The effect of *Diphenyl Dimethyl Bicarboxylate* and *Dexamethasone* on Immunological and parasitological parameters in murine *Schistosomiasis mansoni*

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Abstract: This work aimed to evaluate the effect of Diphenyl Dimethyl Bicarboxylate (DDB) or dexamethasone either alone or combined with praziquantel (PZQ) on different parasitological, immunological, and pathological parameters that reflect disease severity and morbidity in murine schistosomiasis. Diphenyl Dimethyl Bicarboxylate (DDB) or dexamethasone had no effect on worm burden but altered tissue egg distribution. This indicates that under the schedule used, both drugs did not interfere with the development of adult worms or oviposition but it can modulate liver pathology. Meanwhile, dexamethsone showed a marked reduction of granuloma size more than DDB. Dexamethasone-treated mice, also, showed lower levels of serum gamma interferon (IFN-γ), interleukin-12 (IL-12), and IL-4 together with higher IL-10 level compared to infected untreated control animals. These data suggested that dexamethasone is a convenient and promising co adjuvant agent causing decreased morbidity in murine schistosomiasis. [Journal of American Science 2010;6(4):138-145]. (ISSN: 1545-1003).

Keywords: Schistosomiasis – Morbidity – Cytokines – Treatment.

1. Introduction

Schistosomiasis is a chronic and debilitating disease that remains one of the most prevalent parasitic infections in the humid tropics, with an estimated 650 million people at risk of infection and 200 million actually infected in 74 countries (WHO, 2002). It is encouraging that significant progress in the control of schistosomiasis has been achieved over the last several years in Brazil, China and Egypt. However, because of environmental changes linked to water resources development and the rapidly increasing sizes and movements of population, the disease has spread to previously non-endemic or low endemic areas (Engels *et al.* 2002).

The main cause of morbidity and mortality in human schistosomiasis is hepatic fibrosis, which essentially involves portal spaces, without severe lesions in the hepatic parenchyma. Management of schistosomiasis has focused primarily on treating and preventing the complications of portal hypertension. Unfortunately, no therapy has been proved to prevent progressive hepatic fibrosis which is associated with granulomatous hypersensitivity to parasite eggs. A proportion of patients with chronic schistosomiasis retain the hepatic fibrous scarring of the liver, following antihelminthic treatment. This problem leads to the suggestion that addition of anti-fibrotic agents as an adjuvant to anti-schistosomal chemotheraby may be useful in the treatment of *Schistosoma mansoni (S. mansoni)* infection (Mohamed *et al.* 1991).

Praziquantel (PZQ) remains the only antibilharzial drug effective against the four main schistosomes pathogenic to man (Gönnert & Andrews, 1977; WHO, 2002). Although it has been reported that PZQ has minimal side effects, control of schistosomiasis using PZQ at a population level faces some problems. Resistance to PZQ has been recently induced in schistosomes by laboratory selection (Fallon & Doenhoff, 1994). Reduced cure rates and failure of treatment after PZQ have been reported in Senegalese, Kenyan and Egyptian patients (Ismail *et al.* 1999; Fallon *et al.* 2000, Gryseels *et al.* 2001).

DDB (dimethyl – 4 , 4 '- dimethoxy – 5 , 6 , 5 ', 6' -dimethylene dioxybiphenyl- 2,2'-dicarboxylate), a component derived from *Shizandrae,* is a curative agent for the treatment of hepatitis used clinically in East Asia (e.g. China & Korea). It protects liver tissue against carbon tetrachloride-, galactosamine-, thioacetamide- or prednisolone-induced injuries, and enhances antibody production (Liu, 1987; Salama *et al*. 2004). A long term randomized controlled human study has shown DDB to substantially improve the liver function of patients with the hepatitis B virus (Liu, 1987). We have reported that the pharmacological effect of DDB was associated with the inhibition of NF-KB activation and TNFβ (Salama *et al*. 2004).Previous studies on the effect of corticoids in murine schistosomiasis showed variations according to the dose, the type and the schedule of treatment used (Harrison & Doenhoff , 1983, Morrison *et al. 1986,* Hermeto *et al* . 1990). It was proposed that the decrease in worm burden was due to impairment in the initial phase of parasite penetration into host tissues (Hermeto *et al . 1994*). Dexamethasone also decreased the level of collagen synthesis and the level of post-translational enzymes associated with

collagen synthesis (Newman & Cutroneo, 1978; James *et al*. 1983).

This study aimed to explore the effects of DDB or dexamethasone either alone or in combination with PZQ on several parasitological, immunological, and pathological parameters in murine schistosomiasis.

2. Material and Methods

Materials:

Animals:

Male, Swiss albino Laboratory-bred mice, each weighing 18-20 grams were used in this study. They were maintained, in conditioned rooms at 21ºC, on sterile water adlibitum and balanced dry food containing 24% protein. The animal experiment was carried out according to the internationally valid guidelines (Nessim *et al.* 2000) at Schistosome Biological Supply Program Unit of Theodor Bilharz Research Institute (SBSP/TBRI, Giza, Egypt).

Cercariae:

Schistosoma mansoni cercariae suspension (0.2 ml) was obtained from SBSP/TBRI and placed drop by drop on a glass plate. The cercariae on the plate were killed by the addition of one drop of 1% iodine. With the aid of a dissecting microscope, the number of cercariae was determined. Generally five counts were made to calculate the number of cercariae per ml of the suspension and the average number per 0.1 ml was used. Infection was performed by the subcutaneous injection of 100 *S. mansoni* cercariae to each mouse (Stirewalt & Dorsey, 1974).

Drug regimen:

Praziquantel

Tablets (600mg) were grinded as white powder and suspended in 13 ml of 2% cremophore-EL, as it is insoluble in water. The drug was freshly prepared before oral administeration to mice using a stainless steel oral canula. The dose given was 500 mg/kg body weight for two consecutive days.

Diphenyl Dimethyl Bicarboxylate (DDB)

Bimethyl-4,4′-dimethoxy-5,6,5′,6′-dimethylene-dioxybiphenyl-2,2′ dicarboxylate was supplied by Dongkwang Pharmaceutical Co. DDB was administered orally to mice using a stainless steel oral canula. The dose given was 25mg/kg body weight, three times a week until the end of the experiment.

Dexamethasone disodium phosphate

Decadron (Prodome, Brazil) was injected by the intramuscular route at 1 mg/kg body weight three times a week until the end of the experiment.

Experimental design:

Mice (70) were divided into seven groups (each composed of ten mice) as follows:

Group 1: Normal control group.

Group 2: Infected untreated control group, in which mice were infected with 100 *S. mansoni* cercariae.

Group 3: Infected-treated group, with 25 mg/kg DDB orally, three times/ week from the first day of infection to the end of the experiment.

Group 4: Infected-treated group, with 25 mg/kg DDB orally, three times/ week from the first day of infection to the end of the experiment and 500 mg/kg body weight of PZQ orally six weeks post-infection for two consecutive days.

Group 5: Infected-treated group, with intramuscular injection of 1 mg/kg dexamethasone, three times/week from the first day of infection to the end of the experiment.

Group 6: Infected-treated group, with intramuscular injection of 1 mg/kg dexamethasone, three times/week from the first day of infection to the end of the experiment and 500 mg/kg body weight of PZQ orally six weeks post-infection for two consecutive days.

Group 7: Infected with *S. mansoni* cercariae and treated with 500 mg/kg body weight of PZQ orally six weeks post-infection for two consecutive days.

Animals of all groups were killed under anesthesia, 8 weeks post infection.

Methods:

Parasitological Parameters

Worm burden: Hepatic and portomesenteric vessels were perfused to recover worms for subsequent counting (Duvall & DeWitt, 1967).

Tissue egg load: The number of ova/gm intestinal or hepatic tissue was counted after digestion overnight in 5% KOH (Cheever, 1968; Kamel *et al.* 1977).

Percentage egg developmental stages "Oogram pattern": The percentage of eggs at different developmental stages were examined in three samples/mouse and the mean of each stage/animal was obtained (Pellegrino *et al.* 1962).

Immunological study

Serum enzyme assessment

Animals of all groups were weighed, then killed and blood was collected. The serum was separated by centrifugation at 3000×*g* for 10 minutes and stored at −20°C for the assay of ALT [EC 2.6.1.2] (Reitman & Frankel, 1957), GGT [EC 2.3.2.2] using Boehringer reagent kit (Mannheim, Germany), AP [EC 3.1.3.1] (Kind & King, 1954), total protein (Weichselbaum, 1946) and albumin (Doumas et al. 1971).

Cytokine assay

Serum IFN-γ, IL-12, IL-4, and IL-10 levels were measured 8 weeks post-infection 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, plates (Nunc, Roskilde, Denmark) were coated with capture antibodies with 100 ul of serum sample or recombinant cytokine. Following addition of the biotinylated detection antibody and streptavidin-alkaline phosphatase conjugate, the reaction was developed with *para*-nitrophenyl phosphate (Sigma). Absorbance at 405 nm was measured with a Benchmark reader (Bio-Rad Laboratories Inc., Hercules, Calif.). Assays were performed in duplicate. The cytokine concentration was obtained from a regression curve prepared with the help of Microplate Manager software (Bio-Rad).

Histopathological study

Liver specimens were fixed in 10% buffered formalin and embedded in paraffin blocks. The prepared 4μm thick sections were examined by light microscopy using Hematoxylin and eosin and Masson trichrome stains.

Measurement of mean granuloma diameter per group was calculated at a microscopic magnification of X100 using an occular micrometer. Only lobular granulomas containing eggs in their centers and non-confluent ones were measured (Lichtenberg, 1962).

Statistical analysis

The data were presented as mean ± standard error of the mean (X±SE). The means of the different groups were compared globally using the analysis of variance ANOVA. Data were considered significant if p values were less than 0.05. different groups were compared globally using the analysis of variance ANOVA. Data were considered significant if p values were less than 0.05.

3. Results

The Worm burden and tissue egg load in the intestine and liver of each studied group were calculated as the mean ± SE. In the infected control group, the total number of worms counted was 29.6±0.26, divided between liver (43%) and portomesenteric vein (57%). Oral administration of DDB to infected mice with *S. mansoni* reduced the total number of worm burden to 23.3±0.29 (21.37% reduction); treatment of mice with dexamethasone alone reduced the total number of worm burden to 25.6 ± 0.20 (13.5 % reduction) especially those in the liver (Table 1). On the other hand, PZQ caused a marked reduction in worm burden reaching 95.6%, with 60% of the worms shifted to the liver; this inhibition was slightly improved when PZQ was given in combination with DDB (95.9%). The oogram pattern after PZQ treatment showed a complete disappearance of all immature ova from the wall of the intestine, a reduction in the number of mature ova and a four fold increase in dead ova. dexamethsone alone affected the number of dead ova significantly, reduced the number of mature ova (19–20%) while hardly affecting the immature ova. Combination of PZQ with DDB or dexamethasone augmented its effect on the mature ova to reach 85% 95% respectively (Table 2). In principle, the same observation was noted in egg load, where PZQ reduced it in both intestine (95.7%) and liver (96.4%), slightly expanded upon combination with DDB. DDB or dexamethsone alone showed a decrease also in the egg load mounted to 76.8%, 72.7% in the intestine, 76.1% and 66.5% in the liver.

Hepatic granuloma in each studied group was measured as the mean of granuloma diameter± SE and it was 318.8 μm ± 26.3 for the infected control group. Oral administration of DDB to infected mice with *S. mansoni* decreased the granuloma diameter to 194.1 μm ± 21.2 (39.1 % reduction) while administration of DDB in combination with PZQ decreased the mean of granuloma diameter to 168.1μm ± 32.11 (47.3% reduction). Intramuscular administration of dexamethsone to infected mice with *S. mansoni* decreased the granuloma diameter to 142.2 μm ± 25.1 (55.4 % reduction) that reached 119.2 μm ± 29.5 (62.6 % reduction) (Table 3). Accordingly, a significant reduction ($p<0.001$) was observed in granuloma diameter in the two treated groups either alone or combined with PZQ relative to infected control.

Table 1: **Effect of oral administration of DDB and intramuscular administration of Dexamethasone on Worm burden and tissue egg load in different studied groups**

Animal group	Mean No. of worms ± SE	% Reduction	Mean no. of ova count ± SE			
			Intestine	% reduction	Liver	% reduction
Infected Control	29.6 ± 0.26	-	14199±1342	-	2877±411	-
Dexamethson			***		***	
Dexamethson +PZQ	25.6 ± 0.31	13.5 %	3877± 211	72.7%	965±255	66.5 %
DDB	*** 1.6 ± 0.35	94.6 %	*** 715 ± 121	95 %	*** 178± 40	93.8 %
DDB + PZQ	*** 23.3 ± 0.29	21.3 %	*** 3292 ± 233	76.8 %	*** 689 ± 98	76.1 %
PZQ	*** 1.2 ± 0.29	95.9 %	*** 545 ± 133	96.2 %	*** 94±13	96.7 %
	*** 1.3 ± 0.35	95.6 %	*** 612 ± 156	95.7 %	*** 101±13	96.4 %

***** Statistically significant difference at p< 0.001 compared to infected control group.**
***** Statistically significant difference at p< 0.001 compared to infected control group.**

Table 2: Effect of oral administration of DDB and intramuscular administration of Dexamethasone oogram pattern of mice infected with 80 *S. mansoni* cercariae and sacrificed 8 weeks postinfection.

Group Name	**Oogram pattern (% ova)**		
	Immature	**Mature**	**Dead**
Infected Control	65.3 ± 5.4	31.1 ± 2.6	3.6 ± 0.7 *
Dexamethson	50.2± 4.2	30.7 ± 3.4	19.1 ± 1.5
Dexamethson +PZQ	*** 5.3 ± 5.1	*** 4.3 ± 1.4	*** 90.4 ± 8.3
DDB	** 20.2± 5.4	43.2± 6.1	** 36.6± 2.4
DDB + PZQ	*** 10.3 ± 1.9	*** 6.5 ± 1.4	*** 83.2 ± 5.4
PZQ	*** 2.3 ± 0.4	*** 1.9 ± 0.2	*** 95.8 ± 4.9

* Statistically significant difference at p< 0.05 compared to infected control group
** Statistically significant difference at p< 0.01 compared to infected control group
*** Statistically significant difference at p< 0.001 compared to infected control group

Table 3:- Effect of oral administration of DDB and intramuscular administration of Dexamethasone on hepatic granuloma diameter of mice infected with *S. mansoni*.

Group Name	Hepatic granuloma diameter X GD ± SE	% Reduction
Infected Control	318.8 ± 26.3	- ***
Dexamethson	142.2 ± 25.1	55.4 % ***
Dexamethson +PZQ	119.2 ± 29.5	62.6% **
DDB	194.1 ± 21.2	39.1% **
DDB + PZQ	168.1 ± 32.11	47.3 % **
PZQ	201.1 ± 25.3	24.1 %

GD :- Granuloma diameter
SE:- Standard Error.
** Statistically significant difference at p< 0.01 compared to infected control group
*** Statistically significant difference at p< 0.001 compared to infected control group

Table 4:- Effect of oral administration of DDB or intramuscular injection of dexamethasone either alone or combined with PZQ on cytokine production of mice infected with 100 *S. mansoni* cercariae and sacrificed 8 weeks post-infection

Group Name	IL-4 pg/ml X ± SE	IL-10 pg/ml X ± SE	IL-12 pg/ml X ± SE	IFN-γ pg/ml X ± SE
Infected Control	770±160.1	512±19.1	150±10.1	672±74.0
DDB	687±144.1	612±46.5***	131±9.6	607±63.0
DDB + PZQ	712±155.2	668±50.3	138±12.5	613±72.0
Dexamethasone	272±57.1***	711±23.1***	52±16.1***	127±54.0***
Dexamethasone+PZQ	288±100.3	698±33.0	45±14.4	192±94.0
PZQ	711±133.0	588±45.5	163±11.7	633±53.0

X = Mean SE= Standard Error.
**** Significant difference at p< 0.01 compared to infected control group.**
*** significant difference at p< 0.001 compared to infected control group.

Cytokines assy

Cytokines are believed to modulate the amount of fibrosis and granuloma size and play a fundamental role in the pathology of schistosomal infection. In order to investigate if the modulatory effects of both DDB and dexamethasone on granulomas were mediated through alteration of cytokine production, the levels of these mediators in serum were measured. Intramuscular administration of dexamethasone to infected mice induced significant decrease in the levels of IL-4, IFN-γ or IL-12 when compared their levels on infected untreated control group, while treatment of mice with DDB induced insignificant decreases in the levels of IL-4, IFN-γ, and IL-12 (Table 4). On the other hand, significant increases in serum IL-10 levels were detected in groups treated with DDB or dexamethasone comparing to the infected untreated control group.

Liver Enzymes Assay.

Treatment of mice with DDB or dexamethasone was found to reduce serum enzyme levels characteristic of hepatic damage induced by infection, as indicated by a lowering in the raised levels of serum ALT (78%, 85% respectively), GGT (73%, 86% respectively) and AP (76%, 93% respectively). Treatment of mice with DDB or dexamethasone, also tended to normalize the lowered levels of serum albumin. Untreated infected mice showed a two fold elevation of liver enzymes as compared with normal control animals. Treatment with PZQ alone reduced liver enzymes insignificantly compared to untreated infected mice. The highest significant reduction (p<0.100) in liver enzymes was observed in combination of PZQ with DDB or dexamethasone.

4. Discussions

Schistosomiasis is a major public health problem in tropics, with tens of millions infected and many more at risk (Boros, 1999). It has been estimated that greater than 250,000 deaths per year are directly attributable to this disease (Botros et al. 2000), and the subtle morbidities associated with chronic infection have a more serious impact.

Treatment relies on a single drug, praziquantel, to eliminate the adult worms but this has no prophylactic properties and is ineffective against resistant strains (Botros et al. 2000).

Previous studies have been reported using non-steroidal anti-inflammatory drugs (NSAIDs) e.g tiaprofenic acid and piroxicam either alone or as adjuvant to praziquantel in treating hepatic granuloma in *S.mansoni*-infected mice (Hegazy et al. 1997). The possibility of using another NSAIDs namely, ibuprofen (CAS 15687-27-1) and naproxen (CAS 22204-53-1), either alone or in combination with praziquantel (CAS 55268-74-1) has been studied to induce regression of hepatic morbidity or to ameliorate the biochemical and histopathological consequences and intensity of infection (Mahmoud et al. 2002). However, in the current study, we aimed to investigate the possible role of DDB or dexamethasone alone or as co adjuvant therapy in the treatment of murine schistosomiasis.

Oral administration of DDB to *S. mansoni*-infected mice showed insignificant decrease in the worm burden, and alteration of egg load in intestinal and liver tissue (76.8% and 76.1% reduction, respectively). However, combination with PZQ decreased the egg load in the intestinal and hepatic tissue giving 96.2% and 96.7% reduction, respectively. Moreover, reduction in granuloma diameter and cytokine production, indicated that administration of DDB to *S. mansoni*-infected mice may improve disease morbidity.

The protective effects of DDB on chemically induced damage of isolated suspended rat hepatocytes were studied by Fu and Liu (1992) and its reversing effect on the phenotyes of human hepatocarcinoma cell line has been evaluated (Liu et al. 1996). Recently, administration of DDB on tamoxifen-induced liver injury in rats showed that prolonged treatment revealed a potent anti-fibrogenic role (El-Beshbishy, 2005). Moreover, a pharmaceutical composition of garlic oil and DDB, as active ingredients for enzyme induction and liver protection has been used as a curative preparation for patients with acute or chronic viral hepatitis (Park et al. 2005).

On the other hand, dexamethasone was previously investigated as a co adjuvant immunomodulator in treatment of chronic schistosomiasis (Pyrrho *et al.* 2002).

Our results showed insignificant effect of dexamethasone on parasite number which agreed with Lambertucci et al. (1989). However, investigators who used hydrocortisone or a dose of dexamethasone 50 times higher than ours reported decrease in the parasite burden (Coker, 1957, Hermeto *et al. 1993*). Following oviposition, the eggs are carried mainly to intestinal and hepatic veins then to the lungs and other tissues. Although a lack of *S. mansoni* fecundity using dexamethasone in vitro has been described (Morrison, 1986), another decrease in the amount of oviposition was reported after oral administration of dexamethasone in vivo (Lambertucci et al. 1989). Our results demonstrated that with the therapeutic schedule used, neither worm development nor oviposition was significantly modified, but the treatment altered egg distribution in tissue, favoring a more intense deposition in the intestine. The mechanism by which dexamethasone alters the egg distribution in tissue is unknown. Also, little is known about the effect of this glucocorticoid on the migration of female parasites and on the intravascular sites of oviposition. A reduction in the rate of egg excretion following treatment of infected mice with corticosteroids or hydrocortisone acetate was observed (Newsome, 1963; Doenhoff et al. 1978) 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 size in animals treated with dexamethasone showed significant decrease, probably due to the high levels of IL-10 induced by treatment. This observation is in accordance with Franchimont et al. (1999) who showed that administration of exogenous IL-10 resulted in reduction of granuloma size. Furthermore, an opposite effect was seen in IL-10-deficient mice (Wynn et al. 1998). Rezende et al. (1997) 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 IL-10 level.

Cytokines are believed to modulate the amount of fibrosis and granuloma size and play a fundamental role in the pathology of schistosoma infection. In order to investigate if the modulatory effects of both DDB and dexamethasone on granulomas were mediated through alteration of cytokine production, the levels of these mediators in serum were measured. Administration of exogenous IL-4 increases the amount of fibrosis (Yamashita & Boros. 1992), while administration of anti-IL-4 or exogenous IFN-γ decreases the level of collagen deposition (Cheever et al. 1994, Czaja et al. 1989). In murine models, IL-12 was also involved in reduction of the amount of fibrosis and granuloma size (Wynn et al. 1995, Hoffmann et al. 1998). However, compared to wild-type mice, mice lacking IL-4 (IL-4 knockout mice) showed diminution in catalase levels, increased hepatotoxicity that resulted in early

mortality (Fallon et al. 2000, La Flamme et al. 2001). In our study, administration of DDB or dexamethasone to *S. mansoni*-infected mice decreased serum IL-12 and IFN-γ levels and induced a pronounced reduction of IL-4 levels, but it is 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 and has been suggested that it plays an important role in the severity of *S. mansoni* infection and may influence the course of disease (Brunet et al. 1998, Fallon et al. 2000). Since dexamethasone also increased serum IL-10 levels, our data are in agreement with those from previous reports (Hoffmann et al. 1999; 2000; Wynn et al. 1997), 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 disease morbidity.

In conclusion, the use of DDB or dexamethasone as a coadjuvant treatment with praziquantel in murine schistosomiasis, in addition to minimizing the morbidity of infection, may give an insight into the mechanisms involved in its pathogenesis.

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