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PROGRESS REPORT NO. 3

PRODUCTION OF MONOCLONAL ANTIBODIES TO SALMONELLAE THAT CAUSE ENTERIC FEVER

A RESEARCH PROJECT

USAID/PSTC PROGRAM

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PROJECT PROFILE

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BACKGROUND

Enteric fever (typhoid and paratyphoid fever) still remains a major but often underestimated public health problem in less developed countries (LDC). Part of the explaination of this underestimation is due to the difficultries of accurate diagnosis by the Widal test and non-availability of confirmatory hemoculture in much of the rural tropics. In improving the situation, a low cost, simple, rapid and accurate diagnostic test that can be afforded to be applied widely and can be carried out in peripheral health care facilities must be devised such that accurate diagnosis can be made and proper treatment given and that a more realistic epidemiological picture can be monitored.

This diagnostic test should represent an improvement over the standard Widal test and hemoculture, i.e., no cross reaction with other enterobacteriaceae which cause enteric fever like illnesses, the requirement of single specimen in stead of paired specimens, and accurate result in the patients with clinically proved enteric fever but have false negative hemoculture. Hence, this test should provide confident diagnosis with a single sample, preferrably in a rapid, inexpensive and simple way. A logical approach is that it should be accurate, sensitive and specific detection of the bacterium products.

Several groups of investigators have tried several immunological techniques, i.e., coagglutination (1,2), ELISA (3,4) etc, for the detection of bacterial antigens in serum, urine and other body fluids. Unfortunately all of the efforts in this direction have met with the problems of

precision, reproducibility and cross reactivities with other enterobacteriaceae. This problems can be attributed mainly to the antibodies used as only conventional polyclonal antibodies have been employed. The variation of bacterial strains used to produce antisera and the uncertainty of absorption procedures in reducing cross-reactivities make it almost impossible to standardize such polyclonal antisera and the respective tests.

For accurate and specific detection of antigens of Salmonellae causing enteric fever, a secure and consistent source of antibodies will be required. Properly defined clones of hybridoma producing monoclonal antibodies to selected epitopes present a rational approach to meeting these requirements. Once such clones are characterized and established, production of reagents of consistent quality can be ensured and standard kits for a rapid, sensitive, specific and inexpensive dingnostic test, i.e., ELISA can be produced and the molecular components of the antigenic structure among these four species of Salmonellae which are specific to corresponding established monoclonal antibody can also be difined.

OVERALL OBJECTIVES

I. To produce mouse monoclonal antibodies (mAb) directed to species specific antigens of *S.typhi*, *S.paratyphi A*, *S.paratyphi B*, *S.paratyphi C* and common antigens among them but not cross reacting with other enterobacteriaceae that commonly cause enteric fever like illnesses.

II. To develop a rapid, sensitive, specific and inexpensive immunological test detecting salmonella antigens in urine and sera for the diagnosis of enteric fever and identification of the causative species for epidemiological monitoring.

III. To produce test kits that are simple and suitable for use e . in rural areas of LDC where scientific equipments are not readily aviatable.

IV. To identify molecular components of the antigenic structure of salmonellae which are specific to mAb produced in I.

OBJECTIVES OF THIS PERIOD

I. To test the specificity and efficiency of established mAbs to *S.typhi* and to identify molecular components of the antigenic structure of *S.typhi* which are specific to established mAbs.

II. To develope ELISA system using established mAbs for the diagnostic test kit.

III. To develope and produce monoclonal antibodies to *Salmonella paratyphi A*, *S.paratyphi B* and *S.paratyphi C*.

IV. To recruit the sulbjects and collect the blood and urine specimens for the clinical diagnostic trial of the ELISA test kits (cont.).

<u>OBJECTIVE I</u> : To test the specificity and efficiency of established mAbs to *S.typhi* and to identify molecular components of the antigenic structure of *S.typhi* which are specific to established mAbs.

MATERIALS AND METHODS

(Cont. from previous progress report)

1. Specificity determination by ELISA-inhibition

ELISA-inhibition was performed to confirm the specificity of the selected monoclonal antibodies to *S.typhi* protein antigen (BP-ST). The neat monoclonal antibodies from culture supernatant were mixed in equal volume with BP-ST to give final concentrations of 0.1-1000 µg/ml in PBS pH 7.2 and the mixtures were incubated at 37 °C, overnight. After the incubation, they were tested for remaining BP-ST specific antibodies by indirect ELISA as described in the screening test of previous progress report.

2. Efficiency determination for detection of BP-ST by double sandwich ELISA

The selected mAbs from culture supernatants were used to detect BP-ST by double sandwich ELISA (3). The procedure was as follows. Rabbit anti-acetone dried whole cells of *S.typhi* (RS-ST) was diluted 1:500 in 0.05 M carbonate buffer, pH 9.8 and 100 ul per well was added to a Microelisa Immulon I plate (Dynatech,USA). The plate was incubated at 37 °C for 3 hours. Then 100 µl BP-ST which was diluted from 0.1 to 50 µg/ml in 0.05% v/v Tween-saline was added to each well and incubated at 37 °C for 2 hours. At the end of incubation, 100 µl per well of each selected mAbs was added and was incubated at 4 °C, overnight. Then alkaline phosphatase conjugated goat anti-mouse Ig (Southern Biotechnology, Alabama, USA) which was diluted 1:250 in 1% w/v BSA-Tween-saline was added and incubated at 37 °C for 3 hours. A freshly prepared p-nitrophenyl phosphate in

substrate buffer was used as substrate in the concentration of 1 mg/ml. The reaction was allowed to proceed for 60 minutes and the optical density was measured at 405 nm.

During each step before substrate reaction, the supernatant fluid was discarded and the plate was washed 3 times with 0.05% v/v Tween-saline.

3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed for separation of the *S.typhi* protein antigens into small bands on gel prior to transfer these bands to nitrocellulose filter membrane (Western blotting) for using in identification of molecular component of the antigens by reacting with selected mAbs and immunoenzyme staining (5).

3.1 Preparation of a slab gel

A slab gel was performed on a 1.5 x 160 x 180 mm slab using a 14% acrylamide which prepared from a stock solution of 30% w/v acrylamide (Koch Light Ltd, England) and 0.8% w/v N, N-methylene bisacrylamide (LKB, Sweden) in 0.375 M Tris-HCl, pH 8.8. The gel also contained a final concentration of 0.1% w/v SDS, 0.025% v/v tetramethylethylenediamine (TEMED) and 0.05% w/v ammonium persulfate.

When the separation gel was polymerized, the stacking gel was prepared to have a final concentration of 3.5% acrylamide in 0.125 M Tris-HCl, pH 6.8, 0.1% w/v SDS, 0.025% v/v TEMED and 0.05% w/v ammonium persulfate. The stacking gel was loaded on top of the separation gel. The gel was allowed to have a complete polymerization at room temperature for at least 3 hours or overnight at 1°C.

3.2 Sample treatment

Fifty microlitres of protein antigen was mixed with equal volume of sample buffer which contained a final concentration of 0.0625 M Tris-HCl pH 6.8, 2% w/v SDS, 10% v/v glycerol, 5% v/v 2-mercaptoethanol and 0.001%w/v bromphenol blue as an indicator dye. The treated samples were heated in boiling water for 3 minutes before loading.

3.3 Vertical electrophoresis

One hundred microlitre of the treated sample was loaded and electrophoresis was carried out in electrode buffer with 15°C cooling system for approximately 4 hours using a direct current of 30 mA/slab gel. When the indicator dye reached 1 cm from a bottom of gel, the electrophoresis was stopped. The gel was taken out for Western blotting.

4. Western blotting

The separated protein bands from SDS-PAGE were electrophoretically transfered to nitrocellulose filter membranes (Schleicher and Schuell BA 85, Germany) by a Transblot apparatus with 15°C cooling system (LKB, Sweden) at 90 V overnight. A buffer containing 25 mM Trizma base, 192 mM Glycine and 20% v/v methanol was used in this blotting (6).

5. Immunoenzyme staining on nitrocellulose filter membrane

The transfered protein bands on nitrocellulose filter membrane were reacted with the selected monoclonal antibodies or polyclonal antisera and stained by immunoenzyme method as follows. The transfered nitrocellulose filter membrane was soaked in 1% v/v Tween 20-PBS pH 7.2 (Tween-PBS)

at 37°C for 30 minutes. The unoccupied protein binging sites on the filter membrane were blocked by incubation in 3% w/v bovine serum albumin (BSA) in Tween-PBS at room temperature for 60 minutes on a rotated plateform. After rinsing with Tween-PBS, the filter membrane was incubated at room temperature for 2 hours with diluted antibody in Tween-PBS. The antibody was either selected monoclonal antibody or positive control polyclonal antiserum. After incubation, the filter membrane was washed 3 times in Tween-PBS, 10 minutes each time. The alkaline phosphatase conjugated goat anti-mouse Ig (Dakopatts, Denmark) or goat anti-human Ig (Sigma, USA) was applied to the membrane filter depended on the nature of first antibody. It was diluted 1:1000 with 3% w/v BSA in Tween-PBS. The incubation was performed for 60 minutes, at room temperature. After incubation, the filter membrane was washed for 3 times. The substrates used were 4 mg B-naphthyl phosphate (Sigma,USA) and 60 mg O-dianisidine tetrazotized (Sigma, USA), each was dissolved in 10 ml of substrate buffer separately (7, 8). They were mixed together and filtered before use. The substrates were applied to the filter membrane and the reaction was allowed to proceed for 5 minutes, then the filter was rinsed with tap water to stop the reaction.

The precipitate of substrate solution could be wiped out from the filter membrane by cotton wool soaked with Tween-PBS before let it air dry. The red purple color was seen on the reaction band.

6. Molecular weight determination

The molecular weight of any protein band on the separated gel or nitrocellulose filter membrane could be

determined by comparing its electrophoretic mobility with known protein markers (9). A linear relationship should be obtained when the logarithms of the molecular weights of standard polypeptide chains were plotted against their respective electrophoretic mobilities (Rf).

$$Rf = \frac{\text{distance of protein migration}}{\text{distance of tracking dye migration}}$$

The distance was measured from the top of the gel to the center of the protein band or the dye. The protein markers in this experiment were bovine plasma albumin (66.0 Kd), egg albumin (45.0 Kd), bovine pancrease trypsinogen (24.0 Kd) bovine milk B-lactoglobulin (18.4 Kd) and egg white lysozyme (14.3 Kd). All protein markers were purchased from Sigma, USA.

RESULTS

(Cont. from previous progress report)

In the previous progress report there were 10 clones of hybrid selected, but unfortunately we have lost clone number XII:1D6C8. Hence we have 9 monoclonal hybridomas at the present, these hybridomas were divided into 3 groups on the basis of their isotypic characterization and the specificity (Table 1).

One representative clone from each group was chosen for further studies. They were clone XIII:2D1E6, XIV:1D6F7 and XIV:8A2E1 from group 1, 2 and 3 respectively.

1. Specificity of mAbs by ELISA-inhibition

The 3 representative mAbs were used in ELISA-inhibition. Their activities were inhibited by BP-ST at various concentrations, i.e., 100 µg/ml for mAb XIII:2D1E6, XIV:8A2E1 and 1000 µg/ml for mAb XIV:1D6F7. The increasing concentration of BP-ST from 200 to 1000 µg/ml could decrease inhibition of XIV:8A2E1 with no inhibition completely at 1000 µg/ml (Figure 1). The specificity of these mAbs were also tested in Western blotting (immunoblotting) described below.

2. Efficiency of mAbs

The culture supernatant from 3 representative hybrids were used for detection of BP-ST at the various concentrations. The results were shown in Figure 2 that the mAbs from group 1, 2 and 3 could detect BP-ST approximately at the concentration of 10, 5 and 0.1 µg/ml respectively.

3. Western blotting and immunoenzyme staining

The separated protein bands of *S.typhi* and standard protein markers were transfered onto nitrocellulose filter membrane. On the same transfered filter, the protein markers were stained with Coomassie blue dye, the separated protein bands of *S.typhi* were stained with immunoenzyme using mouse anti-*S.typhi* antiserum (MS-ST 1:100), mouse anti-protein antigen antiserum (MS-BP 1:100), mAb XIII:2D1E6 (1:200) and mAb XIV:1D6F7 (1:100) as the specific antibodies (Figure 3). The MS-BP could react with more antigenic determinant than MS-ST indicated that some antigenic determinants in BP-ST preparation hidden inside the bacterial cell. Each mAbs reacted only with one protein band. Both bands had the same molecular weight and could be seen when the BP-ST preparation reacted with MS-BP but not with MS-ST. The protein band which reacted with mAb XIV:1D6F7 was wider than the band reacted with mAb XIII:2D1E6. The difference in size of the reacted bands suggested that both mAbs did not react exactly on the same epitope. The molecular weight of the reacted bands was determined from a standard curve of Coomassie blue dye stained protein markers on the same filter membrane. The result was that both bands had the same molecular weight of 34,000 daltons (34.0 Kd) and had the same relative mobilitly (Rf) of 0.51.

The transfered bands of all protein antigens were revealed by immunoenzyme staining with their homologous antisera (Figure 4, 5A). More than 15 protein bands in each protein antigens were seen.

Both mAbs were tested for their specificity by reacting with protein antigens of *S.paratyphi A*, *S.paratyphi B* and *S.paratyphi C* (Figure 5), *S.cholerasuis*, *S.enteritidis*, *S.krefeld*, *S.panama*, *S.typhimurium*, *E.coli*, *Ps.pseudomallei*

and *Y.enterocolitica* (Figure 6, 7). They could react weakly with a band of protein of the same molecular weight from *S. paratyphi C*, *S.cholerasuis* and *S.typhimurium* although these reactions could not be seen in ELISA for identification test.

DISCUSSION

The specificity of 3 representive mAbs were confirmed by ELISA-inhibition and found that BP-ST could inhibit their reactions. The clone XIV:1D6F7 needed more concentration of BP-ST to complete inhibition, this may due to the low epitope density on the antigen. The clone XIV:8A2E1 which is specific to Vi could be inhibited by BP-ST but the inbition was less at higher concentration of BP-ST. This observation was found even the experiments were repeated two times and this pattern of inhibition was not changed by blocking the remaining sites of ELISA plate after antigen coating with 1% BSA (data not shown). This clone also has a cross reactivity with *S.paratyphi C*. As we know, Vi is present in *S.typhi*, *S.paratyphi C* and rare strains of *S.dublin* (10).

The protein specific clones, XIII:2D1E6 and XIV: 1D6F7, were characterized further by immunoblotting, since proteins can be separated according to their sizes by SDS-PAGE and transfered onto the surface of nitrocellulose filter membrane then immunoenzyme staining or other desirable assays can be performed with the retaining ability of the transfer antigen to bind the specific antibody (11,12, 13). By the immunoblotting technique, each clone was shown to be specific to protein band of BP-ST around the molecular weight of 34 Kd. The pattern of the staining bands were not exactly the same, hence it may be concluded that the XIII: 2D1E6 and XIV:1D6F7 are not exactly specific to the same epitope of the protein antigen since one protein band separated by SDS-PAGE may contain a large number of proteins with the same molecular weight but having different amino acid composition.

To confirm the specificities of these protein specific mAbs, immunoblotting was also performed with a panel antigens of 12 bacteria. The results showed the unexpected weak reactions of both mAbs with BP-SC, BP-SCh and BP-STm, these reactions were not found in the previous ELISA for identification tests. This observation may be due to the more exposure of the hidden epitopes of BP-ST after the protein has been separated by the high resolving power of SDS-PAGE which will increase the sensitivity of immunoblotting in comparison with the simple indirect ELISA on solid phase.

Kanjanaharoutai has studied the characteristic of *Salmonella* Barber proteins by radioimmunoprecipitation and autoradiography. She has found that species specific antigenic component of *S.typhi* might be a single-chain peptide with a molecular weight of approximately 68 Kd. As a result of this observation, she has performed partial purification of BP-ST by Sephadex G-200 gel filtration. The fraction which was proved to contain specific antigen of *S.typhi* by ELISA consisted of several proteins with molecular weight range from 36 to 68 Kd (14). Her finding does not agreed exactly with us, since we have demonstrated that the specific antigen of *S.typhi* is the protein with a molecular weight of 31 Kd. However, the results of both studies supported the idea that the specific antigen of *S. typhi* is possible a protein or a group of proteins with similar size.

In determining the ability of the established mAbs for *S.typhi* antigen detection, double sandwich ELISA was performed with various concentration of BP-ST. The mAb clone XIV:8A2E1 could detect very small amount of BP-ST, its sensitivity is better than using the polyclonal rabbit anti

BP-ST in the previous report (3). However, the opposite results were obtained when using mAb clone XIII:2D1E6 and XIV:1D6F7. These results suggested that the "cocktailed antibody" or the "panel antibodies" consisted of several mAbs specific to different epitopes of *S.typhi* should be used in diagnostic test.

CONCLUSION

Crude protein antigen of *Salmonella typhi* (BP-ST)was prepared according to Barber's method. *S.typhi* strain used in this preparation was freshly isolated from typhoid patient's blood. This BP-ST was proven to contain small amount of Vi capsular polysaccharide and lipopolysaccharide (LPS) by double immunodiffusion method.

Hybridomas producing monoclonal antibodies (mAbs) against specific antigens of *S.typhi* have been established by the fusion of non-producer mouse myeloma cells (P3X63-Ag8.653) with immunoglobulin producing lymphocytes from lymph nodes and spleen of BALB/c J mice that have been immunized with BP-ST. The screening assay for specific mAbs was indirect ELISA using BP-ST as the antigen. The identification assay was also indirect ELISA using a panel of protein antigens extracted from 12 bacteria (*S.typhi*, *S.paratyphi A*, *S.paratyphi B*, *S.paratyphi C*, *S.cholerasuis*, *S.enteritidis*, *S.krefeld*, *S.panama*, *S.typhimurium*, *E.coli*, *Ps.pseudomallei*, *Y.enterocolitica*) and acetone dried cells of *C.jejuni*. All 13 bacteria using in the identification tests can cause enteric fever and enteric fever like illnesses. From 2 fusions (one lymph nodes fusion and one spleen fusion), 111 clones of hybridoma were obtained. Among these 111 clones, 9 clones which were specific to antigens of *S.typhi* were selected. They could be divided into 3 groups on the basis of isotypic characterization and specificity. Group 1 and group 2 consisted of 1 and 3 mAbs respectively, they were specific to protein antigen of *S.typhi* but had different isotype, i.e., IgG2aK for the group 1 and IgG1K for the group 2. Group 3 consisted of 5 mAbs which had IgG1K isotype and Vi antigen specificity. All of them showed no reaction to LPS antigen. One mAbs

from each group was used as a representative. These 3 representatives were tested by ELISA-inhibition and immunoblotting for confirmation of specificity. They could be inhibited by BP-ST at the different concentration. In immunoblotting, two mAbs which were specific to protein antigen reacted with only one band of BP-ST that had 34,000 daltons (34 Kd) molecular weight. Both of them could also react very weakly with a band of the same molecular weight protein of *S.paratyphi C*, *S.cholerasuis* and *S.typhimurium*, although these cross-reactions were not seen in ELISA for identification tests. The efficiency of these mAbs were tested by double sandwich ELISA, the mAbs from group 1, 2 and 3 could detect BP-ST approximately at the concentration of 10, 5 and 0.1 μg/ml respectively. These mAbs have been subjected to a large scale production by injected the hybrid cells into peritoneal cavity of pristane primed BALB/c J mice for obtaining the ascitic monoclonal antibodies. All the valuable hybrid clones have been kept in the liquid nitrogen containers for the future use.

These established mAbs will be of great value for studying the immunological properties and the molecular components of the antigenic structure of *S.typhi* and for using in the development of the diagnostic test for detecting *S.typhi* antigen in the biological fluid of the patients.

Group	Clone number	Subclass	Light chain	Specificity	Source of immune cells
I	XIII:2D1E6	IgG2a	K	protein	lymph node
II	XIV :1D6E5				
	XIV :1D6E9	IgG1	K	protein	spleen
	XIV :1D6F7				
III	XIV :8A2E1				
	XIV :8A2E6				
	XIV :8A2E10	IgG1	K	Vi	spleen
	XIV :8A2F10				
	XIV :8A2F12				

Table 1: Characteristics of monoclonal antibodies specific for protein or Vi antigen of *S.typhi*.

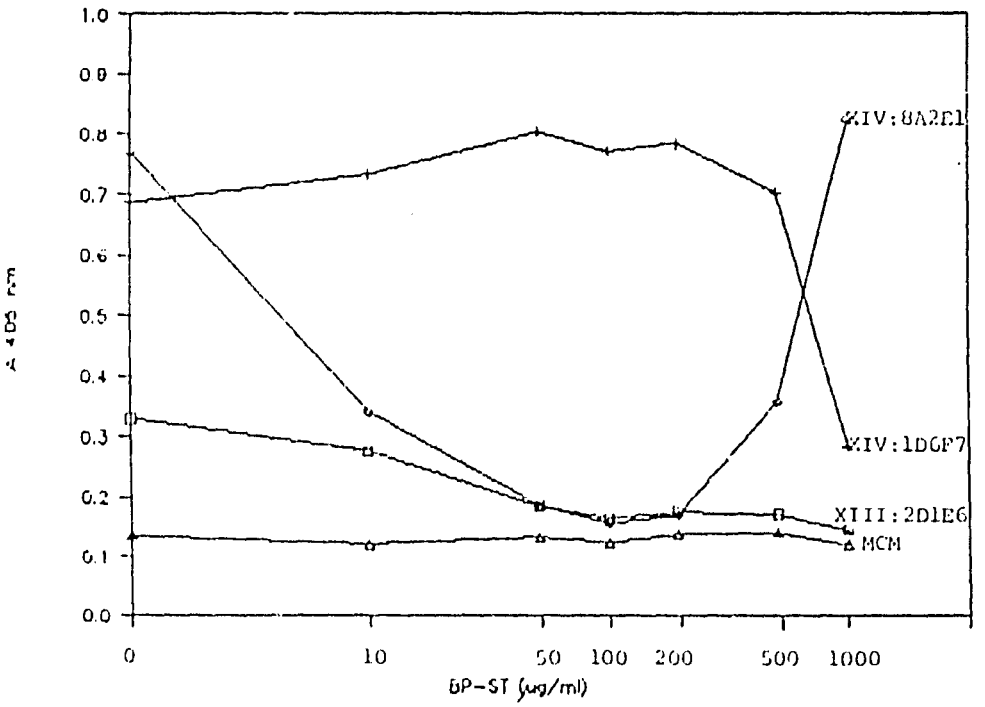

Figure 1: ELISA-inhibition of the three selected mAbs from culture supernatants of clone XIII:2D1E6, XIV:1D6F7 and XIV:8A2E1.
MCM = Myeloma culture medium

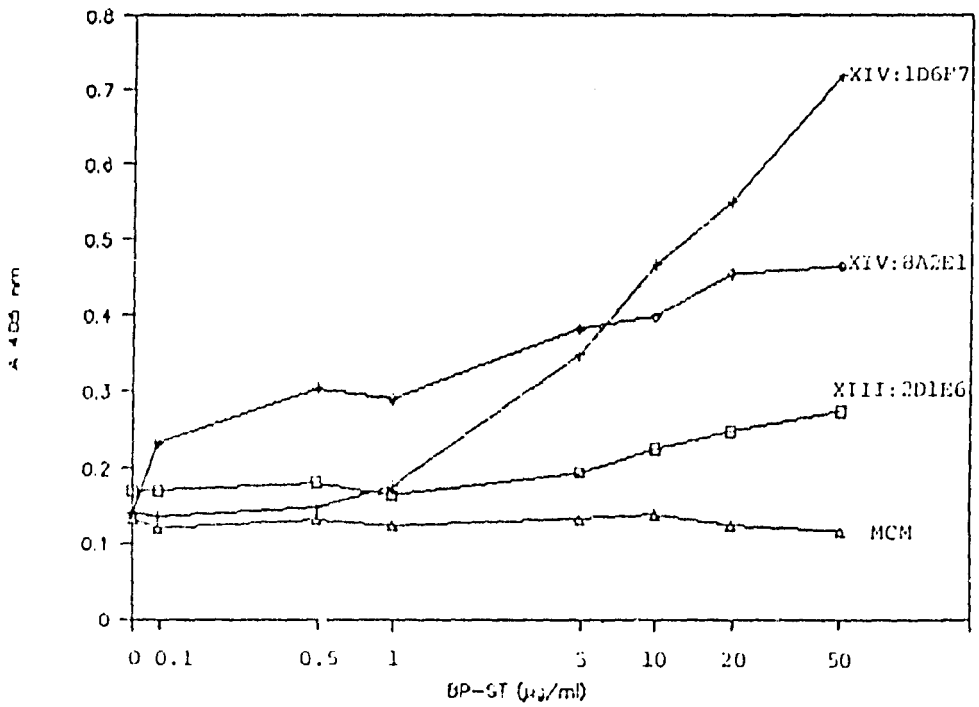

Figure 2: Double sandwich ELISA of the three selected mAbs for detection of BP-ST.
MCM = Myeloma culture medium

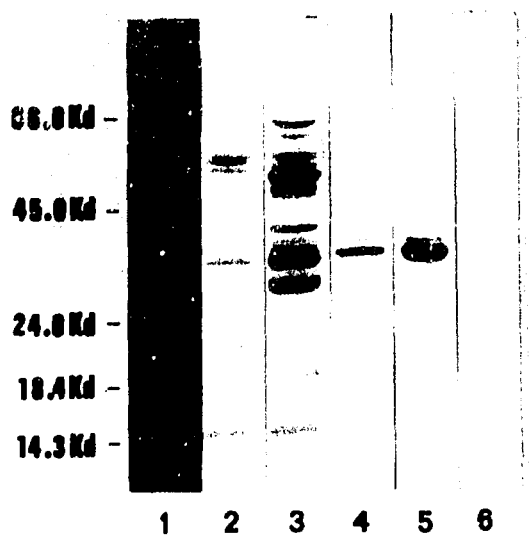

Figure 3 : Western blotting of protein antigen of S.typhi (50 μg/lane) stained or reacted with

1. Coomassie blue dye
2. Mouse anti-S.typhi antiserum (1:100)
3. Mouse anti-protein antigen of S.typhi (1:100)
4. MAB XIII:2D1E6 (1:200)
5. MAB XIV:1D6F7 (1:400)
6. Myeloma culture medium (MCM) as negative control

2-6 followed by immunoenzyme staining.

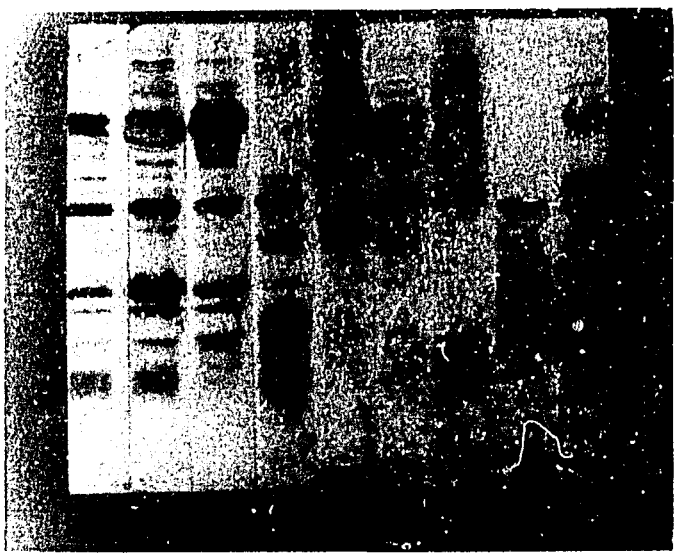

Figure 4 : Western blotting and immunoenzyme staining patterns of protein antigens of

1. S.typhi 50 μg
2. S.cholerasuis 100 μg
3. S.enteritidis 50 μg
4. S.krefeld 100 μg
5. S.panama 50 μg
6. S.typhimurium 50 μg
7. E.coli 50 μg
8. Ps.pseudomallei 100 μg
9. Y.enterocolitica 100 μg

All were reacted with 1:200 dilution of their homologous antisera.

Figure 5 : Western blotting and immunoenzyme staining patterns of protein antigens of

1. S.typhi 50 μg
2. S.paratyphi A 50 μg
3. S.paratyphi B 50 μg
4. S.paratyphi C 50 μg

A. Reacted with their homologous antisera (1:200)

B. Reacted with mAb XIII:2D1E6 (1:200)

C. Reacted with mAb XIV:1D6F7 (1:400)

Arrow indicated protein molecular weight of 34.0 Kd.

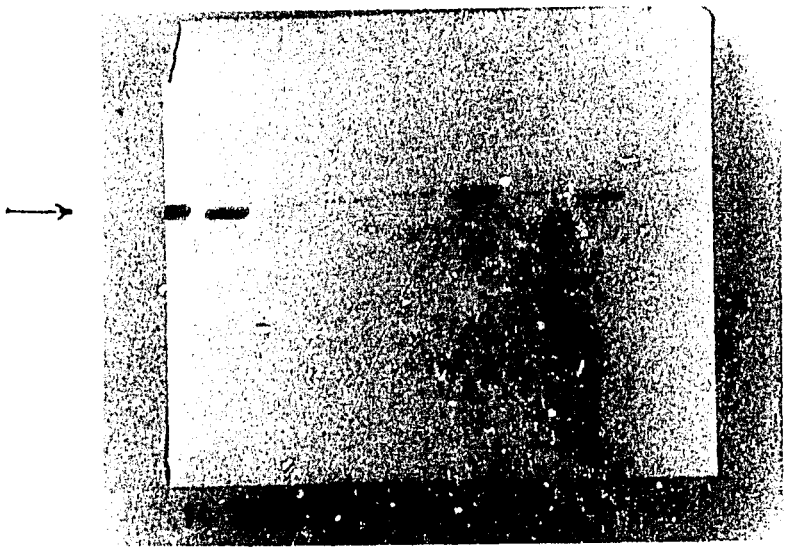

Figure 6 : Western blotting and immunoenzyme staining patterns of the same antigens as Fig. 4 reacted with mAb XIII:2D1E6 (1:200). Arrow indicates protein molecular weight of 34.0 Kd.

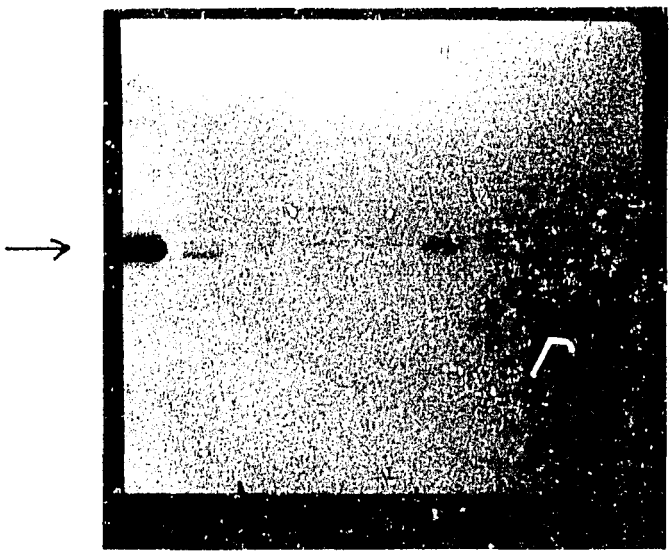

Figure 7 : Western blotting and immunoenzyme staining patterns of the same antigens as Fig. 4 reacted with mAb XIV:1D6F7 (1:400). Arrow indicates protein molecular weight of 34.0 Kd.

REFERENCES

1. Rockhill RC, Rumans LW, Lesmana M, Dennis DT. Detection of *S.typhi* D, Vi and d antigens, by slide conggrutination, in urine from patients with typhoid fever. J Clin Microbiol 1980; 11: 213-5.
2. Sivadasan K, Kurien B, Jacob-John T. Rapid diagnosis of typhoid fever by antigen detection. Lancet 1984; 1:134-5.
3. Appassakij H, Banchuin N and Sarasombath S. et al. Enzyme-linked immunosorbest assay for detection of *Salmonella typhi* protein antigen. J Clin Microbiol 1987; in press.
4. Barrett JJ, Snyder JD, Blake PA, Fuley JC. Enzyme-linked immunosorbent assay for detection of *S.typhi* Vi antigen in urine from typhoid patients. J Clin Microbiol 1982; 15: 225-7.
5. Laemmli Uk. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-5.
6. Toebin H, Staehelin T and Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets : Procedures and some applications. Proc Natl Acad Sci (USA) 1979; 76: 4350-4.
7. O'Connor CG and Ashman LK. Application of the nitrocellulose transfer technique and alkaline phosphatase conjugated anti-immunoglobulin for determination of the specificity of monoclonal antibodies to protein mixtures. J Immunol Methods 1982; 54: 267-71.
8. Turner BM. The use of alkaline phosphatase conjugated second antibody for the visualization of electrophoretically separated proteins recognized by monoclonal antibodies. J Imaunol Methods 1983; 63: 1-6.

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9. Weber K and Osborn M. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 1969; 244: 4406-12.

10. Lindberg AA and Le Minor L. Serology of Salmonella. In Bergan T. ed, Methods in Microbiology Vol.15. London : Academic press, 1984: 1-141.

11. Gershoni JM and Palade GE. Protein blotting : Principles and applications. Anal Biochem 1983; 131: 1-15.

12. Towbin H and Gordon J. Immunoblotting and dot immunobinding : Current status and outlook. J Immunol Methods 1984; 72: 313-40.

13. Mather S and Taylor-Papadimitrion J. Monoclonal antibodies, electrophoretically transferred from polyacrylamide gels, retain their ability to bind specific antigens. Biochem Biophys Res Commun 1985; 133: 1020-5.

14. Karnjanaharcutai S, Sarasombath S and Sirisinha S. Patial purification of Salmonella Barber Proteins. Manuscript in preparation.

OBJECTIVE II: To develope ELISA system using established mAbs for the diagnostic test kit

After several trials, a modified double antibody sandwich ELISA was chosen to be the system used in the diagnostic test kit for detection of *S.typhi*'s antigens. A monoclonal antibody XIV:842E1 which is Vi specific, was used as a model.

MATERIAL and METHODS

Veronal buffer extracted antigen (VBE Ag)

This antigen which was used in the experiment could resemble the antigen in the patient's specimen since it is cruder and contains more Vi antigen than the protein antigen (BP).

This antigen was simply prepared by adding 0.15M Veronal buffer pH 8.1 to acetone dried *S.typhi* bacilli and incubate at 37°C overnight with continuous stirring. The clear extracts were collected by 12,000 x g centrifugation at 4°C for 20 minutes and dialysed against distilled water at 4°C for overnight. The dialysed was filtered through a 0.45 μm millipore filter membrane and was determined for protein concentration by Lowry method as modified by Hartree (1), then stored at -70°C until used.

Preparation of rabbit anti-VBE immunoglobulins

Rabbit anti-VBE antisera were raised in 2 NZW/California rabbits, each weighing approximately 2 kgs. The route of immunization was subcutaneously. Each rabbit was immunized 6 times, at, 1 week interval, with 1.2 mg of VBE Ag in incomplete Freund's adjuvant. The antisera were tested against specific antigen by double immunodiffussion.

Only serum samples having antibody titer ≥1:16 were pooled and used for the preparation of anti-VBE Ig by ammonium sulphate precipitation.

ELISA system developed

The optimum conditions used in various steps of the ELISA were chosen after the standardization of the assay, carried out with rabbit anti-VBE Ig and mAb XIV:8A2E1 as immobilized and second antibody respectively. They were as follows.

	amount/dil	temp	time
1st Ab for coating the plate	10 μg/ml	4°C	over night
Antigen	-	37°C	3 hrs
2nd Ab	5 μg/ml	4°C	2 hrs
Anti-rabbit IgG-alkaline phosphatase conjugate	1:2,000	30°C	over night
Anti-mouse Ig-alkaline phosphatase conjugate	1:250	30°C	over night
Substrate	-	37°C	1 hr

First experiment

Rabbit anti-VBE Ig was diluted in 0.05 M carbonate buffer, pH 9.8, containing 0.1% sodium azide, to a protein concentration of 10 μg/ml. One hundred microlitres of the solution was added into each well of Dynatech Immulon I® microelisa plates (Dynatech Produkta AG, Klonten, Switzerland), which were then incubated at 4°C for overnight. After that, the plates were washed 3 times with phosphate buffered saline-tween 20 (PBST) and tapping dried. One

hundred microlitres of VBE Ag in various concentrations diluted in PBST was added to each well and the plates were incubated at 37°C for 3 hours. Each specimen was assayed in duplicate. After 3 washes with PBST, 100 µl of second antibody which was mAb XIV:8A2E1 diluted in PBST to a concentration of 5 µg/ml was added to each well. The plates were then incubated at 1°C for 2 hours. After washing, 100 µl of rabbit anti-mouse Ig-alkaline phosphatase conjugate diluted 1:250 in PBST containing 1% BSA was added to each well and incubated at 30°C for over night. The excess conjugate was washed out 3 times with PBST and 100 µl of substrate solution (p-nitrophenyl phosplate, Sigma USA) was added to each well. The reaction was allowed to take place at 37°C for 1 hour and stopped by the addition of 25 µl 3 M NaOH. The absorbance value was read spectrophotometrically at 105 nm (Titertek Multiskan®, Flow Laboratory, Germany). In each assay, negative control, conjugate control and substrate blank were also included. One hundred microlitre of PBST was substituted for the antigen in negative control, for both the antigen and mAb XIV:8A2E1 in the conjugate control and also for conjugate in the substrate blank.

Second experiment

The second experiment was performed by using mAb XIV:8A2E1 as the first antibody for coating the plates and rabbit anti-VBE Ig as the second antibody. The procedure was exactly the same as the first experiment. The purpore of this experiment was to see whether which one could give the better result.

RESULTS

The result between 2 modified double antibody sandwich ELISA systems was shown in figure I. It can be seen that the first system, using rabbit anti-VBE Ig as the first antibody coating the plate could detect VBE Ag at the minimal level of approximately 5 μg/ml, while the second system which used mAb XIV:8A2E1 as the first antibody could detect the same antigen at much lower concentration (approximately 0.2 μg/ml). Reference to these results,we have decided to use the second assay system for testing the clinical specimens of the patients.

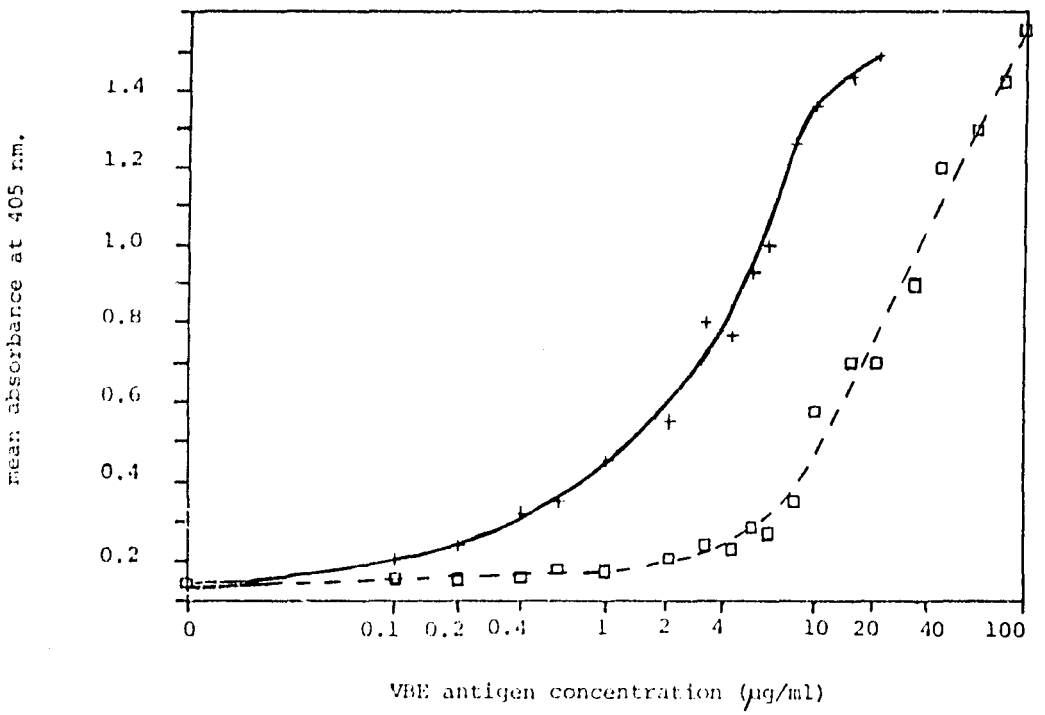

Figure 1 Dose response curves obtained from the assay of VBE antigen at various concentrations, with ELISA using either rabbit anti-VBE Ig as the immobilized first antibody and mAb XIV:8A2E1 Ig as the second antibody (□ - - □ - - □) or vice versa (+ —— + —— +).

Plates were coated with the first antibody concentration 10 μg/ml, at 4°C overnight. The antigen was incubated at 37°C for 3 hours. Second antibody was used at a concentration of 5 μg/ml and was incubated at 4°C for 2 hours. Conjugate and substrate incubation conditions were 30°C overnight and 37°C for 1 hour respectively.

REFERENCES

1. Hartree EF. Determination of protein : a modification of the Lowry method that gives a linear photometric response. Anal Biochem 1972; 48: 422-7.

<u>OBJECTIVE III</u>: To develope and produce mAbs to *Salmonella paratyphi A*, *S.paratyphi B* and *S.paratyphi C*.

The materials and methods used in this experiment is exactly the same as developing and producing mAbs to *S.typhi* preriously described, except the immunization part which 200 μg mixture of equal amount of protein antigen from *S.paratyphi A*, *S.paratyphi C* and *S.paratyphi B* was given to each BALB/c J mice in stead of the protein antigen of *S.typhi*. The lymph nodes and spleen fusion with Ag 8.653 mouse mycloma cells were performed, more than 300 monoclonal hybridomas were observed. We are now in the process of screening to select the clones which have specificity to either *S.paratyphi A*, *S.paratyphi B*, *S.paratyphi C* or the common epitope(s) of these 3 bacilli.

<u>OBJECTIVE IV</u>: To recruit the subjects and collect the blood and urine specimens for the clinical diagnostic trial of the ELISA test kits

At the present, we have sera and urine specimens from hemoculture positive patients as follows, 14 *S.typhi* positive cases, 27 *S.paratyphi A* positive cases, 13 *Salmonella gr. B* positive cases, 7 *Salmonella gr. C* positive cases, 12 cases of gram negative rod other than Salmonella spp., 10 cases of gram positive organisms and 46 healthy normal controls.

We plan to perform this activity further until we can obtain sufficient number of the specimens.

WORKPLANS FOR THE NEXT PERIOD

I. Development and production of monoclonal antibodies to *S.paratyphi A*, *S.paratyphi B* and *S.peratyphi C* (cont.).

II. Identification of molecular components of *S.paratyphi A*, *S.paratyphi B* and *S.paratyphi C*.

III. Further recruitment of the subjects and collection of blood and urine specimens for the clinical diagnostic trial of the ELISA test kits.

IV. Establisment of ELISA test kits (cont.).