ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Immunomodulatory Effect of Schistosomula on Reinfection and Treatment.

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ABSTRACT

We have shown previously that natural extracts increase the efficacy of praziquantel. The present study aimed to evaluate the immunoprotective effect of schistosomula on *Schistosoma mansoni* Egyptian strain re-infection before and after treatment with praziquantel. Mice were immunized subcutaneously with schistosomula (500 schistosomula\mouse) and boosted on the 14th day. After the 2nd immunization mice were challenged by 80 cercariae of *S. mansoni*, on 42nd, 45th and 48th days post infection mice were treated with praziquantel (250 mg\Kg body weights). One week post treatment, mice were re-infected. After six weeks of reinfection the worm burdens were quantified using perfusion technique. The IgM and IgG titers in sera were detected by enzyme linked immunosorbent assay. Thymocytes and mesenteric lymph nodes (MLNs) lymphocytes were counted. Results: The mean percentages of reduction of total worms from mice infected with *Schistosoma mansoni* and treated with praziquantel, *Schistosoma mansoni* infected-treated-re-infected, schistosomula immunized\challenged by 80 cercariae, immunized-challenged-treated and immunized-treated-re-infected by 80 cercariae were 54.6, 43.0, 60.5, 59.2 and 68.5% respectively. Immunized- treated- re-infected mice showed a significant decrease (p<0.05) in the mean percentage of CD8$^+$ T from thymus gland or MLN as compared with mice immunized\challenged or immunized-challenged-treated respectively. **Conclusion:** Our study demonstrated that immunization with schistosomula associated with therapy were able to induce partial reduction in worm burden (68.5%) against *S. mansoni* re-infection and modulate the immunocellular response.
Keywords: Immunomodulatory, Schistosomula, praziquantel, reinfection. CD4$^+$ T. CD8$^+$ T, mesenteric lymph nodes, thymus gland.

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ISSN: 0975-8585

INTRODUCTION

Although praziquantel (PZQ) is an effective chemotherapy for schistosomiasis reinfection occurs rapidly after mass drug administration [1]. Partial resistance can be induced in some adult individuals receiving repeated PZQ treatment and this is correlates with a predominant Th2 response [2]. PZQ treatment induced immunological changes have been associated with resistance to re-infection [3]. The entire population was not re-infected at the same rate, because, it is possible that host factors may play a dominant role in determining resistance or susceptibility to re-infection with schistosomes [4]. Schistosomula are susceptible to humoral and cellular immune responses, but rapidly develop resistance to host immunity [5]. However, antigens obtained from schistosomula were efficient stimulators of lymphocyte proliferation and secretion of Th1 cytokines than those from cercariae and skin-stage larvae [6]. Mice immunized with *S. mansoni* schistosomula tegument (Smteg) in the absence of adjuvant induced no significant reduction in the worm burden [7].The extracts of schistosomula of *S. mekongi* had a protective efficacy (59%) and were able to stimulate the humoral immune responses [8]. These vaccines function mainly through inducing specific antibody responses or activating $CD4^{+}$-T cells against schistosomula or adult worms [9]. Finally, immunization with the tegument nucleotidase; alkaline phosphatase (SmAP) associated with a subcurative PZQ treatment reduced worm burden following *S. mansoni* challenge [10]. Herein, we aimed to evaluate the immuno-protection against *Schistosoma mansoni* re-infection using praziquantel and schistosomula. From our particular point of view such combination may allow better programs to decrease the prevalence of parasite re-infection by low cost.

MATERIALS AND METHODS

Drug and antigens

Praziquantel (Biltricide® - manufactured by Alexandria co. for Pharmaceuticals and Chemical. Ind. Alexandria – Egypt under license of BAYER Leverkusen, Germany) was suspended in 0.01 M Phosphate Buffered Saline (PBS, pH 7.2). The drug was administered to mice by oral administration (250 mg/kg body weight in 200 µl PBS), on 42nd, 45th and 48th days post infection [11]. Schistosomula were prepared from cercariae of *S. mansoni* strain [12]. *S*oluble worm antigen preparation (SWAP) and soluble egg antigen (SEA) were prepared [13, 14].

Animals and infection

Female Swiss albino mice were used (18-20 gram). Animals were fed on standard chew, supplied with water and maintained at ambient temperature 25°C. Ten mice were immunized subcutaneously (Sc) with 500 schistosomula\mouse at the 0 day and boosted at the 14th day. At the 28th day post 2nd immunization (Sc) mice were challenged with 80 cercariae of *S. mansoni* by tail immersion method [15].Ten mice were infected with 80 cercariae of *S. mansoni* strain by tail immersion. On 42nd, 45th and 48th days post infection, mice were treated with PZQ (250 mg/Kg body weight) [16]. After one week of PZQ treatment; the mice were subjected to reinfection with 80 *S. mansoni* cercariae. Ten mice were immunized with 500 schistosomula\mouse and boost on the 14th day. On the 28th day after booster mice were challenged with 80 cercariae of *S. mansoni*. On the 42nd, 45th and 48th days post infection mice were treated with PZQ (250 mg\Kg body weights).Ten mice were immunized with 500 schistosomula\mouse and boosted on the 14th day. After booster (on the 28th day), mice were injected with 3 doses of PZQ (250mg\kg body weight\dose). Ten infected mice were treated with PZQ, one week post treatment mice were re-infected with 80 cercariae/mice. Ten mice were infected with 80 cercariae of *S. mansoni* strain. This group will serve as positive control. Twenty mice were infected by 80 cercariae/mouse and followed by re-infection with 80 cercariae/mice and served as re-infected positive control mice.

Assessment of worm burdens were performed post infection and re-infection using perfusion technique. Worms were recovered by liver perfusion [17, 18]. An esthetic procedures complied with ethical guidelines was approved by the Medial Ethical Committee of the National Research Centre in Egypt with a registration's number (10135).

Detection of the levels of IgM and IgG

The levels of IgM and IgG in sera from mice were detected by ELISA [19].

Phenotypic analysis of mesenteric lymph nodes lymphocytes and thymocytes

Mesenteric lymph nodes (MLNs) and thymus were excised and gently teased in Petri dishes containing (PBST-FCS) using glass slides. Cells from individual mice were washed three times with (PBST-FCS) by centrifugation at 1500 g at 4°C for 10 min. CD4+ and CD8+T-cell subsets were identified using fluorescence isothiocyanate (FITC)-conjugated monoclonal anti-mouse $CD4^+$, $CD8^+$, were obtained from (Biolegend, St. Roselle, San Diego) respectively. While, B-cells, will be detected using FITC labeled anti-mouse IgM µ-chain (KPL, Gaithersburg, MD, USA) [20].

Statistical analysis

The present data has been expressed as mean ±S.D. The statistical comparisons were carried out using one way analysis of variance (ANOVA), followed by Tukey Kramer's multiple comparisons test. The minimal level of significance was identified at $P<0.05$.

RESULTS

Antischistosomal activity:

Infected-treated, infected- treated- re-infected, immunized- challenged, immunized- challenged-treated and immunized- challenged- treated- re-infected mice showed a significant decrease ($p<0.05$) in the mean number of worms burdens as compared with positive infected control mice and the reduction percentage in total worms were 54.6, 43.0, 60.5, 59.2 and 68.5 % respectively (table 1). Infected- re-infected mice died before the time of perfusion technique.

Table 1: Mean worm recovery and percentage (%) worm reduction in the liver of schistosomula immunized and\ or treated with praziquantel (PZQ)

Experimental Animals	Mean worm recovery ± S.D	% Reduction
I	24.571 ± 3.259	-
IPZQ	8.286 ± 4.348*	54.6
IPZQR	14.8 ± 1.05*	43.0
SC	5.6 ± 5.367*	60.5
SCPZQ	6.857 ± 4.488*	59.2
SCPZQR	5.75 ± 2.986*	68.5

I: Mice infected with *S. mansoni* cercariae; used as positive control.
IPZQ: Infected PZQ treated mice.
IPZQR: Infected PZQ treated mice subjected to re-infection.
SC: Immunized challenged mice.
SCPZQ: Immunized challenged and PZQ treated mice.
SCPZQR: Immunized challenged and PZQ treated mice subjected to reinfection.
The percentage of reduction in worm burden was calculated as follows: R = C – V / C x 100. Where, R = reduction (%); C = mean number of parasite recovered from infected animals; and V = mean number of parasite recovered from immunized and/or treated animals.
*Level of Significance $P< 0.05$

Detection of IgM and IgG levels in sera

The IgM and IgG levels in sera from infected- treated mice showed no significant increase against SWAP or SEA as compared with infected ones (tables 2 and 3). The IgM and IgG levels in sera from infected-treated-re-infected mice showed no significant changes against SWAP as compared with infected or re-infected or infected- treated mice as shown in (table 2). The level of IgM in sera from infected- treated-re-

ISSN: 0975-8585

infected mice showed non-significant change against SEA as compared with IgM level in sera from infected or re-infected or infected- treated mice as shown in (table 3). However, the level of IgG in sera from infected-treated-re-infected mice showed a significant decrease (p<0.05) as compared with IgG level in sera from infected control or Infected- treated- mice. There was no significant increase in IgG level in sera from infected-treated-re-infected mice as compared with positive re-infected mice (table 3). The IgM level in sera from immunized- challenged mice against SWAP or SEA showed no significant change as compared with IgM level in sera from infected control mice but, the IgG level showed a significant decrease (p<0.05) against both SWAP and SEA as compared with IgG level in sera from infected mice (tables 2 and 3).

Table 2: Detection of IgM and IgG levels in sera from schistosomula immunized and\or PZQ treated mice challenged with *S. mansoni* cercariae against SWAP

Experimental Animals	IgM *Mean ± SD	Fold change of Normal	IgG *Mean ± SD	Fold change of Normal
N	0.18 ± 0.04	---	0.18 ± 0.03	---
I	0.74 ± 0.17	4.04	0.84 ±0.08	4.69
IR	0.71 ± 0.11	3.88	0.73 ± 0.09	4.10
IPZQ				
1st dose of PZQ	0.80 ± 0.10	4.38	0.82 ± 0.02	4.58
2nd dose of PZQ	0.89 ± 0.11	4.86	0.85 ± 0.05	4.78
3rd dose of PZQ	0.64 ± 0.13	3.5	0.80 ± 0.06	4.51
IPZQR	0.71 ± 0.08	3.9	0.72 ± 0.04	4.04
SC	0.69 ± 0.14	3.76	0.60 ± 0.03**	3.38
SCPZQ	0.82 ± 0.05	4.48	0.68 ± 0.04**	3.79
SCPZQR	0.76 ± 0.06	4.14	0.54 ± 0.05**	3.05

N: Mice injected with PBS; used as negative control group.
I: Mice infected with *S. mansoni*; used as positive control.
IR: Infected challenged mice; positive control re-infection.
IPZQ: Infected treated mice with 1st, 2nd and 3rd dose of PZQ.
IPZQR: Infected mice treated with 3 doses of PZQ followed by re-infection.
SC: Immunized challenged mice.
SCPZQ: Immunized challenged mice treated with PZQ.
SCPZQR: Immunized challenged mice treated with PZQ followed by re-infection.
*Mean ± SD: mean of optical density (OD) recorded at λ max 490 nm
* *Level of Significance P<0.05

Table 3: Detection of IgM and IgG levels in sera from schistosomula immunized and\or PZQ treated mice challenged with *S. mansoni* cercariae against SEA

Experimental animals	IgM Mean ± SD*	Fold change of Normal	IgG Mean ± SD*	Fold change of Normal
N	0.19 ± 0.04	----	0.16 ± 0.04	---
I	0.68 ± 0.13	3.57	0.84 ± 0.06	5.25
IR	0.85 ± 0.11	4.47	0.55 ± 0.06**	3.44
IPZQ				
1st dose of PZQ	0.82 ± 0.13	4.3	0.79 ± 0.11	4.94
2nd dose of PZQ	0.74 ± 0.18	3.8	0.95 ± 0.25	5.94
3rd dose of PZQ	0.77 ± 0.09	4.05	0.80 ± 0.08	5.00
IPZQR	0.86 ± 0.02	4.53	0.51 ± 0.04**	3.19
SC	0.81 ± 0.08	4.26	0.51 ± 0.03**	3.19
SCPZQ	0.90 ± 0.06**	4.73	0.43 ± 0.04**	2.69
SCPZQR	0.75 ± 0.04	34.95	0.51 ± 0.04**	3.19

N: Mice injected with PBS; used as negative control group.
I: Mice infected with *S. mansoni*; used as positive control.
IR: Infected challenged mice; positive control re-infection.
IPZQ: Infected treated mice with 1st, 2nd and 3rd dose of PZQ.
IPZQR: Infected mice treated with 3 doses of PZQ followed by *S. mansoni*re-infection.
SC: Immunized challenged mice.

SCPZQ: Immunized challenged mice treated with PZQ.
SCPZQR: Immunized challenged mice treated with PZQ followed by re-infection.
*Mean ± SD: Optical density (OD) recorded at λ max 490 nm.
**Level of Significance *P*<0.05

The IgM level in sera from immunized-challenged-treated mice against SWAP showed no significant change as compared with IgM level in sera from infected control, as shown in (table 2), the IgG level in sera from immunized-challenged-treated mice showed a significant decrease (p<0.05) as compared with IgG level in sera from infected control mice as shown in (table 2).

The IgM level in sera from immunized-challenged-treated mice against SEA showed a significant increase (p<0.05) as compared with IgM level in sera from positive control mice as shown in (table 3) and the IgG level in sera from immunized-challenged-treated mice showed a significant decrease (p<0.05) as compared with IgG level in sera from positive control mice. There was no significant change in the IgG level in sera from immunized-challenged-treated mice as shown in (table 3). The IgM level in sera from immunized-challenged-treated-re-infected mice showed no significant increase against SWAP or SEA as compared with infected control or positive re-infected or immunized-challenged or immunized-challenged-treated mice as shown (tables 2 and 3). The IgG level in sera from immunized-challenged-treated-re-infected mice showed a significant decrease (p<0.05) against SWAP or SEA as compared with positive control and a significant decrease (p<0.05) as compared with IgG level in sera from positive re-infection mice (tables 2 and 3). The IgG level in sera from immunized-challenged-treated-re-infected mice showed no significant change against SWAP as compared with IgG level from immunized-challenged or immunized-challenged-treated mice as shown (table 3). However, IgG showed no significant change against SEA as compared with IgG level in sera from positive re-infected mice or from immunized-challenged or immunized-challenged-treated mice as shown in (table 3).

Lymphocytes Phenotypic analysis

The mean percentages of $CD4^+$ or $CD8^+$ T from thymus or MLN in infected- treated-re-infected mice showed no significant changes as compared with thymus or MLN lymphocytes from infected or re-infected or infected- treated mice respectively (tables 4 and 5). The mean percentage of MLN-B cells from infected-treated-re-infected mice showed a significant increase (P<0.05) as compared with the mean percentage of MLN-B cells from positive control or infected- treated mice (table 5). Infected- treated-re-infected mice showed a significant decreases (P<0.05) in the mean percentages of $CD4^+$ or $CD8^+$T MLN cells as compared with the mean percentages of positive re-infected $CD4^+$ or $CD8^+$ T MLN cells respectively (table 5). Immunized-challenged mice showed a no significant increase in the mean percentage of $CD4^+$ T from thymus or MLN as compared with positive infected control. However, immunized- challenged mice showed a significant increase (p<0.05) in the mean percentage of $CD8^+$ T from thymus or MLN as compared with positive infected control (tables 4 and 5). Immunized- challenged- treated mice showed no significant increase in the mean percentage of $CD4^+$thymocytes as compared with the mean percentage of $CD4^+$thymocytes from positive infected control. However, immunized- challenged- treated mice showed significant increase (p<0.05) in the mean percentage of $CD8^+$thymocytes as compared with the mean percentage of $CD8^+$thymocytes from infected control. Immunized- challenged- treated mice showed no significant increments in the mean percentages of $CD4^+$ or $CD8^+$T or B-MLN cells as compared with the mean percentages of $CD4^+$ or $CD8^+$T or B-MLN cells from infected control (tables 4 and 5). Immunized-challenged-treated-re-infected mice showed no significant changes in the mean percentages of $CD4^+$ or $CD8^+$thymocytes as compared with infected control $CD4^+$ or $CD8^+$thymocytes. Immunized-challenged-treated-re-infected mice showed no significant decrease in the mean percentage of $CD4^+$ thymocytes as compared with the mean percentage of $CD4^+$ thymocytes from immunized\ challenged and immunized-challenged-treated mice. Immunized- challenged-treated-re-infected mice showed a significant decrease (p< 0.05) in the mean percentage of $CD8^+$ thymocytes as compared with $CD4^+$ thymocytes immunized-challenged mice or immunized-challenged-treated respectively (table 4). Immunized-challenged-treated-re-infected mice showed significant decrease (p<0.05) in the mean percentage of $CD8^+$-T-MLN cells as compared with positive re-infected mice (table 5).

ISSN: 0975-8585

Table 4: Mean percentage (%) of thymocytes from schistosomula immunized and\or PZQ treated mice challenged with *S. mansoni* cercariae

Experimental Animals	$CD4^+T$	$CD8^+T$	$CD4^+ / CD8^+T$
	Mean ± S.D.	Mean ± S.D.	
I	35.5 ± 4.20	26 ± 3	1.365
IR	32.417 ± 4.19	26.5 ± 1.32	1.223
IPZQ	34.5 ± 1.5	34 ± 1*	1.015
IPZQR	34.6 ± 3.67	27.95 ± 0.35	1.238
SC	40.25 ± 3.71	39.125 ± 2.78**	1.029
SCPZQ	35.733 ± 0.25	35.6 ± 0.1**	1.004
SCPZQR	33.6 ± 4.052	28.1 ± 4.75	1.196

I: Mice infected with *S. mansoni*; used as positive control.
IR: Infected challenged mice; positive control re-infection.
IPZQ: Infected treated mice with 1st, 2nd and 3rd dose of PZQ.
IPZQR: Infected mice treated with 3 doses of PZQ followed by *S. mansoni*re-infection.
SC: Immunized challenged mice.
SCPZQ: Immunized challenged mice treated with PZQ.
SCPZQR: Immunized challenged mice treated with PZQ followed by re-infection.
*Level of Significance *P*<0.01
**Level of Significance *P*<0.001

Table 5: Mean percentage (%) of mesenteric lymph node lymphocytes from schistosomula immunized or\and PZQ treated mice challenged with *S. mansoni* cercariae.

Experimental Animals	$CD4^+T$	$CD8^+T$	$CD4^+ / CD8^+T$	B cells
	Mean ± S.D.	Mean ± S.D.		Mean ± S.D.
I	35.5 ± 2.08	22.75 ± 2.22	1.560	34.5 ± 1.5
IR	45.5 ± 0.5*	34 ± 2***	1.338	46.333 ± 1.53
IPZQ	35.5 ± 3.51	22.5 ± 4.20	1.578	34.7 ± 6.43
IPZQR	32.38 ± 2.39	24.333 ± 0.58	1.331	48.867 ± 1.03**
SC	39.833 ± 5.49	30 ± 1**	1.328	39 ± 6
SCPZQ	36 ± 3.65	28.633 ± 0.15	1.257	39 ± 5.43
SCPZQR	39 ± 1	23.5 ± 3.28	1.66	42.5 ± 2.5

I: Mice infected with *S. mansoni*; used as positive control.
IR: Infected challenged mice; positive control re-infection.
IPZQ: Infected treated mice with 1st, 2nd and 3rd dose of PZQ.
IPZQR: Infected mice treated with 3 doses of PZQ followed by S. mansonire-infection.
SC: Immunized challenged mice.
SCPZQ: Immunized challenged mice treated with PZQ.
SCPZQR: Immunized challenged mice treated with PZQ followed by re-infection
*Level of Significance P<0.01
**Level of Significance P<0.05
***Level of Significance P<0.001

DISCUSSION

Our study showed that infected- treated-re-infected mice recorded percentage worm reduction (60.5%). This provided evidence that PZQ treatment of infected mice did not confer additional protection against challenge [21]

In our research the percentage of reduction in the immunized-infected mice was 60.5 %. de Melo et al. [22] demonstrated that Smteg formulated with Freund's adjuvant was able to induce partial protection in mice (43-48%). Our study showed mice that immunized with schistosomula challenged followed by treatment with PZQ or mice immunized with schistosomula challenged treated with PZQ and followed by re-infection recorded percentages of reduction in total worms by 59.2 and 68.5 % respectively. Yole et al. [23] stated that

PZQ seemed to boost protective immunity when administered after vaccination. In our study, it was noteworthy that the survival of immunized challenged mice followed by PZQ treatment and subjected to re-infection were improved as compared with infected challenged mice which could not withstand double infection which did not survive during the course of the experiment.

When we accompanied immunization with PZQ treatment of challenged mice, the IgM levels were enhanced against both SWAP and SEA. However, the IgG levels were decreased as compared with IgG level in sera from infected mice. Li et al. [24] evaluated that antibody against schistosome related closely to the infection, reinfection by schistosome. Boctor and Peter [25] stated that total IgG was increased in *S. mansoni* patients. Tawfik et al. [26]; De-Jonges et al. [27]; Hussein *et al*. [28] and El-Shafie [29] showed that antibodies titer against egg (SEA), cercarial (CAP) and adult worm (SWAP) extracts generally decreased after chemotherapy. Vereecken et al. [30] observed a boost in specific antibody responses against adult worm antigen (SWAP). After 6 weeks treatment with PZQ of Senegalese individuals recently exposed to *S. mansoni* they found that the majority of antibody isotype responses against SEA were not affected by treatment. Although Araujo et al. [7] reported increased production of specific IgG antibodies in sera from mice immunized with Smteg. On the other hand we observed decreased Igs levels against both SWAP and SEA from schistosomula immunized challenged mice as compared with infected mice except for IgM titer measured against SEA.

Also, we observed that infected mice treated with PZQ showed little immunostimulatory effect on the cellular response except for $CD8^+$- thymocytes which were significantly increased in infected\treated mice as compared to infected mice. Also, Wilson et al. [21] observed an increase in parasite-specific T cell proliferation, cytokine secretion, and antibody production in infected mice following PZQ treatment. Furthermore, in the present study infected mice treated with PZQ subjected to re-infection showed a pronounced decrease in $CD4^+$-T and $CD8^+$-T MLN cells accompanied with some decrease in $CD4^+/CD8^+$ T ratio as compared with infected challenged mice. However, we investigated that the schistosomula immunization seemed to evoke the thymus and MLN lymphocytes by increasing the mean number of $CD4^+$, $CD8^+$thymocytes and MLN cells as well as B-MLN cells as compared with infected mice. de Melo et al. [22] attributed that the protection induced by schistosomula tegument formulated with Ferund's adjuvant was due to a predominant Th1 type of immune response, with increased production of IFN-γ and TNF-α, B-cells proliferation and $CD4^+$-T cells and macrophages activation.

Zhou et al. [31] who stated that schistosomula can acquire MHC class I molecules from host tissues and also suggested that some surface antigens on schistosomula could be directly taken up and processed in the endosome and then is cross-presented to $CD8^+$-T cells by DCs.

In our study, the $CD8^+$-T cells from either the thymus or the MLNs of immunized challenged PZQ treated mice were increased in number, whereas the number of $CD4^+$-T cells remained almost unchanged, finally the B-cells were stimulated as well. When immunized challenged mice treated with PZQ were subjected to 2^{nd} challenge, the $CD4^+$ and $CD8^+$thymocytes were slightly increased as compared with infected challenged mice. In contrast to this, the $CD4^+$-T, $CD8^+$-T and B-MLN cells were all decreased as compared to infected challenged mice.

CONCLUSION

Our data cleared that both immunization and therapy were able to induce significant levels of protection against *S. mansoni* re-infection as compared with immunization or treatment only. Our conclusion is agreement with many authors who study the effect of combination of PZQ with chemical or natural extracts to improve the immune-protection efficiency of PZQ as Sudsarn *et al* [32] who demonstrated that, the PZQ with Aspirin improved the Liver Pathology of Hamster *Opisthorchis viverrini* Infection associated cholangio carcinoma. Recently, Almeida *et al*., [33] study the functional analysis of gene interaction networks by showing that PZQ in combination with omeprazole displayed increased efficiency against *S. mansoni* adult worms *in vitro* when compared with either drug alone.

ISSN: 0975-8585

ACKNOWLEDGMENTS

The authors acknowledge the National Research Centres (NRC) of Egypt for Providing all needed facilities and logistics for the work and all the team work in the Immunology and infectious Diseases Group (CEAS).

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