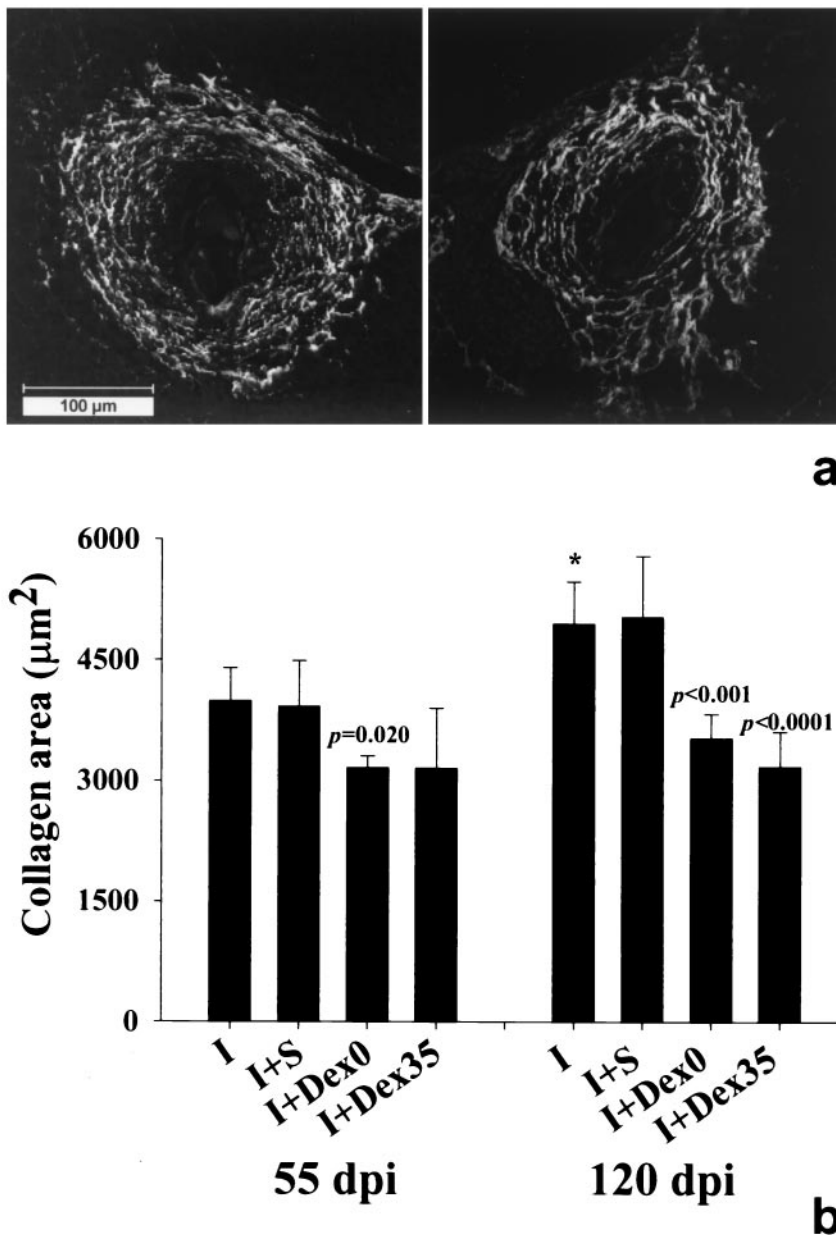


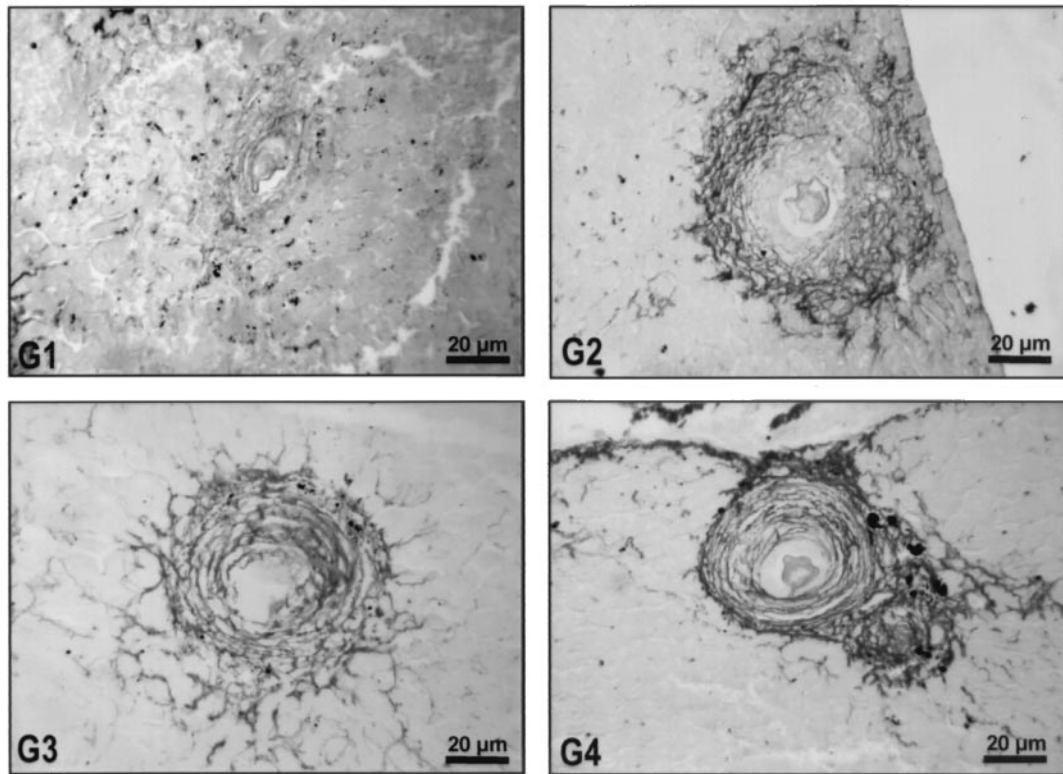
FIG. 3. Dexamethasone decreases the amount of collagen fiber deposition in hepatic granulomas. (a) Examples of images selectively expressing collagen in hepatic granulomas. (b) The area of collagen deposition was determined manually from 20 to 30 granulomas per animal, with at least five animals per group, by using images obtained by laser confocal microscopy from PSR-PMA-stained slides. A statistical difference between acute and chronic phase granulomas is represented by an asterisk ($P < 0.012$), while differences between the nontreated group (group I) and the dexamethasone-treated groups (groups I + Dex0 and I + Dex35) are expressed by P values. dpi, day postinfection.

no decrease in the parasite burden but did show an alteration in the egg distribution in tissue, reductions in the areas of granuloma and the amount of fibrosis, and interference with granuloma maturation and cytokine production, indicating that dexamethasone treatment may lead to mild disease.

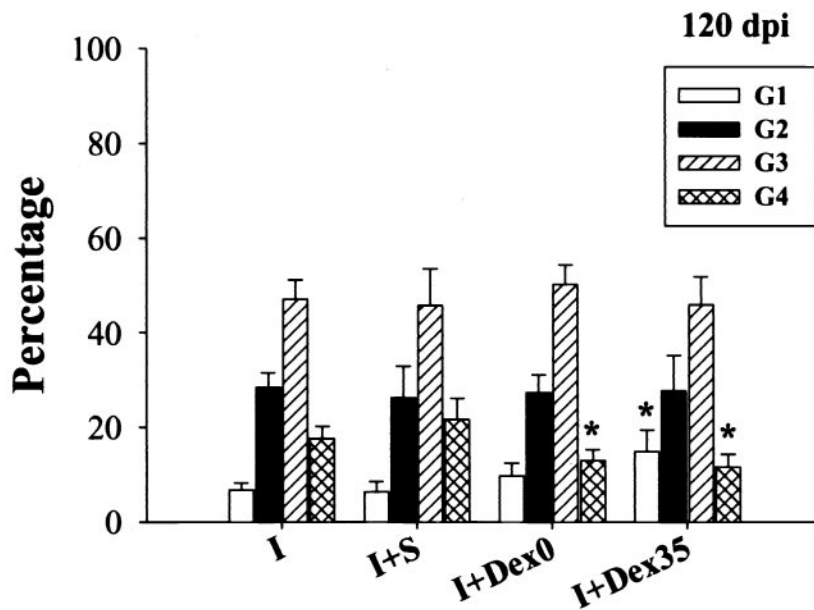
We did not observe any effect of dexamethasone on parasite number, confirming the results of Lambertucci et al. (46). However, investigators who used hydrocortisone or a dose of

dexamethasone 50 times higher than ours reported decreases in the parasite burden (17, 38).

After the start of oviposition, the eggs are carried mainly to intestinal and hepatic veins and also to the lungs and other tissues. Although a lack of effect of dexamethasone on *S. mansoni* fecundity in vitro has been described (54), in vivo a decrease in the amount of oviposition was reported after oral administration of dexamethasone (46). Our results demon-



a



b

FIG. 4. Dexamethasone delays granuloma maturation. (a) Images representing the four stages of granuloma development (stages G1, G2, G3, and G4), as described in Materials and Methods (PMA-PSR staining). (b) An increase in the percentages of G1 stages and a decrease in the percentage of G4 stages were observed in dexamethasone-treated mice (groups I + Dex0 and I + Dex35). The results are expressed as means \pm standard deviations. dpi, day postinfection.

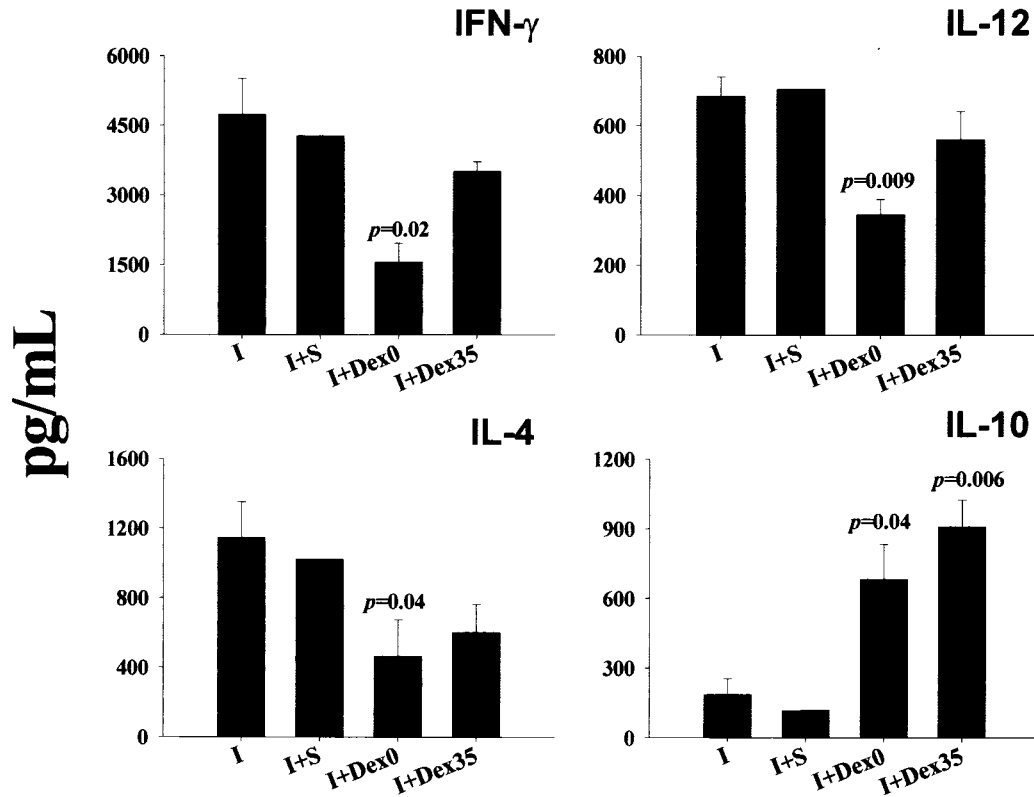


FIG. 5. Effects of dexamethasone on serum cytokine production. Cytokine levels in serum pooled from groups of nontreated and treated mice were measured by enzyme-linked immunosorbent assay as described in Materials and Methods. Asterisks represent statistical differences ($P < 0.05$) between the nontreated group (group I) and the dexamethasone-treated groups (groups I + Dex0 and I + Dex35). The results are expressed as means \pm standard deviations.

strated that with the therapeutic schedule used, neither worm development nor oviposition was significantly modified, but treatment altered the egg distribution in tissue, favoring a more intense deposition of eggs in the intestine instead of the liver. The mechanism by which dexamethasone alters the egg distribution in tissue is unknown. A reduction in the rate of egg excretion following treatment of infected mice with corticosteroids or hydrocortisone acetate was observed (21, 57). Indeed it was shown that in immunosuppressed mice and humans the rate of release of eggs to feces was reduced (21, 22, 44) and the number of eggs retained in intestinal tissue of mice lacking CD4⁺ T cells increased (27). Also, as the migration of eggs through the endothelium depends on intravascular periovular inflammatory cells (49, 50, 69) and/or platelets (58), the possible effects of dexamethasone in modulating the synthesis of molecules by these cells may change the adhesion of eggs to the endothelium, altering the traffic through the endothelium. Also, little is known about the effects of this glucocorticoid on the migration of female parasites and therefore on the intravascular sites of oviposition, with consequent changes in the places where the eggs are trapped in the tissues.

Since granulomas are composed of several cell types and extracellular matrix components, the action of dexamethasone on these elements is pleiotropic and difficult to evaluate in vivo. However, granuloma sizes in animals treated with dexamethasone showed significant decreases, probably due to the

high levels of IL-10 caused by the treatment. This observation is in accordance with those of other researchers, who showed that administration of exogenous IL-10 resulted in reductions in granuloma sizes (30), while an opposite effect was seen in IL-10-deficient (IL-10^{-/-}) mice (78). Rezende et al. (64) suggested that immunocomplexes from patients with chronic intestinal schistosomiasis are able to modulate granulomatous hypersensitivity to *S. mansoni* eggs by inducing prostaglandin E production that augments the IL-10 level. A rise in IL-10 levels in response to dexamethasone treatment was also described for several in vivo and in vitro models (19, 31, 68).

The significant reduction in the area of hepatic granulomas observed in treated animals could be beneficial for the host. Fanning et al. (28) reported that in mice, the diminution in granuloma size was directly correlated with reductions in rates of portal hypertension and morbidity. Otherwise, increased rates of mortality were detected among SCID mice that did not develop a periovular granulomatous reaction (2). The increased rate of mortality was attributed to severe hepatotoxicity caused by the elimination of toxic products released by the parasite eggs (35). In our model the effects of dexamethasone on reductions in the sizes of granulomas and on the maturation of granulomas did not induce hepatotoxic alterations, as the serum AST levels were not significantly different in treated and nontreated animals. This indicates that the periovular granu-

loma occurring in dexamethasone-treated mice serves to protect the host tissues from the miracidial secretions.

Besides the reduction in granuloma size that seems to be beneficial to the host, dexamethasone was also observed to have an antifibrotic effect. This effect could be due either to the direct action of the glucocorticoid in reducing cell recruitment and activation (72, 55, 60) or to its action in promoting the downregulation of several extracellular matrix proteins (67, 33). Thus, by interfering with the expression of costimulatory molecules (B7-1, B7-2, CD-28, CD-40) and cytokine production, dexamethasone may be altering cell activation and recruitment and therefore the outcome of fibrosis (1, 65). Indeed, Pechhold et al. (61) reported that CD80 (B7-1) can be constitutively expressed by murine fibroblasts and upregulated after treatment with IFN- γ and tumor necrosis factor alpha. This molecule was also detected in situ in fibroblasts from *S. mansoni* granulomas (H. L. Lenzi, personal observation). Glucocorticoids are also able to reduce the levels of synthesis of collagen types I and III (43, 56), which inhibit the transcription of collagens in murine schistosomiasis (75). However, this action is counterbalanced by its effect as a metalloproteinase inhibitor, which suppresses extracellular matrix degradation (10, 66, 71). These opposite actions are balanced by cytokines (66, 71).

The role of cytokines in the pathogenesis of *S. mansoni* infection, granuloma formation, and the fibrotic process has been investigated extensively. Administration of exogenous IL-4 increases the amount of fibrosis (80), while the administration of anti-IL-4 or exogenous IFN- γ decreases the level of collagen deposition (14, 18). In murine models, IL-12 was also involved with reductions in the amount of fibrosis and granuloma size (77, 39), while blockage of IL-13 was used to treat progressive liver fibrosis (16). In our study, dexamethasone treatment decreased serum IL-12 and IFN- γ levels and induced a pronounced reduction of IL-4 levels, suggesting that the reduction in the amount of periovular collagen fibers may be correlated with the effects of this glucocorticoid in modulating cytokine production. In addition to its deleterious effect of increasing the fibrotic process in schistosomiasis, a protective role for IL-4 has also been described. Thus, compared to wild-type mice, mice lacking IL-4 (IL-4 knockout mice) showed diminutions in catalase levels, increased levels of hepatotoxicity, and early mortality (26, 45). It is also possible that despite the decrease in the level of IL-4 production, the circulating levels of this cytokine are enough to exert a protective effect, preventing increases in levels of hepatotoxicity and rates of mortality. It has been suggested that this cytokine plays an important role in the severity of the *S. mansoni* infection and may influence the course of disease (9, 27). Since dexamethasone also increased serum IL-10 levels, our data are in agreement with those from previous reports (40, 41, 79), indicating that production of IL-10 is the key factor in preventing the polarization toward a Th1 or Th2 profile and therefore avoiding an increase in rates of mortality and morbidity.

The reasons why dexamethasone delays granuloma maturation are unknown and probably multifactorial. In fact, the modulatory actions of glucocorticoids on cell activation, the cytokine profile, the expression of adhesion molecules, and even on endothelial cells and extracellular matrix synthesis and degradation may interfere with granuloma development.

In general, specific treatment for schistosomiasis results in parasite elimination and, later on, a slight reduction in the amount of hepatic fibrosis (4, 42) that is attributed to parasite eradication. However, recent studies have indicated that despite treatment, part of the population remains infected (32, 70), raising concerns about resistance to praziquantel. In an attempt to improve the efficacies of conventionally used drugs, combined treatments have been investigated (8, 20). Since our results suggest that dexamethasone treatment results in a decrease in the severity of schistosomiasis, they point to dexamethasone as a convenient and promising adjuvant agent in the therapy of this infection, as already proposed by Lambertucci et al. (46) for acute schistosomiasis. In conclusion, this paper showed that the use of drugs with immunomodulatory actions, in addition to minimizing the morbidity from *S. mansoni* infection, may contribute to revealing the mechanisms involved in its pathogenesis.

ACKNOWLEDGMENTS

This work was supported by FAPERJ, CNPq, PRONEX, and FIOCRUZ.

We are grateful to L. Correa for the *S. mansoni* cercariae.

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