

Peptide Mimotopes of Complex Carbohydrates in *Salmonella enterica* Serovar Typhi Which React with Both Carbohydrate-Specific Monoclonal Antibody and Polyclonal Sera from Typhoid Patients

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Received February 13, 2007; in revised form, August 24, 2007. Accepted August 28, 2007

Abstract: Polyclonal sera from typhoid patients and a monoclonal antibody, mAb ATVi, which recognizes the capsular polysaccharide Vi antigen (ViCPS), were used to select for peptides that mimic the ViCPS by using a phage-displayed random 12-mer peptide library. Two major common mimotopes selected from the library carried the amino acid sequences TSHHDSHGLHRV and ENHSPVNIAHKL. Enzyme-linked immunosorbent assays (ELISAs) showed that these peptides carry mimotopes to ViCPS. Phage clones that contained the 12-mer peptides were also tested against pooled/individual typhoid patients' sera and found to have 3 to 5 times higher binding compared to normal sera. By using Phage-ELISA assays, the derived synthetic peptides, TSHHDSHGLHRV and ENHSPVNIAHKL, were tested against a monoclonal antibody mAb ATVi and over 2-fold difference in binding was found between these peptides and a control unrelated peptide, CTLTTKLYC. Inhibition of the mAb's binding to ViCPS indicated that the synthetic peptides successfully competed with the capsular polysaccharide for antibody binding.

Key words: Vi Antigen, Peptide-mimotope, *Salmonella enterica* serovar Typhi, Phage display peptide library

Typhoid fever, a disease caused by *Salmonella enterica* serovar Typhi (*S. Typhi*), remains an important health problem in many developing countries. Antibiotic therapy may considerably reduce mortality but the emergence of multidrug-resistant strains presents an important challenge to public health authorities in combating this disease.

Carbohydrate antigens are immune targets associated with a variety of infectious pathogens, for example, the Vi capsular polysaccharides from *S. Typhi*. One of the problems in developing carbohydrate-based diagnostics is the difficulty involved in synthesizing complex carbohydrate ligands. A possible alternative to the use of carbohydrate would be the development of protein or peptide mimics that could serve the same function. With the development of large random peptide libraries displayed on the surface of filamentous phage (15), it

becomes possible to identify small peptides that could mimic a carbohydrate structure. There have been many successful examples of identifying peptide mimotopes of carbohydrates from phage-displayed peptide libraries (1, 5, 12, 19).

We have previously isolated two populations of phage that react with both carbohydrate-specific monoclonal antibody and polyclonal sera from typhoid patients (18). In this paper, we evaluated the potential of using phage-displayed peptides (phagotopes) as diagnostic reagents by testing the binding of the derived synthetic peptides against monoclonal antibody and their ability to inhibit the reactivity of the mAb with the purified Vi capsular polysaccharide from *S. Typhi*.

Materials and Methods

Sera and antibodies. The sera of 10 individual patients with culture-confirmed typhoid fever were

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Abbreviations: PNS, pooled normal sera; PTS, pooled typhoid patients sera; ViCPS, capsular polysaccharide Vi antigen.

used. Normal human sera (NHS) were obtained from ten healthy, asymptomatic individuals, i.e. blood donors from the University Hospital Blood Bank (Kuala Lumpur). The anti-Vi monoclonal antibody (mAb ATVi) was supplied by Prof. Suttipant Sarasombath, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. Production of mAb ATVi from hybridoma cell line was conducted as described by Pongsunk et al. (13).

Bacterial strain and reagents. The host cell ER2738, an *E. coli* derivative strain [(F' *lacI^q* Δ (*lacZ*)M15 *proA⁺B⁺* *zzf::Tn10* (*Tet^r*)/*fhuA2 supE thi* Δ (*lac-proAB*) Δ (*hsdMS-mcrB*)5(*r_k⁻m_k⁻McrBC⁻*)], was obtained from New England Biolabs. ER2738 is a robust F⁺ strain with a rapid growth rate and is particularly well suited for M13 phage propagation. The F-factor of ER2738 contains a mini transposon which confers tetracycline resistance, so cells harboring the F-factor can be selected by plating and propagating on tetracycline-containing media. Goat anti-mouse IgG horseradish peroxidase (HRP) conjugate and goat anti-human HRP-conjugate were purchased from Sigma Chemical Co. HRP-conjugated anti-M13 monoclonal antibody was purchased from Amersham Pharmacia Biotech UK.

Capsular polysaccharide Vi antigen (ViCPS). The Vi antigen bacteria suspensions were obtained from Newmarket Laboratories, Ltd., United Kingdom. They contain killed bacteria and were used as one of the competitors in the antigen competitive ELISA experiments.

Purified capsular polysaccharide Vi, ViCPS Ag, was obtained from Typhim Vi, Pasteur Mérieux Connaught, France. Typhim Vi is a chemically-defined vaccine against typhoid fever. It does not contain whole bacteria, but consists of a highly purified fraction, the Vi antigen of *S. Typhi* Ty2. It is a polysaccharide which is able to confer protection against the virulent forms of *S. Typhi*. Its O-acetylated groups and its molecular weight were checked by gel filtration.

Reactivity of phage clone against human sera. ELISA testing on the selected phage against individual/pooled typhoid patients' sera as compared to NHS (control) was carried out. Selected phage clones were serially diluted (4-fold dilution of phage concentration from 12.5×10^{11} PFU to 5×10^8 PFU in coating buffer (0.1 M NaHCO₃, pH 8.6) and coated directly to the microtiter plate (100- μ l per wells) for 18 hr at 4 C. The plate was then blocked with TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 0.5% BSA) for 1–2 hr at 4 C. After that, the plate was washed 3 times with washing buffer (TBS/0.1% Tween-20). Then 100 μ l of appropriately diluted human sera (pooled typhoid patients' sera or sera from healthy blood donors) was

dispensed into each well and incubated for 60 min. Sera dilution was carried out at 1/250, 1/500, 1/1,000, 1/2,000, 1/4,000, 1/8,000, 1/16,000 and 1/32,000 using TBS. After that, the plate was washed 4 times with washing buffer, and then 100 μ l of a 1:1,000 dilution of HRP-conjugated anti-human antibodies was dispensed and incubated for another 2 hr. The plate was then washed thoroughly and the substrate ABTS was added. Absorbance readings were read by an ELISA reader at 414 nm (A_{414}).

Preparation of synthetic peptides. Peptides TSHHD-SHGLHRV and ENHSPVNIAHKL were synthesized and supplied by Dr. Stephanovic, Department of Immunology, Institute for Cellular Biology, University of Tuebingen, Germany. All the lyophilized peptides were dissolved in deionized water to give a stock concentration of 10 mM. Peptides were further diluted with TBS to the appropriate concentration for the ELISA tests. An unrelated peptide with the sequence CTLT-TKLYC was used as a negative control. This negative control peptide was synthesized by Genemed Synthesis, Inc., U.S.A.

Synthetic peptide binding assays. Microtiter plates were coated with various concentrations of the synthetic peptides at 2.5–10 μ g per well for 18 hr at 4 C and then blocked with TBS/0.5% BSA for 1–2 hr at 4 C. Varying concentrations of primary Abs (mAb ATVi) were added to wells and incubated for 2 hr at room temperature. mAb ATVi dilution was done at 0.16, 0.32, 0.63, 1.25, 2.5, 5.0, 10.0 μ g/ml in TBS. Plates were washed 5 times (10 min each wash) with TBS 0.5% Tween-20 and HRP-conjugated anti-mouse antibody (1:1,000 in blocking buffer) was dispensed and incubated for 2 hr at room temperature. The plate was then washed thoroughly and the substrate ABTS was added. Absorbance readings at A_{414} were measured.

Peptide inhibition assay. An initial preliminary study of optimal working conditions for the binding of ViCPS to mAb ATVi was carried out. Firstly, 100 μ l per well of appropriately diluted ViCPS was used to coat the microtiter plate overnight (18 hr). Antigen dilution was done at 0.04, 0.08, 0.16, 0.32, 0.63, 1.25, 2.5 and 5.0 μ g/ml of coating buffer (0.1 M NaHCO₃, pH 8.6). The plate was then blocked with 200 μ l of blocking buffer (0.1 M NaHCO₃, pH 8.6, 5 mg/ml BSA, 0.02% NaN₃) for 2 hr at 4 C. The plate was then washed 3 times (10 min each wash) with washing buffer. 100 μ l of appropriately diluted mAb ATVi (2.5, 5.0 and 10.0 μ g/ml) was then dispensed into each well and incubated for 2 hr. The plate was then washed thoroughly 4 times with washing buffer and then 100 μ l of a 1:1,000 dilution of HRP-conjugated anti-mouse antibody was dispensed and incubated for 2 hr at room

temperature. The rest of the steps were the same as described above.

To perform an inhibition-blocking assay, different concentrations of synthetic peptides (0.01–1.0 mM) were premixed with a constant concentration of mAb ATVi (diluted 1:100 in PBS) in a microcentrifuge tube. The mixtures were added to ViCPS-coated microtiter wells (100 µl/well), and the preparations were incubated at 37 °C for 1 hr. After that, the wells were washed six times with PBST (PBS/0.1% Tween-20). 100 µl of anti-mouse HRP conjugate (diluted 1:1,000 in blocking buffer) was added per well at room temperature for 1 hr. This experiment was done in duplicate. BSA was coated as a negative control.

Results

Reactivity of Phage Clones against Pooled Typhoid Patients' Sera and Normal Sera

Previously, two major and minor populations of phage clones bearing peptide sequences TSHHD-SHGLHRV and ENHSPVNIAHKL, respectively were obtained through biopanning of a random 12-mer phage-displayed peptide library against a capsular polysaccharide Vi antigen (18). To further test the antigenicity of these phagotopes, indirect ELISA experiments were conducted. The major population of phage

clones showed a strong binding to pooled typhoid sera as compared to normal sera (Fig. 1). At a serum dilution of 1/250, phage clones showed a response about 5-fold the background signal observed using normal sera. For serum dilution of 1/500, phage clones showed reactivity ranging from 3–4-fold the background signal observed using sera from normal individuals. Similar results were obtained with the minor population group of phage clones (Fig. 2). However the difference between the reactivity of this phagotope against patients' sera and normal sera was not clearly seen at the lower serum concentrations.

When the major population of phage clones (as antigen) (10^{11} PFU) reacted with the individual typhoid patients' sera and normal sera, the mean absorbance reading at 414 nm (A_{414}) was 1.37 ± 0.32 and 0.453 ± 0.06 , respectively, giving a positive to negative ratio of absorbance reading of 3.0. However, when the minor population phage clones (10^{11} PFU) were reacted with the typhoid patients' sera and normal sera, the mean absorbance reading, A_{414} was 1.06 ± 0.33 and 0.430 ± 0.07 , respectively. Hence, it gave a positive to negative ratio of absorbance reading of 2.5. The relatively big difference found between typhoid patients' sera and normal controls in binding to the phage clones suggest specificity of the selected phagotopes.

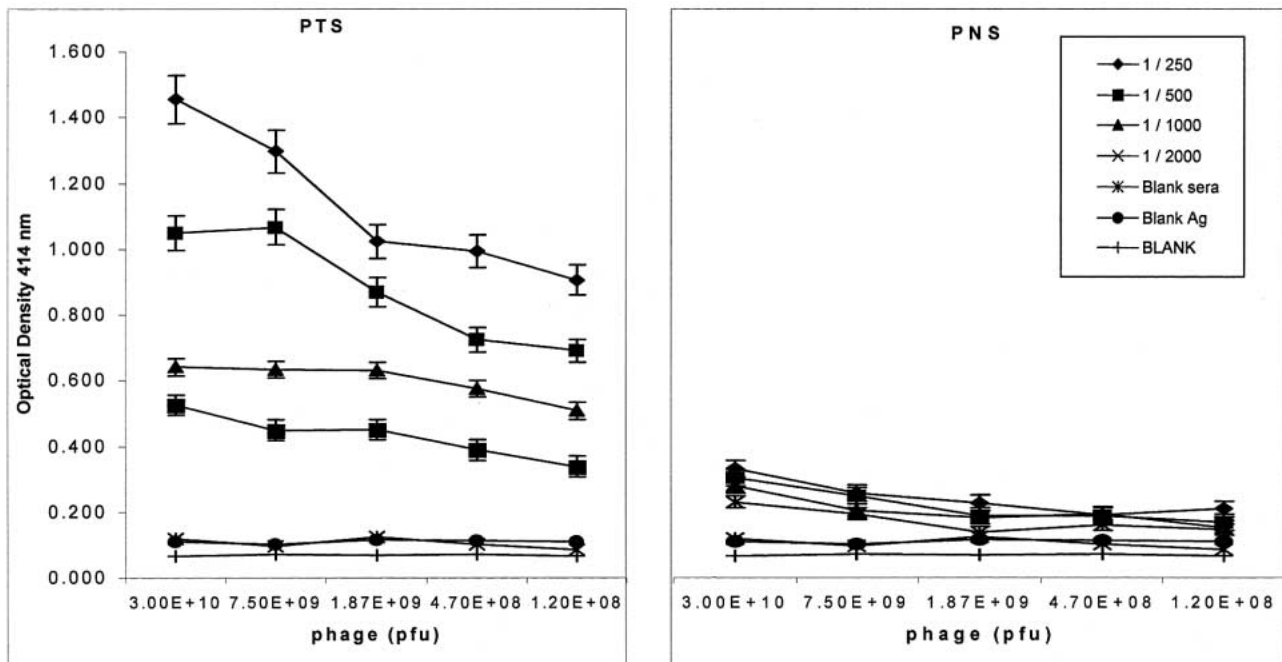


Fig. 1. Comparison of reactivity of pooled patients' sera and normal sera against major population phage clones using indirect ELISA. Plates were coated with serially diluted phage clones (4-fold dilution from 12.5×10^{11} PFU) and then blocked with 0.5% BSA. 100 µl of each serum dilution was added and phage binding to Ab was detected with HRP-conjugated anti-human. The optical density (A_{414}) values are the mean of triplicates and the error bars represent the deviation from the mean (PTS, Pooled typhoid patients sera; PNS, Pooled normal sera).

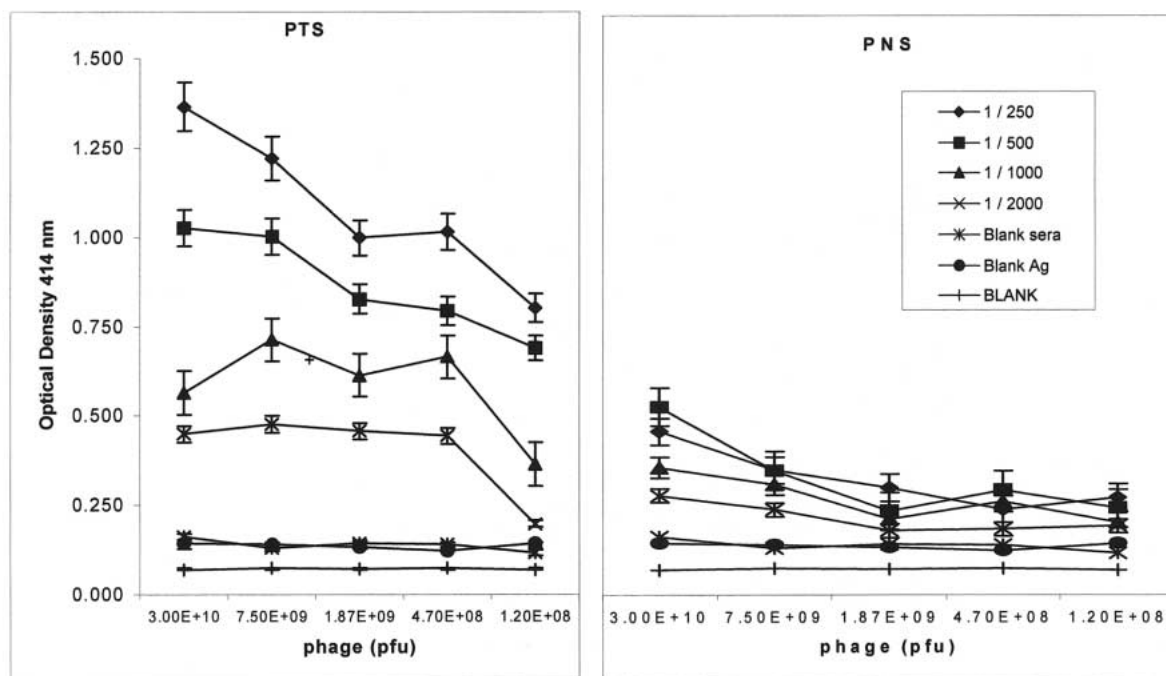


Fig. 2. Comparison of reactivity of pooled patients' sera and normal sera against minor population phage clones using indirect ELISA. Plates were coated with serially diluted phage clones (4-fold dilution from 12.5×10^{11} PFU) and then blocked with 0.5% BSA. 100 μ l of each serum dilution was added and phage binding to Ab was detected with HRP-conjugated anti-human. The optical density (A_{414}) values are the mean of triplicates and the error bars represent the deviation from the mean (PTS, Pooled typhoid patients sera; PNS, Pooled normal sera).

Synthetic Peptide-MAb Binding Assay

Peptides based on sequences of the selected phagotopes were synthesized and peptide-MAb binding assays were performed to determine their specific binding. ELISA plates were coated with representative synthetic peptides derived from the major and minor phage populations. The immobilized peptides were incubated with MAb ATVi, and binding reactivity was measured. The synthetic peptides, TSHHDSHGLHRV (Peptide Vi12A) and ENHSPVNIAHKL (Peptide Vi12B) exhibited binding to the monoclonal antibody in a concentration-dependent manner (Figs. 3–4). Binding of the synthetic peptides parallels the concentration of the MAb coated in the plate while the unrelated peptide CTLT-TKLYC (Peptide CoN) revealed no such reactivity ($P < 0.05$). This indicates that the peptide alone was sufficient for binding to the antibody, independent of the phage structural context.

Peptide Inhibition Assay

To demonstrate that the synthetic peptide mimotopes resemble the carbohydrate epitope of ViCPS, inhibition competitive ELISAs were carried out. Inhibition of the mAb's binding to ViCPS indicated that the derived peptides successfully competed with the CPS for Ab bind-

ing (Fig. 5). In the presence of the synthetic peptide mimotopes, the amount of mAb bound to the ViCPS-coated wells was reduced, as a result of the competition between these two molecules for the same binding site on mAb. For instance, in the absence of synthetic peptide Vi12A, the amount of mAb bound to ViCPS-coated wells gave an absorbance reading of around 0.550 but in the presence of 0.0001 mM of Peptide Vi12A, the absorbance reading was reduced to 0.316, which is about 50% reduction (Fig. 5) ($P < 0.05$).

Similarly, in the absence of the second synthetic peptide (Peptide Vi12B), the amount of mAb bound to ViCPS coated-wells gave an absorbance reading of around 0.600 but in the presence 0.0001 mM Vi12B, the absorbance reading was reduced to 0.472, a 25% reduction ($P < 0.05$). The arbitrary control peptide, CTLT-TKLYC (Peptide CoN), did not produce significant inhibition effect on the ability of mAb ATVi binding to ViCPS (Fig. 5).

Discussion

This study indicates that selected phagotopes reacted strongly with sera from patients with confirmed typhoid fever. This illustrates the potential use of displayed

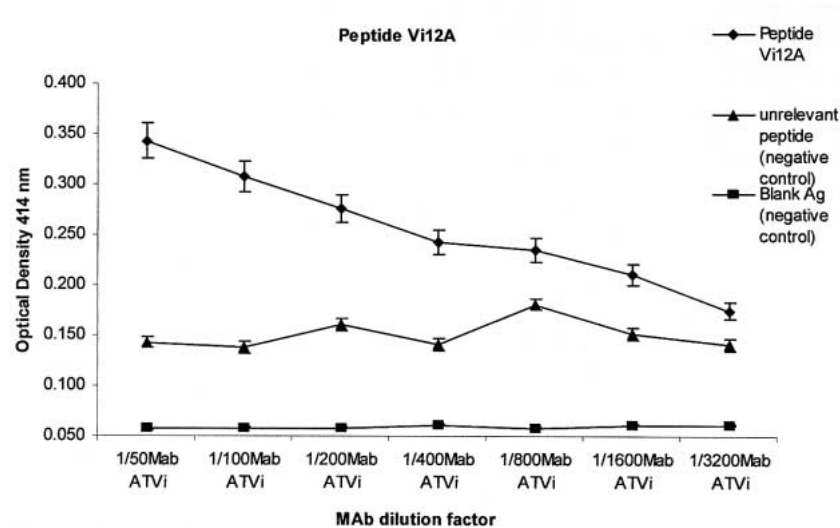


Fig. 3. Binding of anti-Vi monoclonal antibody (MAB ATVi) to peptide Vi12A. Microtiter plates were coated with various concentrations of synthetic peptide (Vi12A) at 2.5–10 μg per well and blocked with TBS/0.5% BSA. The immobilized peptides were incubated with serially diluted MAB ATVi and bound phase was measured by HRP-conjugated anti-M13 antibody. The optical density (A_{414}) values are the mean of triplicate samples and the error bars represent the deviation from the mean.

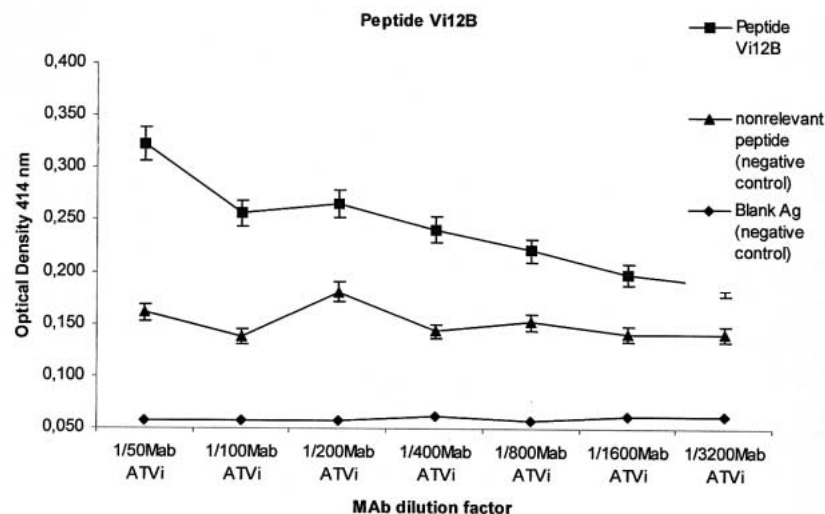


Fig. 4. Binding of the anti-Vi monoclonal antibody, MAB ATVi to peptide Vi12B. Microtiter plates were coated with various concentrations of the synthetic peptide (Vi12B) derived from the minor phage populations at 2.5–10 μg per well and blocked with TBS/0.5% BSA. The immobilized peptides were incubated with serially diluted MAB ATVi and bound phase was measured by HRP-conjugated anti-M13 antibody. The optical density (A_{414}) values are the mean of triplicate samples and the error bars represent the deviation from the mean.

peptide phagotopes as diagnostic reagents. Such application has previously been reported by Poon et al. (14), where the La/SS-B antigen displayed on the phage coat protein aided in the diagnosis of Sjogren's syndrome and systemic lupus erythematosus (SLE). Hyde-DeRuyscher et al. (6) identified phage-displayed peptides that inhibit enzymes and could further be used as

reagents for the detection of small-molecule inhibitors in high-throughput screens.

The advantages of these types of reagents for detection or for diagnosis are the ease with which they can be produced and purified. The possibility of using serum-selected phagotopes as diagnostic reagents depends on the frequency with which they are recognized by dif-

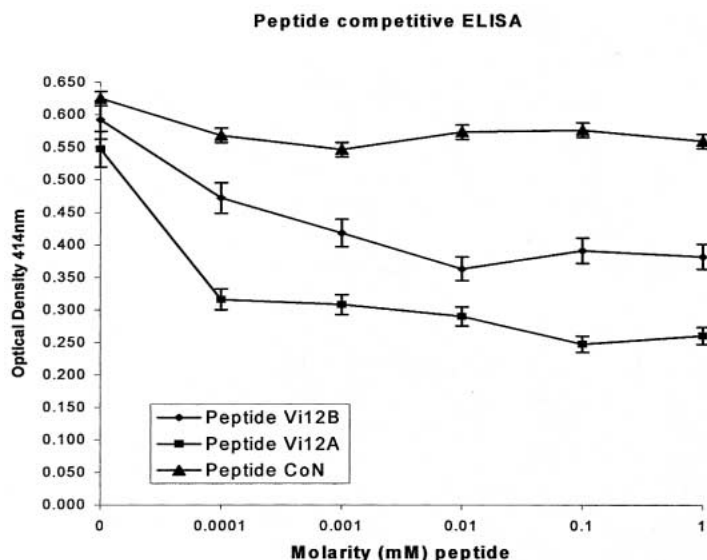


Fig. 5. Inhibition competitive ELISA using peptide mimotope. Various concentrations of inhibitor (synthetic peptide, 0.01–1.0 mM) were premixed with a constant concentration of MAb ATVi (diluted 1:100 in PBS), incubated for 1 hr, and then added onto ViCPS-coated plates. The amount of bound MAb ATVi was measured by anti-mouse HRP conjugate. The optical density (A_{414}) values are the mean of duplicate samples and the error bars represent the deviation from the mean.

ferent patients' sera. In a study by Folgore et al. (4), 80% of the tested immunized population scored positive using just two disease-specific phagotopes. Most studies characterize only a small portion of phage from the enriched phage population (~1%). In this study, about eighty clones were chosen and analyzed. Analyzing larger numbers of phages would undoubtedly identify more disease-specific phagotopes.

Both the synthetic peptides Vi12A and Vi12B could differentiate the sera from typhoid patients and normal healthy individuals. When Vi12A was used as antigen, nine out of ten sera from confirmed typhoid patients were considered as positive at a cut off value fixed at mean plus five times the standard deviation (SD) (mean \pm 5 SD). With the derived peptide Vi12B as antigen, eight out of ten typhoid patients' sera were regarded as positive using the same cut-off value. This cut-off value is considered stringent and discriminative as most published work sets the cut-off value as mean \pm 3 SD only.

Although the study has indicated the possible use of this phagotope (Vi12A or Vi12B) as a diagnostic reagent, this phagotope also reacted with sera from healthy subjects, albeit to a much lesser degree. Klugman et al. (8) defined a serological correlate of protective immunity mediated by anti-Vi antibodies against typhoid fever. Based on this correlate, studies in endemic areas have shown that between 6% (in Kenya)

(9) and 40% (in South Africa) (8) of children already had protective levels of anti-Vi antibody before vaccination. This important observation has been extended to adolescents (16–20 years of age) in South Africa and has been attributed to ongoing antigenic exposure (7). However the data are limited for adults living in endemic areas. Panchanathan et al. (11) showed that a significant proportion (19%) of the adult subjects (aged 18–25 years) who received the Vi vaccine had seroprotective levels of anti-Vi antibody (>1.0 $\mu\text{g/ml}$) even before vaccination. The present study also showed that the phagotope, which is specific to Vi antibodies, had a weak binding to normal sera. This is not unexpected, as portions of the phagotope would most likely bind to other antibodies from a variety of non-related sources. This cross-reactivity is due to common epitopes or antigenic determinants from human or microbial sources that these individuals may have been exposed to (17).

The prevalence of typhoid fever in areas of higher endemicity makes it quite possible that many of the individuals in this area had been exposed to the organism sometime in their life and thus have Vi antibodies. This would account for some of the reactivity seen with these healthy normal sera. This would be an important consideration in applying the peptide in routine serological diagnostic tests. More stringent cut-off values for serology would have to be established. In this sense, using a determinant like peptide mimotope

Vi12A or Vi12B, instead of whole phage antigens, is especially useful because it reduces the background, in that reactivity is limited to only one immunologically important epitope.

The synthetic peptides not only bound to the MAb ATVi but also inhibited the binding of Vi Ag to MAb ATVi. The site of peptide binding on the MAbs ATVi has not been defined. Since the peptides displayed the ability to inhibit binding to ViCPS by MAb ATVi, it is likely that the site is located within the variable regions of the antibody. Bindings to areas outside the antibody-variable regions have not been absolutely ruled out. However the mechanism whereby the peptides inhibit the binding of Ab to Ag is intriguing and is outside the scope of this work.

Peptides that mimic carbohydrate epitopes have also been isolated previously using phage-display libraries (1, 12). Peptides that bind the lectin ConA have been reported (3, 10, 16). Oldenburg et al. (10) demonstrated that peptides containing the consensus sequence (YPY) bind ConA with an affinity comparable to that of the natural ligand (methyl- α -D-mannopyranoside). Phage and synthetic peptides with the motif blocked the binding of ConA to its carbohydrate ligand. Hoess et al. (5) identified a peptide motif (PWLY) that mimics the Ley carbohydrate Ag on the surface of tumor cells and is capable of blocking the binding of Ab to tumor cells and to purified carbohydrate. In this study, the peptide mimotopes have the ability to inhibit the reactivity of the mAb ATVi with the purified Vi capsular polysaccharide in ELISA tests. These peptide mimotopes presumably express important contact residues and intermolecular interactions for the binding of mAb ATVi to the Vi antigen of *S. Typhi*. The unique sequence and specificity of the peptide mimotopes open up new avenues of development for *S. Typhi* diagnostic assays and pave a path for the discovery of potential vaccine candidates.

In conclusion, the isolated phagotopes might be useful for further development as a diagnostic reagent for typhoid fever. Although the specificity of the phagotopes has not been tested in this study, they showed high antigenicity against sera from typhoid fever patients. The inhibition assay showed that the peptides, Vi12A and Vi12B, share a certain degree of structural or functional homology with the ViCPS recognized by mAb ATVi. The binding of ViCPS antigen to mAb was competitively inhibited by the isolated phagotope as well as the synthetic peptide mimotope. The most probable interpretation of the data obtained was that Vi12A and Vi12B are in fact a mimotope, mimicking in part the structure of the Vi capsular polysaccharide antigen of *S. Typhi*. More studies are required to deter-

mine if the peptide mimotopes possess both experimental and therapeutic utility. It may be that the incorporation of such a mimotope peptide into vaccine preparations may enhance the efficacy of vaccines in inducing antibody responses to important carbohydrate epitopes.

We gratefully acknowledge Dr. Stephan Stephanovic (Department of Immunology, Institute for Cellular Biology, University of Tuebingen, Germany) for providing us with the synthesized peptides and Prof. Dr. Suttipant Sarasombath from the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, for supplying the anti-Vi monoclonal antibody (mAb ATVi). We also thank Dr. Linfa Wang, CSIRO, Australia, for technical advice in the initial part of the project. The work was supported by IRPA Grant 06-02-03-0625 from the Ministry of Science, Technology and Innovation, Malaysia and Vote F0053/2001B from the University of Malaya. TSS was financially supported by a tutor fellowship from the University of Malaya, KL.

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