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IMMUNOTHERAPEUTIC EFFECT OF SPIRAMYCIN IN EXPERIMENTAL GIARDIASIS

By

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Abstract

Giardiasis is a major global cause of water borne diarrheal disease, which contributes greatly to the burden of malnutrition and malabsorption especially in children. There is a great demand for a new effective therapeutic agent against giardiasis that can be used safely during pregnancy, lactation and in infants. In the present study, the therapeutic effect of spiramycin as well as its immunomodulatory mechanism of action in giardiasis had been investigated. 90 Swiss albino mice were used in this study and classified into 3 groups: GI: 40 mice infected with *Giardia lamblia* cysts, GII: 40 infected mice that received spiramycin treatment in a daily oral dose of 1000 IU/gm body weight for one week starting one week post infection and GIII: 10 control uninfected untreated mice. 20 mice from each infected group were sacrificed 2 weeks post infection (p.i.) and the remaining mice were sacrificed 4 weeks p.i. Mice of the control groups were sacrificed at one time. The antigiardial therapeutic efficacy of spiramycin was assessed 2 and 4 weeks p.i. by counting of *Giardia* cysts in stool of mice and studying the histopathological changes and disaccharidase activity in small intestine of mice of different groups. Significant reduction in cysts number shedded in stool of treated animals reached 95.73%. The histopathological changes were mild in all infected groups 2 weeks p.i., while 4 weeks p.i. There was also a significant increase in the number of IELs in treated groups denoting the stimulatory effect of spiramycin on lymphocytic proliferation. On studying the disaccharidase activity, there was significant increase in both sucrase and maltase activities in the treated groups as compared with the non-treated groups. The possible immunomodulatory mechanism of action of spiramycin was studied by measuring the local IgA deposition in small intestinal mucosa by PAP technique 4 weeks p.i. The levels of IgA in small intestine were higher in SP-treated group as compared with the non-treated group. The present results suggested that spiramycin has high efficacy as anti-giardial agent possibly by stimulation of local IgA production.

Key words: Spiramycin, Giardiasis, mice, IgA.

Introduction

Giardia lamblia is a protozoan parasite that replicates exclusively in the lumen of the small intestine of a wide variety of mammalian hosts. Giardiasis is a major global cause of water borne diarrheal disease, which contributes greatly to the burden of malnutrition and malabsorption especially in children (Adam, 2001). *Giardia* infections are practically universal in the developing world as Egypt whenever fecal contamination occurs, such as with contamination of water supplies or direct person to-person spread in day care centers (Mohamed *et al*, 2004). Intestinal infection with *G. lamblia* is responsible for decreased absorption of fluids, electrolytes and glucose leading to disaccharidase deficiencies at least in part via diffuse brush border microvillus shortening which may be combined with villus atrophy. These abnormalities lead to malabsorption and maldigestion (Farthing, 1993). The courses of infections are highly variable among individuals as in children, pregnant women and immunocompromised persons. *G. lamblia* appears as an opportunistic parasite with development of various complications as malabsorption syndrome, lactose intolerance, vitamin B12 deficiency and pancreatic insufficiency (Gassama *et al*, 2001). Therefore, host immune status had been found to be the major determinant of the pathogenesis of giardiasis (Farthing, 2003). Both cellular and humoral immune responses are essential for controlling *Giardia* infection. In the early phase of infection, a T-cell dependant mechanism is responsible for controlling acute infection with production of various cytokines as IL-6, IL-4, IL-5 and IFN-γ (Faubert, 2000). When *Giardia* infection persists beyond the initial phase, a second phase of the immune response can occur. This is presented by production of parasite-specific antibodies of the IgA isotype which are found predominantly on mucosal surfaces and react with all parasites present in a population expressing diverse surface antigens (Langford *et al,* 2002).

Although different classes of drugs were used for treatment of giardiasis (metronidazole, quinacrine, furazolidone paromoycin and nitazoxanide), none

of them can be regarded as an ideal drug used safely in infants as well as in pregnant and lactating women (Rosenblatt,1999). In addition, treatment failure and drug resistance are common in most treatment regimens of giardiasis (Harris *et al*, 2001). It was of particular interest to search for an alternative antigiardial drug that relies on the intimate knowledge of biochemistry, molecular biology and immune response against the *G. lamblia.* Spiramycin is a macrolide non-hepatotoxic antibiotic that was used safely in pregnancy, lactation and in immunosuppression (Nucera *et al,* 2001). It has been used effectively in treatment of various protozoal infections as toxoplasmosis and cryptosporidiosis (Giacometti *et al,* 1996). The spiramycin did not act only as antimicrobial drug but also as an immunomodulatory agent by conjunction with cellular host defense mechanisms (Bailly *et al,* 1991). On the other hand, El Basha *et al*. (2016) in Egypt stated that giardiasis is considered the commonest intestinal parasite in humans worldwide and that the children are especially affected with more severe consequences than adults.

The present work aimed to study the immunotherapeutic effect of spiramycin in treating *Giardia lamblia* infection in experimental animals. The antigiardial therapeutic efficacy of spiramycin was assessed by parasitological assay, biochemical assay of disaccharidase activity and examination of the histopathological changes in small intestinal mucosa. The possible immunomodulatory mechanism of spiramycin action was studied by measuring the intensity of local IgA deposition in small intestinal mucosa of experimentally infected mice.

Materials and Methods

Giardia lamblia cysts were separated from stool of heavily diarrheic children attending the Outpatient Clinic of Pediatric Hospital, Tanta University. Cyst suspension was prepared according to Cheesbrough (1987), in which cysts were separated from stool samples by a modified formol ether technique where normal saline was used instead of formalin. The cyst suspension was adjusted to contain 400.000 cysts/ml. In *G.lamblia* infected groups, each mouse was infected by intraoesophageal inoculation of 0.25 ml cyst suspension containing 100.000 *G.lamblia* cysts.

Drug regimen: Spiramycin (SP), (Rovamycin, 3.000.000 IU, Alex Pharm, Co.) was given as antigiardial agent in a dose of 1000 IU/gm body wt. for each mouse. Each mouse received a daily oral dose of SP for one week starting one week post infection; peak of intestinal *Giardia* colonization (Buret *et al,* 1990).

Experimental design: 90 laboratory-bred, parasite-free Swiss albino mice (18-20gm, 6-8 weeks old) were used in this study. They were classified into 3 groups: GI (infected, non SP treated): 40 mice, infected with *G. lamblia* cysts. Mice received no spiramycin treatment. GII (infected, SP treated): 40 mice infected with *G. lamblia* cysts. Mice received spiramycin treatment. GIII (control group):10 mice uninfected and untreated.

Two weeks post infection abs a day after last spiramycin dose: Two subsequent stool samples were collected from all mice of the infected groups for cyst counting. Thereafter, 20 mice from each infected group were sacrificed. The upper 10 cm of small intestine of each mouse were dissected, removed and divided into 3 parts. One part was fixed in 10% formalin and embedded in paraffin wax for histopathological studies. Third part was blotted dry, weighed, homogenized in 2.5mM EDTA (4 ml/one gram of tissue) and stored at -70^{o}C till use for assay of disaccharidase activity.

Four weeks post infection: The remaining mice* of each of the infected groups were sacrificed. The upper 10 cm of small intestine of each mouse were removed , fixed in 10% neutral buffered formalin (pH 7.3) and embedded in paraffin wax for histopathological examination as well as for immunostaining of local small intestinal IgA (which is released later during the chronic phase of giardiasis (Langford *et al,* 2002).

Mice of the control GIII were sacrificed at same time and processed as before for comparison.

*Number of mice died during the course of the experiment: one mouse died from GI and GII.

Parasitological assay: Cyst counting in stool of mice: A two-hour fecal collection was obtained from each mouse by isolating it in a plastic cage for 2 hours. Feces were broken up in tap water and the fecal suspension was centrifuged at 400g for 15 minutes. The sediments were resuspended in a known volume of water before counting in a haemocytometer (Moitinho *et al,* 1999). Results were

presented as the mean number of cysts recovered from infected treated and non-treated groups, and statistically analyzed. The reduction percentage was calculated (Abou Gamra and El Hosseiny, 2003) reduction% = a-b (a = mean cysts number in infected non-treated groups, b =mean number of cysts in infected treated groups).

Small intestinal histopathological examination and trophozoite counting: Formalin-fixed, paraffin-embeded sections (5µm) were prepared and stained with hematoxylin-eosin. The five random sections/mouse was examined (Scott *et al,* 2004).

Crypts and villi were measured by a calibrated eyepiece micrometer and the intraepithelial lymphocytes (IELs) were counted along villus units. IELs were expressed as number per 100 epithelial cells (Scott *et al,* 2000). Colonization in small intestine was assessed by counting trophozoites/villous by oil immersion lens and heaviness of *Giardia* infection was made and scored in each section (Deyab and Nosseir, 2000) as containing many trophozoites (more than 2/villus), few trophozoites (1or 2/villus) or no trophozoites (6 to 8/villi).

Biochemical assay of disaccharidase activity: Sucrase and maltase activities were used as a functional marker of *Giardia* induced mucosal injury. Their activities were determined (Belosevic *et al,* 1989) and expressed as units per gram of protein. Total protein content was determined by the Bradford protein assay (Bradford, 1976).

Immunostaining of local IgA in small intestine: Paraffin-embedded, formalin fixed sections were stained with peroxidase-antiperoxidase (PAP) using monoclonal antibodies to detect local IgA in small intestines. Scoring each section was made (Deyab and Nosseir, 2000) as containing no IgA deposits (-), mild IgA deposits (+), moderate IgA deposits (++) and marked IgA deposits (+++).

Statistical analysis: The result were expressed as M±SD. The differences were statistically analyzed and compared for significance at $P \leq 0.05$ using student's t test and analysis of variance (ANOVA) test by using SPSS program version 13.

Results

The parasitic load of *Giardia lamblia* in mice showed a significant reduction ($P<0.001$) in the cyst shedding two weeks post infection (p.i.) in the stool of infected treated group as compared with infected non treated one, with 96.52% cyst reduction.

The immunostaining of local IgA by PAP technique showed that four weeks p.i., there was marked deposition of IgA (+++) in GII (Fig. 1) and moderate deposition (++) in GI.

The histopathological changes in small intestinal sections 2 weeks p.i. showed non-significant shortening in villus height and non-significant increase in crypt depth in all infected groups. Trophozoites were many in sections examined for GI and none in examined sections for GII. 4 weeks p.i., without significant difference in villus height or crypt depth between infected groups.

Intraepithelial lymphocytic (IEL) count: 2 weeks p.i., there was non-significant increase in number of IELs in GI when compared to GIII. 4 weeks p.i., there was non-significant increase in number of IELs in both GI when compared to GIII. Disaccharidase activity in small intestinal mucosa: Two weeks p.i., there was a significant increase ($P<0.001$) in both sucrase and maltase activities in treated groups (GII) as compared with GI. But, the mean values of both sucrase and maltase in the infected treated groups were lower than in GIII.

Details were given in tables (1, 2, 3, 4, 5 & 6) and figures (1, 2, 3, 4 & 5).

Table 1: Number of shaded *G. lamblia* cysts in stool of mice 2 weeks p.i.

Items	GI	GII
Range	32.0-41.0	0.5-2.0
Mean ± SD	36.75±2.69**	1.28±0.35
Percentage of Reduction	96.52%	
t	58.43	
P-value	***P <0.001*	

Table 2: Intensity of local IgA deposition in small intestinal mucosa 4 weeks p.i.

Experimental group	Local IgA 4 weeks post infection
GI (IC, infected, non SP treated)	++
GII (IC, infected, SP treated)	+++
GIII (control IC group)	-

Table 3: Changes in villus height and crypt depth in small intestinal mucosa in µm

Experimental group	2 weeks post infection (M±SD)			4 weeks post infection (± SD)		
	No.	villus height	crypt depth	No.	villus height	crypt depth
GI	20	502+45.49	123+34.33	19	498+38.66	126+35.38
GII	20	512+64.33	121+39.28	19	516+72.51	114+23.42
GIII	10	520+76.61	103+19.55	10	520+76.61	103+19.55
P. value	*P <0.05					

Table 4: Count of intraepithelial lymphocytes/100 epithelial cells in small intestinal mucosa of different groups.

Experimental group	IELs 2 weeks p.i.		IELs 4 weeks p.i.	
	No.	(M± SD)	No.	(M ± SD)
GI	20	7.66 ± 1.42	19	8.31 ± 2.55
GII	20	8.72 ± 2.56	19	12.93 ± 1.67
GIII	10	6.58 ± 1.46	10	6.58 ± 1.46
P<0.001				

Table 5: Maltase activity in small intestinal mucosa of mice 2 weeks p.i.

	Maltase		
	GI	GII	GIII
Range	376 - 385	304 - 506	625 - 639
M±SD	381.15±2.70	489.30±43.72	631.65±3.92
F	1048.422		
P-value	<0.001		

Table 6: Sucrose activity in small intestinal mucosa of mice 2 weeks p.i.

	Sucrase		
	GI	GII	GIII
Range	62 - 68	74 - 82	91 - 101
M±SD	65.35±1.66	78.35±2.08	95.65±2.91
F	1192.2		
P-value	<0.001		

Discussion

Giardia lamblia is one of the first enteric pathogens of infants in the developing world, with a prevalence peak of 15-20% in children younger than 10 years, mainly among nursery and primary school children (Seidel, 2004). Although *Giardia* can infect all people, young children and pregnant women may be more susceptible to different complications resulting from dehydration and lactose intolerance (Huston and Gurrant, 2002). There was a great need for giardiasis effective therapeutic drug that can be used safely during pregnancy, lactation and in infants.

In the present study, therapeutic effect of spiramycin (SP) and its immunomodulatory mechanism of action in giardiasis were investigated. It was found that there was significant reduction in number of cysts shaded in stool of SP-treated animals with a percentage of reduction reached 95.73%. This percentage of cure with spiramycin was higher than that with metronidazole, which commonly used as first-line agent for treatment for giardiasis, and showed cure rates reaching 85-90% in patients (Gardner and Hill, 2001). Spiramycin has the advantage over metronidazole in safety even in repeated courses. Zaat *et al*. (1997) declared that repeated chronic courses of metronidazole predisposed to many side effects as pancreatitis, peripheral neuropathy and carcinogenicity in experimental animals. Metronidazole was contraindicated during pregnancy especially in first trimester and in infants (Tracy and Webster, 1996). Other antibiotics used during pregnancy as paromomycin has a relatively low efficacy as antigiardial drug with 55-7 % (Sandford *et al*, 1997).

In this work, the spiramycin effect on humoral immune response was investigated 4 weeks p.i. by immunostaining of local IgA in small intestine of different groups. There was marked deposition of local IgA in treated group as compared to moderate deposition in non-treated one. Bienz *et al.* (2003) found that IgA responses require Th-2 cells producing cytokines which control IgA B-cell differentiation.

The histopathological examination revealed that 2 weeks p.i., there was absence of trophozoites in infected and treated group denoting the high therapeutic efficacy of spiramycin as antigiardial drug.

Regarding the intraepithelial lymphocytic (IEL) infiltration, there was a non significant increase in the number of IELs in the treated group at the second week p.i., whereas a highly significant increase in the number of IELs in the treated group was noticed at the fourth week p.i, when compared to non treated groups. These findings could be referred to the stimulatory effect of spiramycin on intra-epithelial lymphocytic proliferation that play an important role in controlling giardiasis. These results agreed with Singer and Nash (2000) and Echmann (2003) as they showed that IELs influx in giardiasis is responsible for the elimination of the infection and their origin is both thymic and extrathymic. Also, there was no significant difference in villus architecture between infected groups 2 weeks p.i., as compared to control group. Four weeks p.i., there was preservation of the villus architecture in both infected treated and untreated groups groups. Ebert (1999) showed that brush border injury and increased crypt/villus ratio causing malabsorptive diarrhea in giardiasis, are dependent on cytotoxic-T cells. As *Giardia* trophozoites cause T-helper cells in intestine to proliferate and produce IFNγ that in turn increase epithelial cell permeability, permitting more *Giardia* antigens to enter the mucosa and further sensitize the cytotoxic T lymphocytes. The results agreed with Wolfe (1992) who showed that patients with symptomatic giardiasis had a normal jejunal histological picture without inflammatory exudates.

The disaccharidase activity is considered as a functional marker of the small intestine digestive epithelium. The alteration of enzymatic activity in infected group was significant as compared to the control group which showed the parasite itself potential to cause damage to brush border membrane (Daniels and Belosevic, 1995). Disaccharidase deficiency had markedly improved with spiramycin therapy in treated mice. This could be attributed to its antiparasitic effect on *Giardia* trophozoites and eliminating their injurious effect on brush border membrane of small intestine.

Conclusion

The outcome data showed that existence of T-cell dependent pathways and their effects in controlling giardiasis allow the development of novel immunotherapies for giardiasis.

In this respect, spiramycin has been shown to have a high efficacy as anti-giardial agent, possibly by enhancing the immune response of the host that stimulates differentiation of IgA producing B-lymphocytes into IgA producing plasma cells. The immunomodulatory mechanism of action of spiramycin was evidenced by highly significant increase in intraepithelial lymphocytes as well as local IgA in small intestine of spiramycin treated mice.

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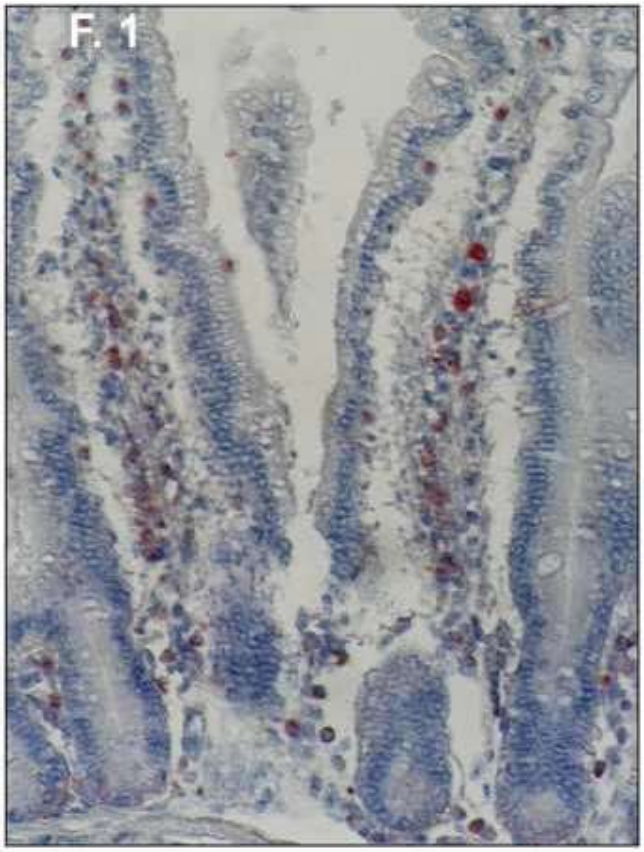
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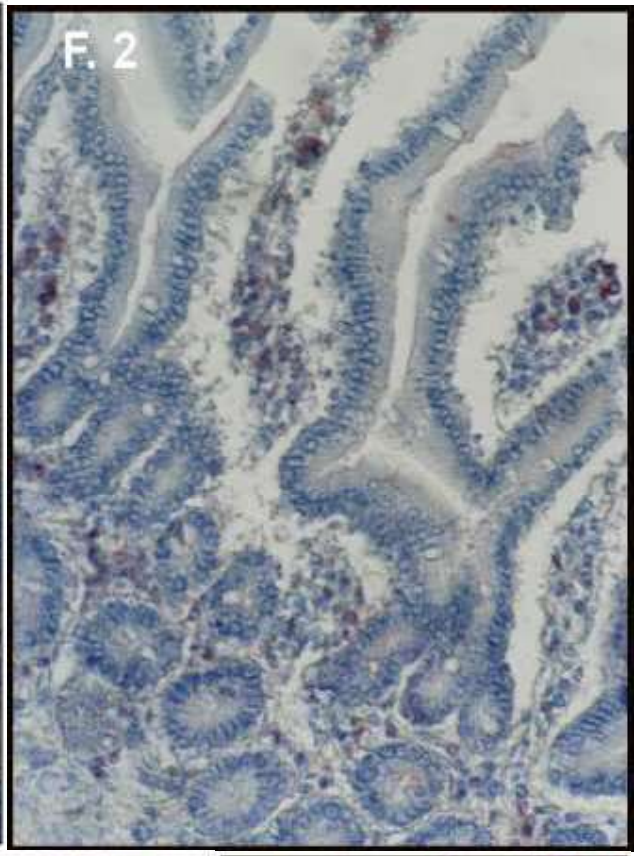
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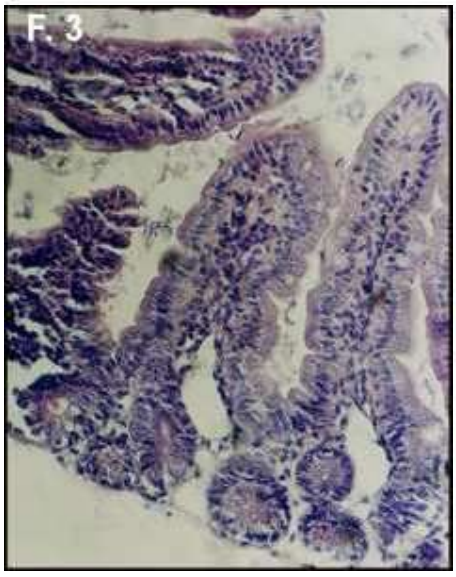
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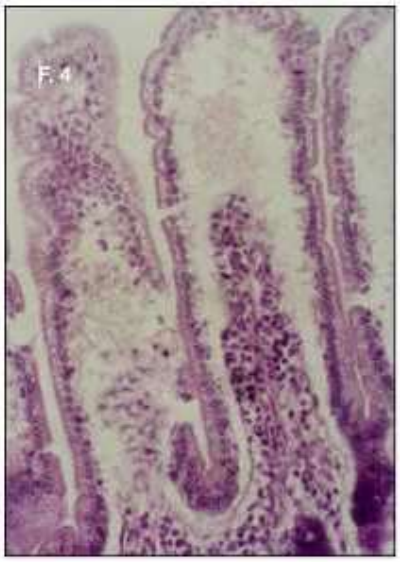
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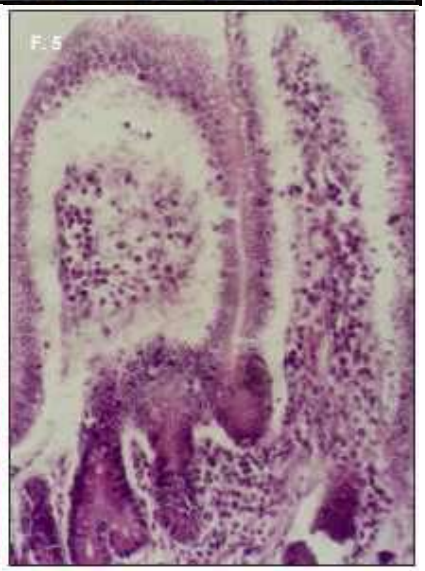
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