STUDIES ON THE 52 kDa ANTIGEN OF *SALMONELLA TYPHI*: PHYSICOCHEMICAL STABILITY, PURIFICATION BY AFFINITY CHROMATOGRAPHY AND IMMUNOCHEMICAL SPECIFICITY

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Abstract. The 52 kDa specific protein antigen of *Salmonella typhi*, as identified by monoclonal antibodies (Ekpo *et al*, 1990) has been studied with respect to its physicochemical stability, purification by affinity chromatography and immunochemical specificity. It was found that the 52 kDa protein was degraded into smaller antigenic fragments of MW 30-51 kDa when treated with acetone, ethanol, sodium thiocyanate, 0.3M sodium chloride and Veronal and Tris buffers. The exact chemical nature of the degradation of the protein under these conditions is not known but digestion by conventional proteases and dissociation of the non-covalent subunit type have been ruled out. It is proposed that the degradation may be the result of yet unidentified enzyme(s) which become activated by various physical or chemical treatments. Affinity chromatography using a specific monoclonal antibody has been carried out in an attempt to purify the 52 kDa protein. The binding of *S. typhi* protein to the column was saturable at 65.6 μg protein/ml gel. The amount of *S. typhi* protein adsorbed on the column was 0.51% of the total sonicated cell protein. SDS-PAGE of the immunoadsorbent purified protein revealed bands at Mr 15-58 kDa, indicating that the protein obtained had been severely degraded. However, Western blot of the purified protein stained with a specific monoclonal antibody and with rabbit polyclonal antibody against *S. typhi* showed striking similarity, indicating that the protein obtained was close to immunochemical purity. The 52 kDa protein purified by affinity adsorbent was used as an antigen for the detection of specific IgM in sera of patients. It was shown that sera of patients infected with *S. typhi* as well as those infected with other bacteria, contained specific IgM against the 52 kDa protein. Thus, it appears that the 52 kDa protein contains species specific as well as cross-reacting epitopes. The possible development of specific diagnosis of *S. typhi* based on the present experimental results in discussed.

INTRODUCTION

In an attempt to develop specific immunodiagnosis of typhoid fever, highly specific monoclonal antibodies against *S. typhi* have been prepared by Ekpo *et al*, (1990). The mAbs were specific to a 52 kDa protein of *S. typhi* and showed no cross-reactivity by indirect ELISA and by immunoblotting with proteins of other bacteria causing enteric fever and enteric fever-like illness. They reacted most strongly with a 52 kDa protein band, less strongly with a 48 kDa band and faintly with bands at 40, 29 and 25 kDa. When these mAbs were allowed to react with freshly prepared *S. typhi* whole cell (WC) antigen on immunoblots, an immunoreactive band was observed only at 52 kDa (Ekpo *et al*, 1990). Thus, it appeared that the 52 kDa protein was highly labile and that degraded products of smaller molecular weights were generated, possibly by proteases, during the preparation of Barber protein (BP). The 52 kDa protein was found to be highly immunogenic in both mice and man.

The 52 kDa protein, against which specific IgM antibodies were detected in the majority of patients (Ekpo *et al*, 1990), could be an antigen useful for the development of specific serodiagnosis of typhoid fever. However, several potential problems are apparent in any attempt to purify

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the protein. This protein is present in trace amounts in *S. typhi* antigen preparations (Ekpo *et al,* 1990). It is also highly labile and easily breaks down into smaller components. Thus, conditions will have to be found to achieve the highest stability and recovery of the required protein antigen.

The aims of the present study were 1) to study the stability of the 52 kDa protein under various physical and chemical conditions, 2) to attempt to purify the 52 kDa protein by affinity chromatography using previously prepared specific monoclonal antibodies against the protein, and 3) to test whether the purified 52 kDa protein could be used as a specific antigen for the detection of IgM antibodies from sera of patients with typhoid fever.

MATERIALS AND METHODS

Chemicals

Chemicals and biochemicals were purchased from Sigma Chemical Co, St Louis, Mo, unless otherwise indicated.

Preparations of crude antigens of *S. typhi*

Acetone dried cells, Barber protein (BP) and Veronal buffer extract (VBE) antigens were prepared as previously described (Barber *et al,* 1966).

Sonicated cell (SC) antigen. Harvested *S. typhi* from trypticase soy agar was disrupted by Sonicator Model W-380 (Heat-Systems, Ultrasonics, Inc, Farmingdale, NY, USA), using a 1/2 inch Disruption Horn (Standard) with the output control knob set at No. 7 to give double amplitude of ultrasonic vibration of 80 μm for 250 μl each sample volume. The percent duty cycle selector switch was adjusted to give a variable pulse rate of 50% at a 5 second cycle rate and sonication was carried out for 10 minutes. The disrupted cells were stained by Gram stain and examined microscopically to confirm complete disruption before centrifugation at 12,000 *g* at 4°C for 30 minutes. The supernate of the sonicated cells was collected and stored at -20°C for further study.

Crude S. typhi whole cell homogenate (WC) antigen. WC antigen was freshly prepared for each use by mixing a loopful of viable cells of *S. typhi* grown on a trypticase soy agar plate with 600 μl of SDS-PAGE sample buffer and heated for 2.5 minutes in boiling water.

Antibodies

In the present study, monoclonal antibodies (clones 2C1E10 and 5B6E3) were produced and characterized as described by Ekpo *et al* (1990) and were specific for the 52 kDa protein antigen of *S. typhi.* Polyclonal rabbit antisera to *S. typhi* was also prepared as described previously (Ekpo *et al,* 1990).

Purification of the 52 kDa protein antigen by affinity chromatography on a Sepharose 4B-monoclonal antibody column

The coupling reaction between monoclonal antibody and CNBr- activated Sepharose 4B was carried out essentially as described previously (Kukongviriyapan *et al,* 1982). The affinity adsorbent was packed in a 0.9 × 5.0 cm column equilibrated with 0.15 M PBS, pH 7.2. The appropriate amount of crude antigen (20 mg for VBE or 40 mg for SC of *S. typhi*) dissolved in 0.15 M PBS, pH 7.2 was circulated 5 times through the column at a flow rate of 12 ml/hour. The column was then washed exhaustively with 10 column volumes of the equilibrating buffer until the absorbancy of the eluent reached the baseline. The bound protein antigen was eluted with 12 ml ice-cold 3M sodium thiocyanate (Merck, Darmstadt, Germany) directly into a dialysis bag immersed in ice-cold 25 mM PBS pH 7.2 or in 25 mM ammonium bicarbonate pH 8.3. The dialysis was done at 4°C overnight and the dialysate was pooled and concentrated by Diaflo filtration (Cherdchu *et al,* 1978). Otherwise the eluent was dialysed against ice-cold 25 mM ammonium bicarbonate at 4°C overnight and the dialysate was pooled and lyophilized.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and immunoenzyme staining

SDS-PAGE was performed according to Laemmli (1970). The preparation of samples involved mixing of the protein solution with an equal volume of sample buffer (0.0625 M Tris HCl, pH 6.8 containing 2% SDS, 10% mercaptoethanol, 20% glycerol and 0.002% bromophenol blue). The amount of protein sample loaded was between 50-100 μg/well. The mixture was heated in a boiling waterbath for exactly 2 1/2 minutes prior to application to the gel. Western blotting and

immunoenzyme staining were carried out essentially as described previously (Towbin *et al,* 1979; Sarasombath *et al,* 1988). For mAb staining, either mAb 2C1E10 or mAb 5B6E3 was used since they gave indistinguishable results.

Preparation of serum samples from patients

Serum samples were collected from seventeen patients with fever of undetermined origin at Siriraj and Bamrasnaradura Hospitals, Bangkok. Serum was collected and hemocultures were performed on the first day of the patient's admission. All sera were kept at -20°C until used.

Testing of clinical specimens with purified proteins

The immunoadsorbent purified VBE protein after SDS-PAGE and Western blot (14.8 μg/lane), was reacted with a 1:200 dilution of each of the 17 serum samples. The nitrocellulose filter membrane was then incubated with goat-anti-human IgM conjugated with alkaline phosphatase at dilution 1:100. When the immunoadsorbent purified SC protein was used, the purified protein on a nitrocellulose filter membrane (45-50 μg/lane) was reacted with a 1:50 dilution of each of the 17 serum samples from patients described above.

Protein determination

Protein concentration was determined as described by Lowry *et al* (1951) using bovine serum albumin as the standard.

RESULTS

Preparation and SDS-PAGE study of the crude antigens of *S. typhi*

Four preparations of crude antigen of *S. typhi* were studied. They were sonicated viable cells (SC), Veronal buffer extract antigen from acetone dried cells (VBE), Barber protein (BP) and crude *S. typhi* whole cell homogenate (WC) antigen. The result of Coomassie blue staining is shown in Fig 1. Each of the crude antigen preparations gave many polypeptide bands of various molecular weights. The SC preparation showed a comparable number and range of polypeptides to those of WC. Both crude antigen preparations gave more polypeptide components than observed with VBE and BP. However, careful inspection of the protein

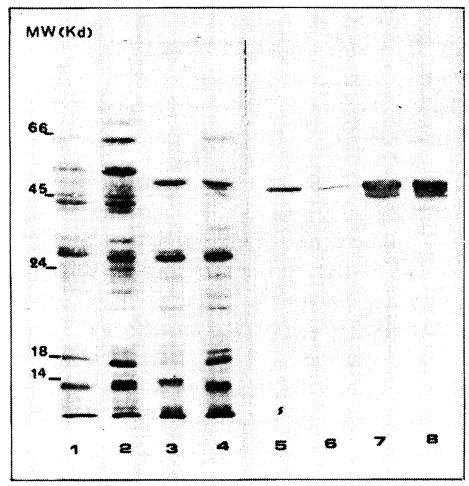
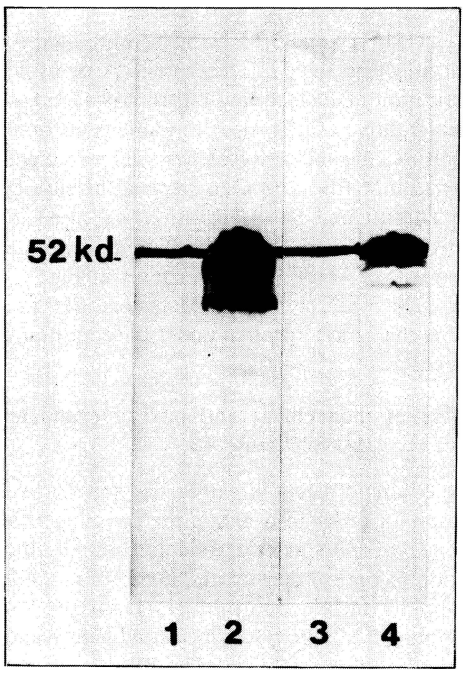


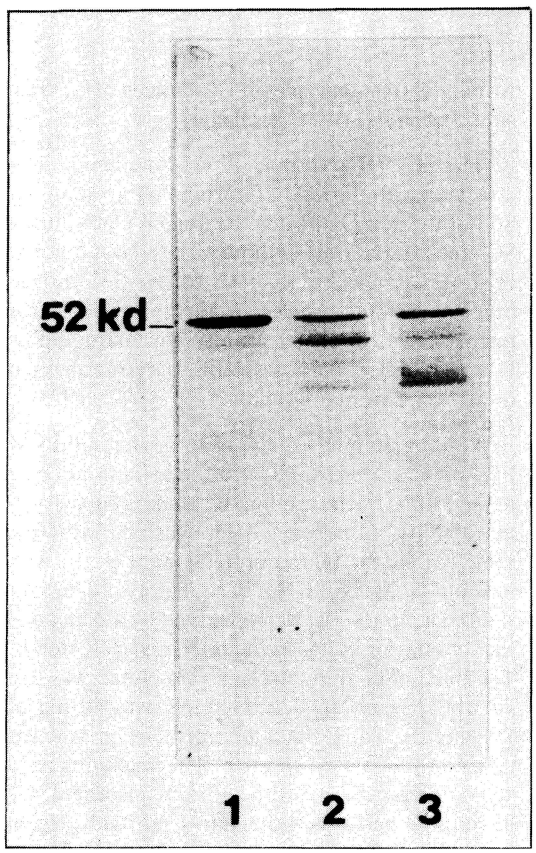
Fig 1—SDS-PAGE (lanes 1-4), Western blot and immunoenzyme staining (lanes 5-8) of various preparations of crude *S. typhi* antigens reacted with mAb. Lanes 1, 5: WC; Lanes 2,6: SC; Lanes 3,7: VBE; Lanes 4,8: BP.

bands of these four antigen preparations revealed major differences in some protein components. For example, a protein band of approximately 18 kDa was absent in the VBE preparation but was quite prominent in the remaining three preparations. This difference in pattern was also observed with proteins of approximately 40 kDa and 70 kDa. Of interest was the enrichment in the VBE and BP preparations of a 52 kDa protein which was not prominent in WC and not at all visible in the SC preparation.

Western blotting and immunoenzyme staining of crude antigens

The SDS-PAGE separated protein bands of WC, SC, VBC and BP of *S. typhi* were transferred onto a nitrocellulose filter membrane and reacted with 2C1E10 mAb (dilution 1:1000). The mAb reacted with only one band of approximately 52 kDa from the WC and SC antigens (Fig 1). However, with VBE and BP antigen preparations, the mAb reacted not only with the 52 kDa protein but also with additional protein bands of slightly lower molecular weights. Thus, the procedures used in the preparation of VBE and BP had somehow altered the structure of the 52 kDa protein to give





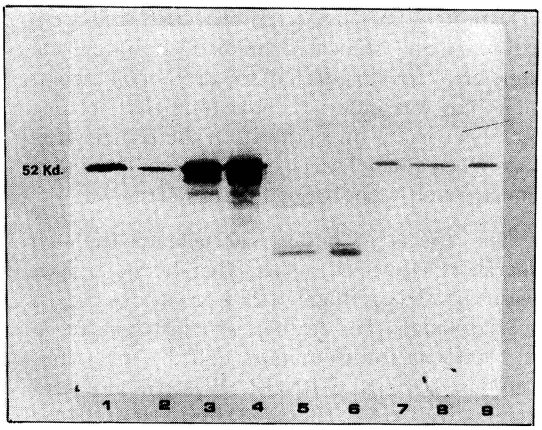


Fig 2—Western blot and immunoenzyme staining patterns of various physicochemically treated antigens. All blots were stained with mAb 2C1E10.

(A) Effect of sonication. Lane 1, WC; Lane 2, VBE; Lane 3, SC sonicated in 0.15 M NaCl for 5 minutes Lane 4, acetone dried cells sonicated in distilled water for 5 minutes.

(B) Effects of Veronal and Tris buffers. Lane 1, SC in 0.15 M sodium chloride; Lane 2, SC in 0.15 M Veronal buffer pH 8.4 (25°C, overnight); Lane 3, SC in 0.15 M Tris buffer pH 8.4 (25°C, overnight). The amount of protein was 70-80 μg/lane. PMSF (0.1 mM) was added to viable whole cells before the preparation of SC and during incubation with buffers.

(C) Effects of NaCl, ethanol, NaSCN and glycine. Lane 1, WC ; Lane 2, SC ; Lane 3, VBE ; Lane 4, BP; Lane 5, acetone dried cells treated with 0.3 M NaCl, overnight; Lane 6, acetone dried cells treated with 0.3 M NaCl, overnight followed by dialysis in 25 mM PBS at 4°C; Lane 7, SC in ethanol, 15 minutes at 25°C; Lane 8, SC in 3M NaSCN 15 minutes at 25°C; Lane 9, SC in 0.2 M glycine pH 2.0, 15 minutes at 25°C.

antigenically active components with lower molecular sizes.

Various physical and chemical effects on the degradation of the 52 kDa protein antigen of *S. typhi*.

The effects of sonication, SDS, acetone and ethanol treatments. The results obtained in Fig 1 with SC antigen indicated that sonication and treatment with SDS did not affect the 52 kDa protein. By contrast, the VBE and BP preparations obtained via acetone precipitation and Veronal

buffer extraction showed degradation of the 52 kDa protein. When the acetone dried cells were extracted and sonicated in distilled water, the same breakdown pattern of the protein on the immunoblot was observed, indicating that acetone treatment had a direct effect on the protein (Fig 2A). When ethanol was used in place of acetone, a similar result was obtained (data not shown).

The effects of buffers, phenylmethylsulfonyl fluoride (PMSF) and sodium chloride: SC antigen was incubated overnight at 25°C in various buffers or salt solutions. The mixtures were then centrifuged at 2,500 rpm for 10 minutes and the supernates were used for SDS-PAGE, Western blot and staining with mAb. The 52 kDa protein in the SC preparation treated with Veronal buffer or Tris buffer at the same pH (pH 8.4) was partially broken down to give bands in the molecular weight range of 39 to 52 kDa (Fig 2B). It should be noted that the degradation of the 52 kDa protein at pH 8.4 in two buffers (Veronal VS Tris) gave quantitatively different protein patterns. When 25 mM of PMSF was present during the preparation of SC and during incubation with buffer, it did not prevent the degradation of the 52 kDa protein (Fig 2B).

Interestingly, 0.3 M sodium chloride drastically reduced the molecular size of the 52 kDa protein to 30-35 kDa in acetone and ethanol dried cells preparations (Fig 2C). Normal saline solution (0.15 M) did not have such an effect. Hence, the effect of sodium chloride on these antigen preparations was studied further by removing 0.3 M NaCl by dialysis in 25 mM PBS at 4°C overnight. The degraded bands did not revert to the 52 kDa component (Fig 2C).

The effect of temperature and time of storage of SC

The SC antigen preparations were kept in 0.15 M NaCl at 25°C, 4°C, -20°C and -70°C for 3 days; then they were subjected to SDS-PAGE and reacted with mAb 2C1E10 after Western blotting. Storing the supernate of SC for 3 days at 25°C, 4°C, -20°C, and -70°C, had little effect on degradation of the 52 kDa protein (data not shown).

The effect of affinity chromatography eluting buffers on the 52 kDa protein

Two of the eluting agents described by Twining and Atassi (1979) for the affinity column (0.2 M glycine pH 2.0 and 3 M NaSCN) were used to test for effect on the 52 kDa protein. A two fold concentration of each eluting buffer was added to an equal volume of SC. After 15 minutes at room temperature, the mixture was dialysed overnight against 25 mM PBS at 4°C to remove the eluting agent. This treated SC preparation was used in SDS-PAGE, Western blotting and immunoenzyme staining with mAb 2C1E10. As shown in Fig 2C, neither 3 M NaSCN nor 0.2 M glycine pH 2.0, degraded the 52 kDa protein under the conditions described.

Coupling of monoclonal antibody to cyanogen bromide-activated Sepharose 4B

The coupling reaction was carried out on two occasions. In order to evaluate the coupling efficiency of mAb to activated-Sepharose 4B, the reduction of optical density at 280 nm of mAb in the coupling buffer (100 mM $NaHCO_3$ pH 8.0 containing 0.5 M NaCl) was measured before and after the coupling reaction. The results for the immunoadsorbent preparations are summarized in Table 1.

Affinity chromatography of SC antigen of *S. typhi* on a Sepharose 4B-mAb column

The SC preparation exhibited one band when it reacted with the mAb, showing that no degradation of the 52 kDa protein had taken place during SC preparation. Furthermore, since a large amount of protein could be obtained easily, this antigen preparation was used as a starting material for further purification by affinity chromatography. For comparative purposes, VBE was also subjected to affinity chromatography.

Varying amounts of SC (5 to 75 mg in 0.15 M PBS pH 7.2) were applied to the column in order to determine the maximum SC binding capacity of the affinity adsorbent. After washing with the same buffer, the bound proteins were eluted with ice-cold 3 M NaSCN. The affinity adsorbent showed saturation as demonstrated by a plateau in the amount of eluted protein at higher SC loads. The maximum amount of protein eluted was 210 μg when the column was loaded with 40 mg of SC (Fig 3). Thus, the eluted protein represented 0.51% of the total SC protein. The maximum antigen binding capacity of the affinity adsorbent was 65.6 μg/ml gel. When the eluted purified protein from SC (PP-SC) was concentrated by Diaflo

Table 1

Some properties of the synthesized mAb-Sepharose 4B affinity chromatographic column.

No.	Sepharose 4B	mAb (2C1E10)		OD 280	Coupling	Ligand density	
	weight	Lot date	Protein content	Before / after coupling	efficiency	mg mAb/ml gel	nmole mAb/ml gel
1	2 g	21/10/88	40 mg	3.12 / 0.21	86.5%	2.16	14.4
2	1 g	20/06/88	20 mg	3.28 / 0.09	94.4%	4.72	31.46

filtration, 59.5% of the protein was recovered. The problem of protein lost during concentration by Diaflo filtration was avoided by lyophilization of the PP-SC in 25 mM sodium bicarbonate. The protein of PP-SC after lyophilization was found to be completely soluble in distilled water. It was calculated that 8.76% of the immobilized mAb was capable of binding the PP-SC. When VBE was used in the affinity chromatography, similar results were obtained (Fig 3). However, the overall yield of purified protein (PP-VBE) eluted from the column was 0.87% of the total VBE protein loaded.

Characterization of the purified protein from SC (PP-SC)

The SDS-PAGE profiles of SC, PP-SC and the unadsorbed component of SC are shown in Fig 4. The protein patterns under reducing and non-reducing conditions were similar after staining with Coomassie blue. The PP-SC preparation contained 8-10 bands of polypeptide with molecular weights between 15-58 kDa. Two prominent bands were noted at Mr 45 and 52 kDa. These polypeptides were minor components in the SC preparation. Thus, affinity chromatography selectively enriched these two bands. When compared to the SDS-PAGE patterns of unbound components of SC, there was a reduction of polypeptide at 45 and 52 kDa positions.

Western blotting and immunoenzyme staining of various SC preparations were carried out before and after affinity chromatography. They were reacted with mAb (2C1E10) or rabbit-polyclonal anti-SC antibody. When the Western blot was reacted with mAb (Fig 5), PP-SC showed more than one polypeptide band with MW's ranging from 29 to 52 kDa. Major bands were at 45 to 52 kDa, as was shown in the Coomassie blue staining (Fig 4). Uncentrifuged SC showed one band at 52 kDa even though it was frozen (-20°C) for 1 month (lane 1). For the SC centrifuged supernate (lane 2), which was frozen at -20°C for 1 month, traces of low molecular weight polypeptides were observed, but the major component was still at 52 kDa.

When the same panel of antigens was reacted with rabbit polyclonal anti-SC antibody, the staining pattern of PP-SC was very similar to the PP-SC reacted with mAb (Fig 5). There might be some low molecular weight bands faintly stained by the polyclonal antibody. In order to find out why the PP-SC contained many peptide bands, the effect of 3 M NaSCN on SC was tested for a period 1 hour incubation. The results showed that treatment with NaSCN for 1 hour caused break down of the 52 kDa protein antigen from SC resulting in a protein pattern similar to that of PP-SC after affinity chromatography (data not shown).

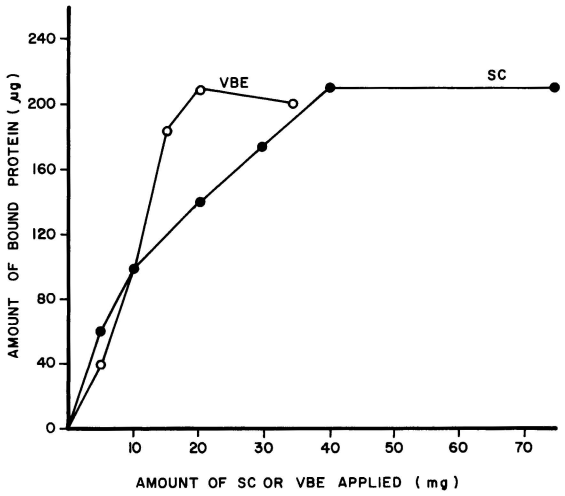


Fig 3—The amount of protein bound to Sepharose 4B-mAb column loaded with varying amounts of SC (—●—●—) or VBE (—o—o—).

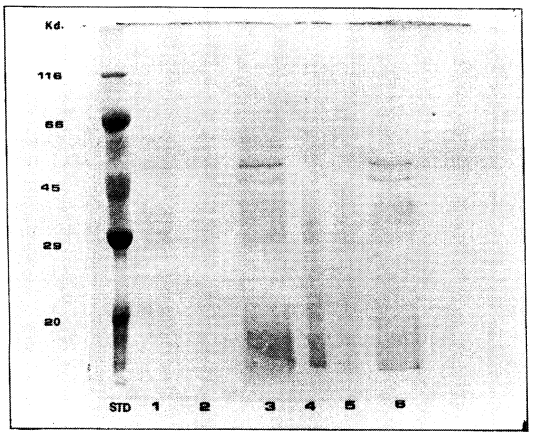


Fig 4—Coomassie blue staining of SDS-PAGE of SC preparations before and after affinity chromatography. Lanes 1,4: SC; Lanes 2,5: unbound component of SC; Lanes 3,6: purified protein from SC (PP-SC), 100 µg. Lanes 1,2,3 samples were prepared in sample buffer with 2-mercaptoethanol. Lanes 4,5,6 samples were prepared in sample buffer without 2-mercaptoethanol.

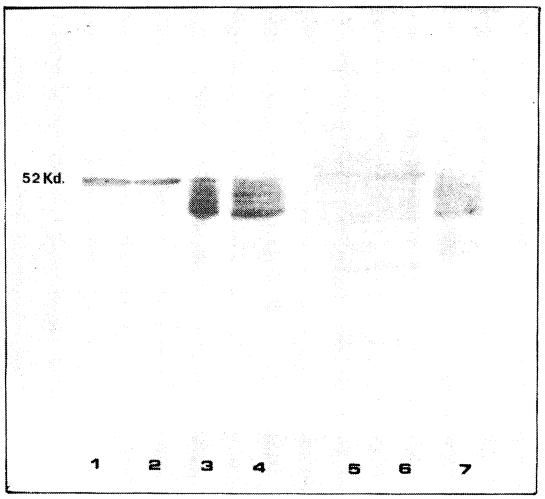


Fig 5—Western blot and immunoenzyme staining of SC preparations before and after affinity chromatography; Lanes 1,5: SC (uncentrifuged), 100 µg; Lanes 2,6: supernate of centrifuged SC, 100 µg; Lane 3: purified protein from SC in PBS, 120 µg; Lanes 4,7: purified protein from SC lyophilized in 25 mM ammonium bicarbonate, 120 µg. Lanes 1,2,3,4 reaction with mAb (2C1E10). Lanes 5,6,7 reaction with rabbit polyclonal anti-SC antibody.

The specificity of the PP-VBE and PP-SC in the diagnosis of typhoid fever

Seventeen sera from patients with hemocultures positive for *S. typhi* and other enteric bacteria were reacted with PP-VBE immobilized on nitrocellulose filter membranes. After reaction with goat-anti-human IgM antibody conjugated to alkaline phosphatase, an IgM band at 52 kDa was observed for sera of typhoid patients as well as for that of patients infected with other gram negative bacteria (Table 2). Similar results were obtained when PP-SC was used as the antigen.

DISCUSSION

The present study has conclusively shown that the 52 kDa antigen of *S. typhi,* as identified by mAbs of Ekpo *et al* (1990) is extremely labile and is easily degraded into smaller immunochemically active fragments. It was found that the protein was degraded to varying degree when subjected to the following reagents and/or conditions: acetone (25°C, overnight), ethanol (25°C, overnight), Tris.HCl buffer (150 mM, pH 8.4, 25°C, overnight), Veronal buffer (150 mM, pH 8.4, 25°C,

Table 2

The number of acute sera from patients who had hemoculture positive for various bacteria and had positive specific IgM to the purified protein antigen (PP-VBE) of *S. typhi,* as demonstrated by Western blot and immunoenzyme staining.

Organisms in hemoculture	No. of sera test	No. of sera positive for IgM to 52 kDa band
S. typhi	5	4
S. paratyphi A	3	2
Salmonella group B	3	3
Salmonella group C	3	1
Klebsiella pneumoniae	1	0
Pseudomonas maltophilia	1	1
Escherichia coli	1	0

overnight), sodium thiocyanate (3 M, 25°C, 1 hour) and sodium chloride (0.3 M, 25°C, overnight). The 52 kDa protein was stable to storage in 0.15 M sodium chloride for at least 3 days at -70°C, -20°C, 4°C and 25°C, and it was stable to sonication and lyophilization, to SDS (1%, 100°C, 2.5 minutes), to ethanol (25°C, 15 minutes), 3 M sodium thiocyanate (25°C, 15 minutes) and to 0.2 M glycine.HCl pH 2.0 (25°C, 15 minutes).

Although the exact nature of the chemical reaction(s) involved in the degradation of the 52 kDa protein antigen is not yet known from the experiments carried out in the present study, various types of reaction can be excluded while some possible reactions can be discussed.

Proteolytic digestion of the 52 kDa protein, as originally suggested by Ekpo *et al,* (1990) can now be ruled out as a cause of the degradation for the following reasons. First, the degradation was observed even in the presence of PMSF, indicating that serine protease(s) were not involved. Second, storage of the SC preparation in the absence of protease inhibitor over a prolonged period at 25°C, did not result in smaller protein fragments.

Also, the generation of smaller molecular weight components of the 52 kDa protein is not due to a non-covalent type of subunit dissociation. This conclusion is reached from the observation that treatment of WC or SC preparations with SDS (1%, 100°C, 2.5 minutes) in the sample preparation for and during the running of SDS-PAGE had no effect on the 52 kDa band. Judging from the slight decrease in molecular weight of the degraded fragments (except in the case of 0.3 NaCl treatment), the degradation seemed to involve a gradual shedding of small parts of the 52 kDa molecule. When incubated with 0.3 M NaCl, a substantial part of the protein was removed.

Most surprising, the 52 kDa protein while stable to 0.15 M sodium chloride over an extended period of time, was degraded into fragments of about 30-35 kDa in 0.3 M sodium chloride. This result was repeatedly observed. The reaction was not reversible, since removal of the salt by dialysis did not result in restoration of the 52 kDa protein.

Taken together, the results obtained from these experiments are extremely puzzling and do not allow one to reach a definitive conclusion. It seems that the breakdown of the 52 kDa involves cleavage of covalent bonds. If this is the case then two possible types of reaction can be envisaged. First, are reactions that involve cleavage of extremely unstable chemical bonds such as thioester and acyl phosphate bonds, etc. This possibility seems unlikely since heating the protein at 100°C for 2.5 minutes in the presence of SDS dit not degrade the protein. An alternative possibility is the involvement of yet unidentified hydrolytic enzyme(s) which can be activated by treatments with various organic solvents and chemicals mentioned in this study. Further study on these degradation reactions are necessary to arrive at a difinitive answer.

It should be mentioned in this connection that, in spite of the chemical lability of the 52 kDa protein, its immunochemical activity remained surprisingly stable. This is evident from the experiments with immunoblots, where the protein was subjected to SDS treatment and yet retained full antibody binding capacity. This observation strongly suggests that the epitope involved in the binding of the monoclonal antibody is predominantly of the linear type. If the epitope were conformational in nature, it would not have been expected to survive the treatment with SDS and 2-mercaptoethanol. This conclusion may have positive implications for the development of specific serodiagnostics for typhoid fever (see below).

Considering the lability of the 52 kDa protein, its low abundancy and the availability of specific monoclonal antibodies against the protein, affinity chromatography becomes a favorable choice for its effective purification. Preparation of immunoadsorbents by coupling one of the two specific monoclonal antibodies to CNBr-activated Sepharose was accomplished with reasonably high coupling efficiencies (85-95%). The ligand density of the immunoadsorbent was intentionally kept low (2-4 mg antibody per ml gel) to increase the antigen binding efficiency and also to minimize non-specific binding of bacterial protein to the immunoadsorbent.

Binding of the SC protein to the affinity column was saturable indicating that specific interaction was involved. The results obtained from various chromatographic runs gave 0.51 μg of bound protein per 100 μg of the SC protein loaded. This should be considered satisfactory in view of the

fact that the 52 kDa protein is present in trace amounts, ie, at most 1% in the crude antigen preparations as observed by SDS-PAGE.

From the maximum antigen binding capacity of the immunoadsorbent of 65.6 μg protein/ml gel, it was calculated that approximately 8.76% of the total immobilized monoclonal antibody was capable of antigen binding. This may be considered low. However, most immunoadsorbents exhibit only 1-30% of the theoretical antigen binding efficiency (Kukongviriyapan *et al,* 1982; Pfeiffer *et al,* 1987; Matson and Little, 1988). Various factors such as steric hindrance, muliple point attachment of the antibody to the matrix, random orientation of the antibody toward the matrix, etc, can all contribute to low binding efficiency.

The major difficulty encountered in the attempt to purify the 52 kDa antigen was the lability of the protein. Even with a relatively stable protein, there is a limited choice of eluting buffers available that can effectively dissociate the antigen-antibody complex and yet denature the proteins involved minimally. Guanidine hydrochloride (8M), sodium thiocyanate (3M) and 0.2 M glycine hydrochloride, pH 2.0 are most often used (Twining and Atassi, 1979). Sodium thiocyanate was found to give the best result in terms of eluted protein recovery and effect on stability of the immunoadsorbent (Twining and Atassi, 1979). In the present study, a preliminary experiment was carried out which indicated that the 52 kDa was not degraded when incubated with ice-cold 3M sodium thiocyanate for 15 minutes. However, under actual chromatographic conditions the eluted 52 kDa protein was degraded into several smaller components. This occured in spite of efforts to minimize the exposure time of the 52 kDa protein to the ice-cold chaotropic agent by immediate dialysis of the eluted fraction. Glycine buffer at pH 2.0 was also used as an eluting agent, but still the recovery of the 52 kDa protein was very poor.

When the protein eluted from the immunoadsorbent was subjected to SDS-PAGE followed by Coomassie blue staining, several protein bands with molecular weights ranging from 52 kDa to 15 kDa were observed. Comparisons of this protein staining pattern with the immunoblots scored with monoclonal and polyclonal antibodies allowed certain conclusions to be made. The presence of the low molecular weight components in the Coomassie stained gel and the absence of these bands in the monoclonal antibody stained immunoblots indicated that the affinity column purified protein was not yet homogeneous. These small proteins must have been the result of non-specific binding on the immunoadsorbent or from degradation during affinity chromatography, or both. It should be pointed out that high salt (0.5N NaCl), usually used in this chromatography to minimize non-specific ionic interaction, could not be used because the salt caused severe breakdown of the 52 kDa protein as mentioned earlier. The immunoblots of the affinity adsorbent purified protein, when stained with monoclonal and polyclonal antibodies, showed striking similarity. The only difference was the probable presence of extremely faintly stained bands at about 15 kDa. These results indicated that the purified protein, though not chemically homogeneous, was closed to immunochemical purity.

The proteins purified from affinity chromatography using VBE or SC as the starting materials were used as antigens for the detection of IgM antibody in sera of patients infected with various bacterial pathogens. It was observed that the IgM antibody against the purified 52 kDa protein was present in sera of patients infected with *S. typhi* as well as with other bacteria. It seems, therefore that there is present on the 52 kDa protein, epitope(s) which are common to bacterial proteins from various sources. If this is in fact the case, the usefulness of this 52 kDa protein as a specific antigen in serodiagnosis of typhoid fever might be limited.

The existence of a specific epitope on the 52 kDa protein, as revealed by the specific monoclonal antibody established previously (Ekpo *et al,* 1990) may be useful in development of an immunodiagnostic test for *S. typhi* infection. As mentioned earlier, this specific epitope recognized by the monoclonal antibody is most likely a sequential (linear) epitope of about 5-6 amino acids. Thus, if the amino acid sequence of this epitope is elucidated, then a short synthetic peptide could be synthesized and used to develop an immunodiagnostic test to detect specific IgM in body fluids of patients with *S. typhi* infection.

ACKNOWLEDGEMENTS

The authors thank Dr Napatawn Banchuin, Dr Leera Kittikul and Dr Tim Flegel for valuable suggestions. This study was supported by USAID grant 936-5542-G-00-5059-00.

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