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Schistosoma japonicum: Isolation and Identification of Peptides Mimicking Ferritin Epitopes from Phage Display Library

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Abstract In an attempt to isolate and identify the antigenic epitopes on ferritin of *Schistosoma japonicum* (*Sj*Fer) and to test their protective potentiality against *Schistosoma japonicum* (*S.j*), polyclonal antisera against *Sj*Fer was prepared to screen a 12-mer random peptide library. Three rounds of biopanning were performed and resulted in an enrichment. Six peptides selected randomly from the third elute were all found to be positive by evaluating the binding to anti-*Sj*Fer sera by ELISA and Western blotting. Three amino acid sequences were deduced from the six phage clones by sequencing. When they were used to vaccinate mice, the three peptides could induce significant reduction in adult worms (26.7%, 20.4%, and 25.9%) as well as in liver eggs per gram (LEPG) (40.0%, 38.2%, and 40.8%). This result showed that three mimotopes on *Sj*Fer were obtained and they could induce significant protective efficacy against *S.j*.

Key word phage displayed peptide library; epitope; *Schistosoma japonicum*; ferritin

Although schistosomicidal drug and other control measures (including hygiene and snail control) are available, schistosomiasis continues to afflict an estimated 200 million and kill 20 thousand people every year, new approaches for controlling this disease are urgently needed.

Cocktail vaccine, such as multiple antigen peptide vaccine, emerged as the most potentially powerful means. For multiple antigen peptide vaccine that bases on domains or subdomains of antigens, the key is to obtain antigenic epitopes. It has been shown that screening the phage display peptide library is a powerful tool for the identification of epitopes or mimotopes on antigens [1,2].

Although little is known about its immunological feature, ferritin as a vaccine candidate, has drawn more and more attention because of its anti-fecundity ability. In this study, we have used a 12-mer peptide library displayed on filamentous bacteriophage to obtain mimotopes on *Sj*Fer. for its further characterization and efficacious antigenic epitope candidates.

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Materials and Methods

Materials

Phage library and bacteria The phage display library and *E. coli* host strain ER2738 were kindly provided by Dr. Larry McReynolds, New England Biolabs. Inc..

Parasites and experimental animals Cercarie of *S.j* were obtained from *Oncomelania hupensis* purchased from Jiangsu Institute of Parasitic Disease, China. Female Kunming mice were provided by the Department of Experimental Animal, Central South University Xiangya School of Medicine.

Antisera preparation Polyclonal mouse antisera against recombinant *Sj*Fer were collected after the third immunization. Specific IgG was purified by saturated ammonium sulphate precipitation.

Panning the phage library

The panning technique [3] was used to screen the phage peptide library. Briefly, each microtiter well was first coated with 10 μg of IgG in 100 μl of coating buffer (0.1 M $NaHCO_3$, pH 8.5) overnight at 4 °C in sealed

humid container, then blocked with 350 μl of 50 g/L skimmed milk powder in TBS (50 mM Tris, 150 mM NaCl, pH 7.5) for 2 h at room temperature. Afterwards, 100 μl of original phages (containing 10^{12} phage particles) were added to the wells and incubated for 1 h at 37 °C with gentle agitation. The wells were washed eight times with TBS-T (5 ml Tween-20 per liter TBS) to remove unbound phages. Phages specifically bound to IgG were eluted with 100 μl of 0.2 M glycine-HCl (pH 2.2) for 8 min at room temperature. Neutralized with 15 μl 1 M Tris-HCl (pH 9.1), the eluted phage were titered and used to infect *E. coli* 2738 cells for amplification for the next round of panning. Three rounds of panning were performed.

Monoclonal phage amplification

Monoclonal phage randomly chosen from phage pools were amplified by infecting a log-phase culture of *E. coli* 2738 and shaking for 4.5 h at 37 °C in Luria-Bertani medium containing 0.02 g/L tetracycline. The supernatant was clarified by centrifuging at 10,000 r/min for 15 min and 1/6 volume of PEG solution (200 g/L polyethylene glycol 8000, 2.5 M NaCl) was added. After incubated at 4 °C for at least 2 h, the sample was centrifuged at 10,000 r/min for 15 min to precipitate the phage. The phage pellet was suspended in 1 ml of TBS and stored at 4 °C.

Western blot

10^{12} phage particles from each selected clone were incubated in boiling water for 3 min in the sodium dodecyl sulfate (SDS) loading buffer and applied to 10% SDS-polyacrylamide gel. Separated proteins were then transferred to nitrocellulose membrane which was then blocked for 2 h at room temperature in 50 g/L skimmed milk powder in PBS. The membrane was rinsed three times with PBS and probed with mouse anti-*Sj*Fer sera. HRP-linked anti-mouse IgG was used as a secondary antibody. Binding was visualized by incubating the membrane in diamino-benzidine (DAB).

Phage ELISA

ELISA plates were coated with mouse anti-*Sj*Fer IgG (10 μg/well) overnight at 4 °C and then blocked with 50 g/L skimmed milk powder for 2 h at room temperature. 10^{12} phage particles diluted in TBS were added to the wells and incubated at room temperature for 2 h. The wells were subsequently washed three times with TBS-T, followed by addition of anti-M13-HRP antibody. Specifically bound phages were visualized by the addition of 3,3,5,5-tetramethylbenzidine (TMB). The absorbance was determined at 490 nm after adding the stop solution (1 N H_2SO_4).

Nucleotide sequencing

Single-stranded DNA was prepared from the purified phages as described by Sambrook *et al.* [4]. The nucleotide sequence of the gene III inserts was sequenced with -96gIII sequencing primer 5′-^{HO}CCC TCA TAG TTA GCG TAA CG-3′ by automated dye terminator cycle sequencing (ABI 100). The amino acid sequence of the insert was deduced from the nucleotide sequence.

Immunization and parasite challenge

75 female Kumming mice of 4-week-old were randomly divided into 5 groups. In the first three groups, mice were injected subcutaneously at three sites with 10^{12} phage particles for three times. In control groups, mice were injected only with TBS or original library phage. Two weeks after the third immunization, mice were challenged with 40 cercariae percutaneously on abdominal skin. All mice were perfused at day 42 after infection. The number of adult worms was determined and the liver eggs per gram (LEPG) were calculated. One-Way ANOVA was used to evaluate the statistical significance in worm burden and LEPG between vaccinated and control groups.

Results

Panning

To isolate peptides that mimic epitopes on *Sj*Fer, we used the anti-*Sj*Fer sera to pan a phage display random 12-residue library with 1.5×10^{13} clones. After the third round of panning, a significant enrichment of phage with affinity for the anti-*Sj*Fer sera was observed (Fig. 1, Table 1).

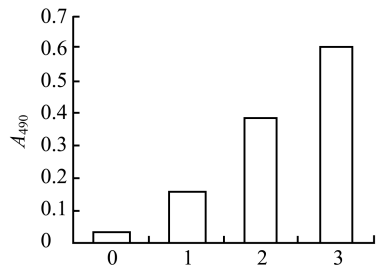

Fig. 1 Phage ELISA of pools of clones after each round of panning

Pooled phages were amplified and 10^{12} phage particles were added to each well of microtiter plate precoated with IgG. Phage were detected with an anti-M13-HRP antibody. A_{490}, absorbance at 490 nm.

Table 1 Enrichment of specific phages during panning

Round of panning	Phage added	Phage eluted
1	1×10^{12}	2.1×10^{5}
2	1×10^{12}	3.3×10^{7}
3	1×10^{12}	5.8×10^{8}

Titers of phages added to and eluted from wells at each round of panning. Comparing to the first panning, the third was of 2000-fold enrichment.

Specific binding of individual phage clones to anti-*Sj*Fer sera IgG

Phage ELISA Six randomly selected phage clones were tested by ELISA for their binding specificity to anti-*Sj*Fer IgG. The phage clones showed high-affinity binding. In contrast, wild-type M13 showed no binding (Fig. 2).

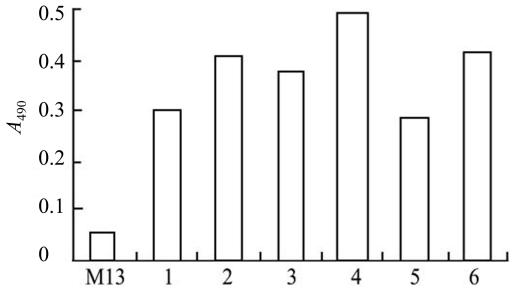

Fig. 2 Phage ELISA of binding of clones to anti-SjFer IgG

Equal numbers of phage particles were incubated with wells coated with anti-*Sj*Fer IgG. For comparison, binding of M13 is shown under the same conditions. The six selected phage clones (1–6) showed high-affinity binding, while M13 did not.

Western blotting The peptide fused gene III products were recognized by anti-*Sj*Fer sera; while wild-type M13 without any foreign peptide, was not recognized (Fig. 3).

Amino acid sequences of peptide

Three sequences were obtained from the six phage clones (Fig. 4). There was no identity between the peptides and *Sj*Fer. Peptides F1 and F2 shared two

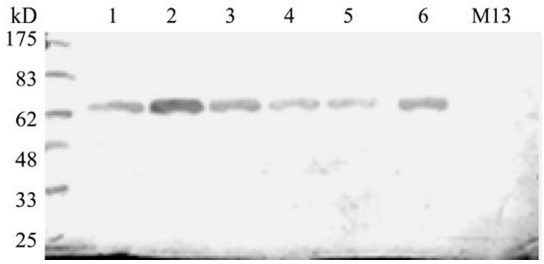

Fig. 3 Interaction of selected clones with anti-*Sj*Fer IgG was analyzed by Western blotting

Six selected clones (1–6) and wild-type M13 phage as a control were separated by SDS-polyacrylamide gel electrophoresis (100 g/L polyacrylamide), transferred to nitrocellulose membranes, and probed with anti-*Sj*Fer IgG. The positions of peptides fused to gpIII were visualized. No binding was observed to gpIII on wild-type M13 phage.

F1 (F3)	W	P	R	L	L	F	D	A	S	A	N	H
F2 (F6)	T	R	E	F	M	K	T	A	S	R	C	P
F4 (F5)	N	V	Q	F	L	E	I	A	R	R	Y	S

Fig. 4 Deduced amino acid sequences of the six phage clones

Similary regions in the peptides were boxed.

amino acids Ala and Ser. F4 do not have any homology to F1 and F2.

Protection against *S.j* of the three mimic peptides

Amplified phage clones F1, F2 and F4 were used to immunize Kunming mice respectively. Two weeks after the third immunization the mice were challenged and perfused six week later. The reduction in worm burden (Table 2) and LEPG (Table 3) was calculated. The reduction in worm burden and LEPG in mice immunized with phage clone F1, F2 or F4 was significant.

Discussion

Iron, playing a role in DNA synthesis, electron transfer

Table 2 Worm reduction in mice vaccinated with each peptide

Groups	Mice	Worms obtained	Reduction vs. TBS (%)	*P*
F1	15	21.07 ± 2.09	26.7	<0.001
F2	15	22.87 ± 1.96	20.4	<0.001
F4	15	21.29 ± 2.52	25.9	<0.001
Original phage	15	26.00 ± 4.07	9.6	>0.05
TBS	15	28.73 ± 2.12	–	–

There is significant reduction of the three experiment groups ($P<0.05$), while the original phage group is not ($P>0.05$).

Table 3 LEPG reduction in mice vaccinated with each peptide

Groups	LEPG ($\times 10^2$)	Reduction vs. TBS (%)	*P*
F1	88.4 ± 1.76	40.0	<0.001
F1	91.0 ± 2.89	38.2	<0.001
F1	87.3 ± 1.39	40.8	<0.001
Original phage	132.6 ± 1.95	10.0	>0.05
TBS	147.3 ± 1.69	–	–

Compared with TBS group, reduction of three experiment groups is significant ($P<0.05$), while that of original phage group is not ($P>0.05$).

and oxygen activation, is required for animals, plants, and micro-organisms [5]. It is so insoluble (10^{-18} M) [6] under physiological conditions that special proteins, ferritins, need to maintain iron in a soluble form and also protect against the toxic effects of excess iron [5]. Ferritin may be classified as one of two types based on whether the iron is stored for intracellular purpose (housekeeping ferritin, plays a role in normal metabolism) or for other kinds of cells (specialized-cell ferritin, e.g. ferritin in hepatocyte which store iron for the entire organism for the long term) [5,7]. In *Schistosoma mansoni* (*S.m*), there are two isoforms of the iron storage protein ferritin, soma ferritin (Fer2) and yolk ferritin (Fer1). The Fer2 occurs at a low level in most cells of both gender, whereas the Fer1, which is a female-specific gene product, is much more abundant than Fer2 (about 15 fold) [8]. *S.j,* gene of ferritin, which had 70 percent homology to Fer1 of *S.m*, had been cloned and the recombinant protein had been expressed and purified [9].

Evidence from several studies [10,11] has indicated that ferritin plays an important role in early growth and development of schistosomula and is essential for embryonic development of *S. mansoni.* These results had led to the idea that ferritin could be a useful immunogen in developing a vaccine against *S.japonicum*. To *S. japonicum*, there is not circumstantial evidence of the function of ferritin. Although the immunological mechanism of *Sj*Fer is not well understood, ferritin could induce significant protective immunity by vaccination of mice [12,13]. This confirmed that ferritin could be a vaccine candidate.

In an effort to identify peptides that mimic epitopes on *Sj*Fer, we have panned a 12-mer random peptide library displayed on the surface gene-3 protein (pIII) of filamentous phage M13 with polyclonal anti-*Sj*Fer antibodies. In this study, we have used two methods to examine the enrichment of specific phages during panning. One is to titer eluted phages, the other is to test the binding specificity of eluted phage to anti-*Sj*Fer IgG. Three rounds of panning resulted in an approximately 2000-fold enrichment of eluted phages (Table 1). Correspondingly, phage pools after three rounds of panning exhibited enhanced binding to anti-*Sj*Fer IgG (Fig. 1).

Six clones bound specifically to anti-*Sj*Fer IgG were selected by phage ELISA. The selected clones showed high-affinity binding, about 10 fold higher than that of M13 control (Fig. 2). Further evidence was from Western blot analysis. At the site of 67 kD the gene III protein fused peptides were recognized by anti-*Sj*Fer sera while M13 was not. The results strengthened the proposal that the six selected phage clones bound specifically to the antigen-binding site of anti-*Sj*Fer IgG and the peptides mimick the epitopes on *Sj*Fer.

Three sequences were deduced by DNA sequencing (Fig. 4). Although F1, F2 contained a common AS motif, there was no identity between the three sequences and *Sj*Fer sequence, suggesting that the peptides isolated were mimotopes.

We vaccinated mice with F1, F2, and F4 individually. A significant protective immunity against *S.j* challenge infection was induced by each of the three peptides, indicating that they were all mimotopes of *Sj*Fer. We believe that development of a vaccine against schistosomasis, in the long term, is feasible through obtaining more antigenic epitopes.

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