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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 15 (2005) 2415–2419

Synthesis and bioactivities of two multiple antigen peptides as potential vaccine against schistosoma

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Received 26 September 2004; accepted 14 January 2005
Available online 25 March 2005

Abstract—Based on the two antigenic peptides, 26–43 (P_{26}) and 116–131 (P_{116}), derived from 28 kDa glutathione *S*-transferase of *Schistosoma mansoni* (Sm28GST), two multiple antigenic peptides (MAPs), $(P_{26})_4$-MAP and $(P_{116})_4$-MAP with the same oligomeric lysine core, were synthesized by stepwise solid-phase peptide synthesis method. The antigenicities and protective effects of these two MAPs were examined on experimental animals. As shown in the dot-ELISA result, the synthetic MAPs could be recognized and bound by immunoglobins in both patient's and infected-rabbit's sera. After Kunming mice were immunized with $(P_{26})_4$-MAP, the worm burden reduction rate and the liver egg reduction rate were 59.9% and 61.1%. In $(P_{26})_4$-MAP or $(P_{116})_4$-MAP immunized BALB/c mice, the worm burden reduction rates were 37.5% and 62.5%, respectively, and the liver egg reduction rates were 35.1% and 54.0%, respectively.

1. Introduction

Schistosomiasis is a severe disease caused by different species of schistosomes, but most of the human infections are caused by *Schistosoma mansoni*, *Schistosoma haematobium*, and *Schistosoma japonicum*, which have been estimated to infect approximately 200 million people in 74 countries and cause 1 million deaths annually.[1,2] Cercaria infects human through skin, and then transforms into schistosomula that migrates into host's lung and liver. Enormous eggs laid by the mature worm stay in the liver and other tissues and lead to severe syndrome including inflammation, granuloma, and tissue fibrosis. Therefore, reducing eggs and schistosomulae in human tissues can significantly relieve the syndrome of schistosomiasis. Vaccine, as a potent measure to prevent many infectious diseases, will play an important role in controlling schistosomiasis through reducing the eggs and schistosomula burden.[3]

Because the glutathione *S*-transferases in both *S. mansoni* (28 kDa, Sm28GST) and *S. japonicum* (26 kDa, Sj26GST) are highly homologous, with the aid of computer epitope modeling, we designed, synthesized, and screened three Sm28GST peptide fragments, namely P_{26}, P_{116}, and P_{141}, and one Sj26GST peptide fragment J_{187} in vitro.[4] In order to increase the immunogenicity of the synthetic peptide, the approach of multiple antigen peptide (MAP) was adopted. We designed and synthesized two tetravalent monoepitopic MAPs [$(P_{141})_4$-MAP and $(J_{187})_4$-MAP], two tetravalent diepitopic MAPs [$(P_{26})_2(J_{187})_2$-MAP, $(P_{26})_2(P_{141})_2$-MAP], one octavalent monoepitopic and four octavalent diepitopic MAPs [$(P_{26})_8$-MAP, $(P_{26})_4(P_{141})_4$-MAP, $(P_{26})_4(J_{187})_4$-MAP, $(P_{116})_4(P_{26})_4$-MAP and $(P_{116})_4(P_{141})_4$-MAP].[5–7] Antigenicity test indicated that MAPs were stronger agents than monomeric peptides in both specificity and immunogenic activity against schistosoma infection. Based on our previous research, we synthesized another two MAPs, $(P_{26})_4$-MAP and $(P_{116})_4$-MAP (Fig. 1), and investigated their antigenicity, immunogenicity, and protective immunity against schistosoma infection in testing animals.

Keywords: Schistosomiasis; Synthetic peptide vaccine; Multiple antigenic peptides.
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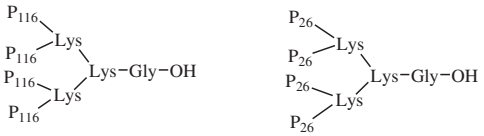
doi:10.1016/j.bmcl.2005.01.040



$(P_{116})_4$-MAP **$(P_{26})_4$-MAP**

P_{116}: Pro-Gln-Glu-Glu-Lys-Ile-Thr-Lys-Glu-Ile-Leu-Asn-Gly-Lys

P_{26}: Ala-Ala-Gly-Val-Asp-Tyr-Glu-Asp-Glu-Arg-Ile-Ser-Phe-Gln-Asp-Trp-Pro-Lys

Figure 1. Schematic presentation of the structure of the MAPs containing two different antigenic peptides derived from Sm28GST.

2. Experimental

2.1. Materials and instruments

N-protected amino acids, *N*-Boc-glycine Pam resin (0.34 mmol Boc-glycine/1 g resin, 1% cross-linked, 200–400 mesh), DCC, HOBt, TFA, BOP, *N*,*N*-diisopropylethylamine (DIPEA), Sephadex G-50 were purchased from Sigma. *N*-Fmoc protected amino acids were purchased from Shanghai Gil Biochemical. The side chains of Boc protected amino acids were protected either with benzyl (Asp, Glu, Ser, and Thr) or with tosyl (Arg) and Cbz (Lys). Enzyme-labeled Protein A was purchased from Sino-America Biotech.

Crude peptides were purified by MC99-3 automatic liquid chromatography (Shanghai Huxi analytical instrument) on Sephadex G-50 column; MAPs were analyzed on a Agilent 1100 HPLC system equipped with a G1311A Quatpump, G1365B MWD UV detector at wavelength 220 nm, and Zorbax SDW-C18 reverse phase chromatography column (1 mm × 15 cm). The MALDI-TOF MS data of two MAPs were obtained on an AXIMA-CFP plus mass spectrometer (KRATOS Analytical, Shimadzu Group Company). The amino acid composition of MAPs was determined on a Hitachi-835-50 amino acid auto analyzer.

2.1.1. Experimental animals. Kunming mice and BALB/c mice (6–8 week old) weighing about 20 g were assigned randomly to several groups.

2.2. Testing objects

Human serum was collected and mixed for testing from 10 patients in schistosomiasis epidemic district in Hubei province. China rabbit serum was collected from 10 rabbits infected of *S. japonicum* and mixed.

2.3. Solid phase peptide synthesis (general method)

$(P_{26})_4$-MAP and $(P_{116})_4$-MAP were synthesized on the preloaded PAM resin. First, the oligomeric lysine matrix was synthesized on *N*-Boc-Gly-PAM resin. In the peptide synthesis reactor, a certain amount of *N*-Boc-Gly-PAM resin was sufficiently solvated in DCM/DMF (2:1, v/v) overnight. After removal Boc group with 50% TFA/DCM, the resin was then neutralized with 10% DIPEA/DCM. Three equivalents of dicyclohexylammonium N_α,N_ϵ-di-Boc-lysinate was added to couple with the amino group on the resin using BOP as activator in DMF. The quaternary antennal oligomeric lysine was obtained in the same way.

The schistosoma antigen peptides linked to oligomeric lysine were synthesized stepwise adopting the standard procedure of solid phase peptide synthesis: the coupling was performed in the solution of DMF/DCM (1:1, v/v) using DCC + HOBt as activator, the amount of Boc-protected amino acid used in the coupling was 3 equiv of the free amino group on the resin. The following steps were repeated until the whole peptide was completely synthesized: (1) TFA/DCM (1:1, v/v) was added to the reactor containing the Boc-protected oligomeric lysine and vibrated twice (10 min and 30 min, respectively), to remove Boc group; (2) washed with DCM (3 × 1 min); (3) washed with methanol (3 × 1 min); (4) Neutralized with 10% DIPEA/DCM (2 × 2 min); (5) washed with DCM (3 × 1 min); (6) washed with methanol (3 × 1 min); (7) washed with DCM (3 × 1 min); (8) Three equivalents of Boc-protected amino acid together with DCC + HOBT in DMF were added to the reactor, and the mixture was vibrated at room temperature overnight; (9) washed with DCM (3 × 1 min); (10) washed with methanol (3 × 1 min); (11) washed with DMF (3 × 1 min); (12) washed with methanol (3 × 1 min); (13) washed with DCM (3 × 1 min); (14) monitored by ninhydrin assay.[8] If the color was light yellow, the result was judged as negative and the next coupling step was continued. If the color was purple (positive result), steps 6–14 were repeated until the result turned negative.

After the coupling of the whole peptide was completed, N_α-Boc protective group was removed with TFA/DCM (1:1, v/v), then protective groups on the side chain and the resin were cleaved altogether with low–high HF method.[9] The residue was extracted with 10% acetic acid and frozen dry to obtain the crude peptide, which was then purified with Sephadex G-50. The desired product was analyzed with automatic amino acid analyzer, HPLC (see Fig. 2), and MALDI-TOF MS (see Table 2).

2.4. Dot-ELISA

The sample of the synthetic peptide was diluted with phosphate buffer in four different concentrations: 2000, 1000, 500, and 250 $\mu g\,ml^{-1}$. Celloidin film was washed with distilled water, air dried, and then divided into small pieces. To one piece of film, 2 μl of peptide solution of one concentration was added, and then dried in the air. All films were soaked in 1% BSA phosphate buffer solution for 2 h, and air dried. Serum from patient or rabbit was diluted and added to the film. The films were incubated at 37 °C for 30 min, washed with PBS and air dried. The films were soaked in 1:1000 enzyme-labeled Protein A solution, incubated and washed. Finally the films were treated with substrate to determine the result.

2.5. Mice immunization experiment

Each mouse in experimental group was injected with 200 μg of synthesized peptide in 0.1 ml PBS sc, and

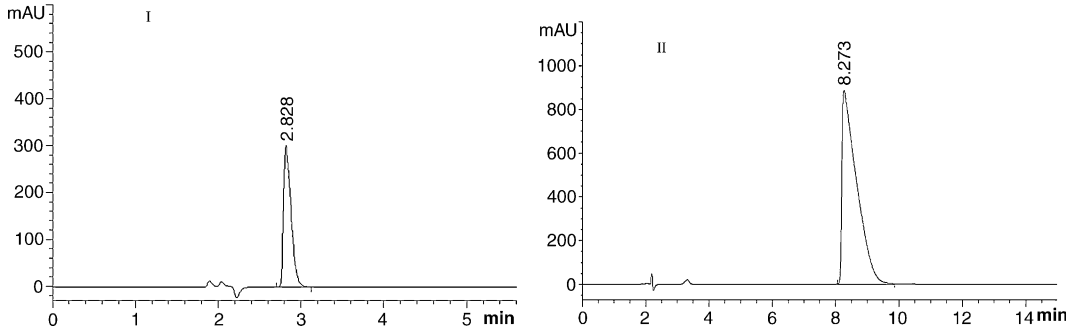


Figure 2. Analytical HPLC profiles of the synthetic MAPs.

enhanced immunization in day 7 and 14, while each mouse in control group was injected with 0.1 ml PBS sc. The serum samples were collected from the tails of the mice before immunization and 10 d and 17 d, respectively, after the last immunization. Coating with the mixed antigens of *S. japonicum* and *S. mansoni*, the antibody was detected with ELISA. Three weeks after the last injection, each mouse was infected with 40 ± 1 normal cercariae of *S. japonicum* through abdominal skin. Six weeks after the infection, the mice were anatomized and count the number of schistosoma via a procedure of douche. Liver tissue (0.5 g) was taken from each mouse, and the tissue was digested with 10% KOH. The number of eggs was counted through microscope. The rate of worm and egg reduction was calculated.

3. Results and discussion

Using glycine as the linker to connect the antigen peptide and resin, we synthesized the dendritic oligomeric lysine matrix on PAM resin. Exploiting BOP as an activator in our experiment, we found that dicyclohexylammonium N_{α},N_{ϵ}-di-Boc-lysinate can be used directly in the coupling reaction without the pretreatment of acid. Then we synthesized the antigen peptides $(P_{26})_4$-MAP and $(P_{116})_4$-MAP in yields of 38.5% and 29.3%, respectively, adopting the protocol of solid phase peptide synthesis. The result of amino acid analysis was consistent with the theoretical values (Table 1). The results of HPLC and MALDI-TOF spectra were given in Table 2.

Dot-ELISA was used to determine the antigenicity of the synthesized MAPs. When the concentration of MAPs reached 250 $\mu g\,ml^{-1}$. Both $(P_{26})_4$-MAP and $(P_{116})_4$-MAP showed positive responses to 1:100 and 1:50 of diluted patient serum, as well as 1:100 of diluted infected rabbit serum. To anti-31/32 kilo Dalton antigen monoclonal antibody, the results were also positive. In contrast, the corresponding mono-antigen peptide required higher concentration to display the positive response (Table 3). These results indicate that the synthesized MAPs possess relative stronger antigenicity toward *S. japonicum*.

To investigate the potential of the synthesized MAPs to generate specific antibody response toward certain antigen in mice, MAPs were injected hypodermically to the experimental BALB/c mice without any adjuvants to induce immunoreaction. ELISA results showed that mice in experimental group engendered the antibody at relatively low level 10 days after the last immunization. And the antibody level increased significantly in both groups 17 days after last immunization (Table 4). The results indicated that the synthesized MAPs induced an explicit antibody response in mice, and the serum from the immunized mice could cross response to the natural antigen of *S. japonicum*.

In the recent research of schistosoma immunity, the rate of worm and egg reduction is an important indicator to evaluate the protective activity of the potential vaccine. Our experiment for this purpose showed the efficacy of MAPs (Table 5). Without adjuvant, in the immunized Kunming mice with $(P_{26})_4$-MAP much fewer worms and eggs in the liver were detected compared to the mice in control group. The rates of worm and egg reduction were 59.9% and 61.1%, respectively. But in BALB/c mice, the protective efficacy was only medium with the corresponding rates of 37.5% and 35.1%. In the BALB/c mice immunized with $(P_{116})_4$-MAP, the worm and egg reduction rates were 62.5% and 54.0%,

Table 1. Amino acid composition analysis of synthetic MAPs[a]

Peptide	Asx[b]	Thr	Ser	Glx[b]	Gly	Ala	Val	Ile	Leu	Tyr	Phe	Lys	Arg	Pro	Trp
$(P_{26})_4$-MAP	3.0 (3.0)	—	1.0 (1.0)	3.3 (3.0)	1.3 (1.0)	2.0 (2.0)	1.3 (1.0)	1.0 (1.0)	—	0.8 (1.0)	1.0 (1.0)	1.8 (2.0)	1.0 (1.0)	1.0 (1.0)	—[c] (1.0)
$(P_{116})_4$-MAP	1.0 (1.0)	1.0 (1.0)	—	5.4 (5.0)	1.3 (1.0)	—	—	2.0 (2.0)	1.0 (1.0)	—	—	4.8 (5.0)	—	0.9 (1.0)	—

The numbers in parentheses correspond to the expected composition of synthetic MAPs.'—' means that the synthetic peptide does not contain this amino acid residue.

[a] The synthetic peptides reacted with 6 $mol\,L^{-1}$ HCl for 24 h at 110 °C.

[b] Asx: Asp and/or Asn; Glx: Glu and or Gln.

[c] Trp was decomposed in the course of acid hydrolysis.

Table 2. The condition of analytical HPLC and their MALDI-TOF MS data

Peptide	Eluotropic condition	Flow rate (ml/min)	Retention time (min)	$[M + H]^+$	
				Calc.	Found
$(P_{26})_4$-MAP (I)	99.9% A[a]-0.1% B[a]	0.8	2.828	8894.6	8891.1
$(P_{116})_4$-MAP (II)	97.0% A[a]-3.0% B[a]	0.8	8.273	7926.1	7930.0

[a] Solution A: 0.1% TFA/H_2O; B: 0.1%TFA/CH_3CN.

Table 3. Antigenicities of the synthetic peptides detected by Dot-ELISA

Peptide	Patient sera (1:100)	Patient sera (1:50)	Infected rabbit sera (1:100)	Enzyme-labeled anti-31/32 kDa mAb (1:500)
P_{26}[a]	2+[*]		2+	2+
P_{116}[a]	1+	1+	3+	3+
$(P_{26})_4$-MAP	4+	4+	4+	4+
$(P_{116})_4$-MAP	4+	4+	4+	4+

[*] 4+, 3+, 2+, 1+ represent the concentrations of the peptide solutions showing positive reaction: 250 μg/ml, 500 μg/ml, 1000 μg/ml, 2000 μg/ml, respectively. Two micro litres of each dilution was dotted on the nitrocellulose sheet.
[a] See Ref. 4.

Table 4. Antibody responses in BALB/c mice immunized with the MAPs

Peptide	Number of animals	Serum antibody (OD)		
		A[a]	B[a]	C[a]
$(P_{26})_4$-MAP	9	0.28	0.46	0.68
$(P_{116})_4$-MAP	8	0.27	0.37	0.53
Control	9	0.23	0.21	0.25

[a] A: prior to injection; B: 10 days after the last injection; C: 17 days after the last injection.

Table 5. Protective efficacy in mice vaccinated with the monomeric peptides and MAPs

Peptide	Mouse	Number of animals	Mean worm burden ± SD	WRR[a] (%)	*P* Value[c]	Mean egg number/1 g liver(±SD)	ERR (%)[b]	*P* Value[c]
P_{26}[d]	Kunming	9	21.0 ± 7.3	37.1	<0.001	17,427 ± 9977	54.0	<0.001
$(P_{26})_4$-MAP	Kunming	10	13.4 ± 2.7	59.9	<0.001	14,718 ± 4906	61.1	<0.001
Control	Kunming	10	33.4 ± 2.1			37,884 ± 7546		
$(P_{26})_4$-MAP	BALB/c	9	18.0 ± 4.6	37.5	<0.001	35,067 ± 13,710	35.1	<0.05
$(P_{116})_4$-MAP	BALB/c	8	10.8 ± 4.6	62.5	<0.001	24,850 ± 6881	54.0	<0.001
Control	BALB/c	9	28.8 ± 4.9			54,089 ± 14,026		

[a] Worm reduction rate (WRR)% = (1 − A/B), A: mean number of worms in vaccinated mice; B: mean number of worms in control mice.
[b] Egg reduction rate (ERR) % = (1 − A/B), A: mean egg number per gram of liver in vaccinated mice; B: mean egg number per gram of liver in control mice.
[c] *T*-test was used for comparisons of data. *P* value was calculated, comparing the mean number of worms or the mean egg number in per gram of livers in the control group to that obtained in the vaccinated group in each experiment.
[d] See Ref. 4.

respectively. The protective efficacy of MAPs was much higher than that of the monopeptide. The egg reduction is probably due to the immune inhibition of the worm growth, and the subsequent low egg-disposition by the female worms.

4. Conclusion

Schistosoma do not reproduce in human host. The severe syndrome related to schistosomiasis occurs only in those who have heavy worm and egg burden. Epidemiological research has revealed that vaccine against schistosoma does not need to have 100% protective capacity. If a vaccine can reduce the worm burden by 50% or more, it can apparently decrease the pathological impairment and the disease promulgation.[10,11] The two MAPs reported in this paper showed promising protective activity in Kunming and BALB/c mice, and thus could be potential vaccine candidates against the dreadful schistosomiasis. Though, their protective efficacy is lower than that of the octavalent diepitopic MAPs reported in our previous paper, their relative simpler structure and lower cost make it more valuable in the future research.

Acknowledgements

This work was supported by National Nature Sciences foundation of China (Grant No. 30371631) and special fund for promotion of education by the Ministry of Education of China.

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