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AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON D.C. 20523

DATE: 8/4/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 5. 227
PR #2
PR #3

Attachment

PN-AAA-540
32846

PROGRESS REPORT NO. 2

PRODUCTION OF MONOCLONAL ANTIBODIES TO SALMONELLAE THAT CAUSE ENTERIC FEVER

A RESEARCH PROJECT

USAID/PSTC PROGRAM

GRANT NO. : 936-5542-G-00-5059-00

SUBMITTED BY

DR. SUTTIPANT SARASOMBATH, M.D., F.C.A.P.

DEPARTMENT OF MICROBIOLOGY

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THAILAND

Rec'd in AUG 11 1986

PROJECT PROFILE

Country	:	Thailand
Grant No.	:	936-5542-G-00-5059-00
Program	:	Program on Science and Technology Cooperation
Project Title	:	Production of monoclonal antibodies to Salmonellae that cause enteric fever
Project Leader	:	Dr. Suttipant Sarasombath, M.D., F.C.A.P.
Organization	:	Mahidol University, Thailand
Co-investigators	:	1. Dr. Napatawn Banchuin, M.D., Ph.D. 2. Mrs. Tassanee Sukosol, M.Sc.
Project Consultants	:	1. Dr. Pornchai Matangkasombut, M.D., Ph.D. 2. Dr. J.F. Kearney, D.D.S., Ph.D.
Authorized Officer	:	Dr. Natth Bhamarapravati, M.D., Ph.D. Rector, Mahidol University
Total Project Budget	:	US$ 147,740.00 (฿ 3,988,980.00)
Project Duration	:	3 years
Reporting Period	:	January 1, 1986 - June 30, 1986
Budget Allocation for This Period	:	US$ 15,990.00 ฿ 431,730.00

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 difficulties 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 even in rural areas of LDC where scientific equipments are not readily avialable.

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

OBJECTIVE OF THIS PERIOD

I. To develope and produce monoclonal antibodies to *Salmonella typhi*.

II. To recruit the subjects and collect the blood and urine specimens for the clinical diagnostic trial of the ELISA test kits.

III. To identify molecular components of the antigenic structure of *S. typhi* which are specific to established mAb.

OBJECTIVE I : To develope and produce monoclonal antibodies to *S. typhi*

MATERIALS AND METHODS

Bacterial strains The strains used in this study, *Salmonella typhi*, *S. paratyphi A*, *S. paratyphi B*, *S. typhimurium*, *S. choleraesuis*, *S. enteritidis*, *S. panama*, *S. krefeld*, *Escherichia coli*, *Campylobacter jejuni*, *Pseudomonas pseudomallei* and *Yersinia enterocolitica*, were all isolated from the patients suffered from enteric fever and enteric fever like illnesses. *S. paratyphi C* (BR 1751) was kindly provided by Dr. B. Rowe, Central Public Health Laboratory, London, U.K.

Production of antigen The protein antigen was extracted from the bacteria by the method described by Barber et al.[5,6] with slight modifications. Briefly, washed and acetone dried bacterial cells were extracted with veronal buffer pH 8.4 and the protein was precipitated out of the extract by trichloroacetic acid. The precipitate was washed, resolubilized, lyophilized and store at 4 C until used. The protein content of these preparations was measured by the method of Lowry.[7]

Immunization of mice

Female BALB/cJ mice, 6 to 10 weeks of age, were immunized once every 3 days for 15 days. For the first immunization, mice were given 200 μg per mouse of *S. typhi* protein antigen diluted to 0.25 ml in normal saline solution (NSS) emulsified with an equal volume of complete Freund's adjuvant (Difco Laboratories, Mich., USA) subcutaneously into 2 footpads, 2 axillary and 2 inguinal areas. The second immunization was similar to the first immunization except incomplete Freund's adjuvant was used in stead of complete Freund's adjuvant. For the 3rd to 6th immunization, each mouse was given 200 μg of *S. typhi* protein antigen in NSS without adjuvant subcutaneously into the 6 areas mentioned above with the exceptional of the 6th immunization which the

antigen was divided and half of the amount was given intravenously.

Cell fusion and cloning One day after the final administration of antigen, mice were sacrified by cervical dislocation. The axillary and inguinal lymph nodes and the spleens were aseptically removed. A single cell suspension was prepared by teasing the lymph nodes or spleen in the sterile petri-dish containing PBS. The cells were passed through a sterile glass wool column once to obtain a fine suspension. The cells were washed three times in serumless RPMI 1640 at 4 ^{o}C and counted. The mouse myeloma cells (X63-Ag8.653, kindly provided by Dr. J.F. Kearney, University of Alabama in Birmingham, USA) was also washed three times in serumless RPMI 1640 at 4 ^{o}C and counted. To 3.5×10^{6} lymph node cells, 3.5×10^{6} myeloma cells were added and to 7.5×10^{7} spleen cells, 4×10^{7} myeloma cells were added. The cell mixture was washed once with serumless RPMI 1640 at room temperature. After the supernatant was removed, a 1 ml of polyethylene glycol solution (PEG-4000, Accurate Chemical and Scientific Co., NY, USA) was added dropwise to the cell mixture for 1 minute at 37 ^{o}C. After waiting for 30 seconds, the 20 ml serumless RPMI 1640 was added dropwise at 37 ^{o}C, then 30 ml of serumless RPMI 1640 was added and the cells were gently centrifuged at room temperature for 10 minutes at 300 x g. The supernatant was removed and the cells were very gently resuspend in 37 ^{o}C complete culture medium (RPMI 1640, 15% fetal calf serum, glutamine, 2-mercaptoethanol, penicillin-streptomycin and fungizone) containing hypoxanthine, aminopterin and thymidine[8] to obtain the final concentration of 5×10^{5} spleen cells/ml and 1×10^{5} lymph node cells/ml. The peritoneal feeder cells were added and 1 ml of the cell mixtures were plated per well into 24 well Costar plates (Costar, Massachuset, USA). The plates were incubated in a tissue culture incubator at 37 ^{o}C with 80% humidity and 7% CO_2. Around 10 days after fusion, the cells were switched

to complete culture medium without HAT gradually. Three weeks after fusion, the supernatantfluid from culture wells displaying hybrid growth was initially screened for the desired antibodies by enzyme-linked immunosorbent assay (ELISA) described below.

Hybrid cells producing antibodies of interest were cloned by limiting dilution method. Briefly, the 1-5 µl of agitated cell suspension from well was diluted into 30 ml complete culture medium containing peritoneal feeder cells. The 200 µl of cell suspension was plated per well into one 96-well Costar plate. The remaining 10 ml of cell suspension was further diluted into 30 ml of complete culture medium containing peritoneal feeder cells and plated as above. This cloning procedure was repeated 1-2 times to ensure that each well containing one clone which was from a single cell.

Antibody screening

1. Antibody to protein antigen Antibodies in the supernatant fluid from culture wells containing hybrid growth were screened by ELISA performed in 96-well microtiter Immulon® plates (Dynatech Laboratories, Virginia, USA). The procedure was as described earlier.[9] In brief, 10 µg/ml of protein antigen (in 0.05 M carbonate buffer, pH 9.8) extracted from each of bacterial strains previously mentioned except Campyrobacter jejuni was added to the wells (one antigen each per plate) and incubate at 37°C for 5 hours. After the antigen solution was removed, the plates were washed three times with normal saline solution and 0.05% Tween 20 (NSS-Tween 20) and tapped dry. One hundred microlitres of supernatant fluid was added in duplicate to each well and incubate at 4°C overnight. After 3 washes with NSS-Tween 20, 100 µl of goat anti-mouse immunoglobulins labelled with alkaline phosphatase (Southern Biotechnology Assoc., Alabama, USA) was added and incubated at 37°C for 3 hours. Excess

conjugate was washed out 3 times with NSS-Tween 20. One hundred microlitres of p-nitrophenylphosphate (Sigma Chemical Co., MO, USA) dissolved in diethanolamine buffer (1 mg/ml), pH 9.5, was then added to each well as the substrate. The enzyme-substrate reaction was allowed to take place at room temperature for 60 minutes before being terminated by the addition of 25 μl of 3 M NaOH. The optical density at 405 nm was measured in a Titertek Multiskan® (Flow Laboratories, Bonn, West Germany). An optical density above 0.2 (derived from the mean of the optical density values of 115 negative controls plus three standard deviations.[10] was considered a positive result.

In each plate, a direct conjugate control (DCC), a substrate blank (SB), a negative control and a positive control were included. One hundred microlitres of NSS-Tween 20 was substituted for supernatant fluid in the DCC and for both supernatant fluid and conjugate in the SB. One hundred microlitres of mouse myeloma cells Ag8.653 culture medium and mouse polyclonal antiserum against strain of bacteria corresponding to the bacterial antigen coated in each plate was substituted for supernatant fluid in the negative and positive control well respectively.

II. Antibody to Vi antigen The screening method used in this study for detection of anti-Vi antibody in the supernatant fluid was ELISA which originally described by Barrett et al.[11] Briefly, rabbit anti-S. typhi Ty2 serum was diluted 1:20,000 in 0.06 M carbonate buffer, pH 9.6, and mixed with purified Vi capsular polysaccharide from Citrobacter freundii (kindly provided by Dr. Ed Robbins, National Institute of Health, Maryland, USA) at a final concentration of 3 μg/ml. One hundred microlitres of this mixture was added to alternate rows of the 96-well microtiter Immulon® plates. Rabbit anti-S. typhi Ty2 serum without Vi antigen was added to the other rows of wells.

The plates were incubated for 1 hr at 37°C and at 4°C overnight and then were washed three times with NSS-Tween 20. One hundred microlitres of the supernatant fluid was added to both the Vi and non-Vi containing wells in duplicate. The plates were incubated for 1 hr at 37°C and then washed three times in NSS-Tween 20.

Alkaline phosphatase-conjugated goat anti-mouse immunoglobulins was diluted 1:250 in NSS-Tween 20 with 1% bovine serum albumin, one hundred microtitres of this conjugate was added to each well. After incubation for 1 hr at 37°C, the plates were washed three times with NSS-Tween 20. Enzyme substrate (p-nitrophenylphosphate in diethanolamine buffer, pH 9.5, 1 mg/ml) was added and the plates were incubated for 60 minutes at room temperature, the enzymatic reaction was then terminated by adding 0.025 ml of 3 N NaOH to each well. The absorbance of the colour developed was read at 405 nm with a Titertek Multiskan®.

A direct conjugate control, a substrate blank, a negative control and a positive control which was polyclonal mouse anti-Vi antibodies were also included in each plate.

Isotype assay. Immunoglobulin class, subclass and light chain was determined by ELISA as mentioned above. The goat antibodies to mouse IgM, IgA, IgG2a, IgG2b, IgG3, kappa and lambda light chains (Southern Biotechnology Assoc., Alabama, USA) were substituted for goat anti-mouse immunoglobulins in the assay.

Antibody production. To obtain high yields of antibodies, the hybridomas were grown as ascitis tumours in pristane (2, 6, 10, 14-tetramethylpentadecane, Aldrich Chemical Co., WI, USA) -primed BALB/cJ mice. The 0.5 ml pristane was injected into mice intraperitoneally. At 1 week after the pristane injection, the mice were given 2×10^7 hybrid cells in 3 ml of NSS intraperitoneally.

RESULTS

Production of hybridomas Ten hybridomas producing specific antibodies against protein or Vi antigen of S. typhi were established. These clones were isolated from 2 fusion experiments. They are designated as XIII:1D6C8, XIII:2D1E6, XIV:8A2E1, XIV:8A2E6, XIV:8A2E10, XIV:8A2F10, XIV:8A2F12, XIV:1D6E5, XIV:1D6E9 and XIV:1D6F7.

Immunoglobulin isotype The results of immunoglobulin isotypic assay are presented in Table 1.

Specificity of the monoclonal antibodies The specificity of the monoclonal antibodies for protein or Vi antigen of S. typhi was investigated by ELISA by using a panel of protein extracted from 12 bacterial strains (S. typhi, S. paratyphi A, S. paratyphi B, S. paratyphi C, S. typhimurium, S. choleraesuis, S. enteritidis, S. panama, S. stanley, E. coli, Y. enterocolitica, Ps. pseudomallei), the acetone dried whole cell of C. jejuni and purified Vi antigen. The results are shown in Table 2. One monoclonal antibodies (XIII:1D6C8) exhibited reactivity to Vi antigen and S. typhi while another five monoclonal antibodies (XIV:8A2E1, XIV:8A2E6, XIV:8A2E10, XIV:8A2F10, XIV:8A2F12) were reactive to Vi antigen, S. typhi and S. paratyphi C. The last four monoclonal antibodies (XIII:2D1E6, XIV:1D6E5, XIV:1D6E9, XIV:1D6F7) showed strongly reaction to S. typhi only.

The immunoblotting (Western Blot) and ELISA-inhibition experiments to demonstrate further the specificity of the monoclonal antibodies are currently being performed. The results are not yet completed.

Antibody production

All pristane primed mice which were injected with our hybrid cells are currently being observed daily for ascites development.

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

Clone number	Class or subclass	Light chain	Specificity	Source of immune cells
XIII:1D6C8	IgG3	κ	Vi protein	lymph node
XIII:2D1E6	IgG2a	κ		
XIV :8A2E1	IgG1	κ	Vi	Spleen
XIV :8A2E5				
XIV :8A2E10				
XIV :8A2F10				
XIV :8A2F12				
XIV :1D6E5	IgG1	κ	protein	
XIV :1D6E9				
XIV :1D6F7				

Table 2. Reactivity of monoclonal antibodies to a panel of protein extracted from 12 bacterial strains, acetone dried whole cells of *Campylobacter jejuni* and purified Vi antigen.

Clone Number	S. typhi	S. paratyphi A	S. paratyphi B	S. paratyphi C	[illegible]	[illegible]	S. enteritidis	S. panama	S. kralefeld	E. coli	Y. enterocolitica	Ps. pseudomallei	C. jejuni	Vi antigen
XIII:10b6B	0.74	0.13	0.12	0.17	[illegible]	[illegible]	0.12	0.11	0.13	0.12	0.13	0.12	0.14	1.12
XIII:20H6	1.13	0.15	0.11	0.15	[illegible]	[illegible]	0.12	0.11	0.12	0.12	0.15	0.14	0.17	0.13
XIV :8A2G1	1.15	0.17	0.17	0.26	[illegible]	[illegible]	0.19	0.16	0.17	0.19	0.12	0.13	0.16	1.89
XIV :8A2H6	0.89	0.14	0.13	0.2[illegible]	[illegible]	[illegible]	0.14	0.13	0.15	0.15	0.14	0.14	0.16	1.96
XIV :8A2E10	0.74	0.16	0.17	0.33	[illegible]	[illegible]	0.20	0.20	0.16	0.19	0.14	0.15	0.15	1.86
XIV :8A2F10	0.59	0.14	0.20	0.31	[illegible]	[illegible]	0.22	0.19	0.18	0.21	0.12	0.14	0.13	1.92
XIV :8A2F12	0.64	0.13	0.16	0.26	[illegible]	[illegible]	0.16	0.16	0.15	0.16	0.12	0.12	0.14	1.93
XIV :10bE5	1.54	0.14	0.12	0.16	[illegible]	[illegible]	0.13	0.12	0.13	0.12	0.13	0.14	0.13	0.12
XIV :10bE9	1.58	0.12	0.11	0.13	[illegible]	[illegible]	0.11	0.11	0.12	0.13	0.12	0.13	0.13	0.13
XIV :10bF7	1.61	0.16	0.13	0.17	[illegible]	[illegible]	0.13	0.13	0.13	0.12	0.13	0.13	0.15	0.17

Absorbance at 405 nm.

DISCUSSION

Ten monoclonal antibodies specific to S.typhi established in this study can be divided into 4 groups on the basis of isotypic characterization and the specificity. The first group (XIII:1D6C8) has isotype IgG3k with Vi specificity. The second group consisted of 5 monoclonal antibodies (XIV:8A2E1, XIV:8A2E6, XIV:8A2E10, XIV:8A2F10, XIV:8A2F12), all have IgG1k with Vi specificity. These 2 groups react differently with S.paratyphi C as shown in Table 2. The first group has no specificity with S. paratyphi C while the second group can react strongly with S. typhi and weakly with S. paratyphi C. This result suggests that the Vi polysaccharide remains in the protein antigen preparation of S. typhi and S. paratyphi C and these 2 groups of mAb are specific to different epitope on the Vi antigen, therefore S. typhi and S. paratyphi C should have some difference in the components of the antigenic structure of Vi capsular polysaccharide.

The third group (XIII:2D1E6) and the fourth group (XIV: 1D6E5, XIV:1D6E9, XIV:1D6F7) are both specific to protein antigen of S. typhi but have different isotype, i.e., IgG2ak for the third group and IgG1k for the fourth group. The specificity of these monoclonal antibodies for the same or different epitopes on the protein antigen are currently being investigated by the immunoblotting technique.

CONCLUSION

A hybridoma technology has been established and 10 monoclonal antibodies specific to *S. typhi* were obtained. They can be divided into 4 groups, in which 2 groups are specific to Vi antigen and other 2 groups are specific to protein antigen of *S. typhi*. Such antibodies will be of great value for studies of 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.

OBJECTIVE II : To recruit the subjects and collect the blood and urine specimens for the clinical diagnostic trial of the ELISA test kits.

This is currently being performed. At the present, the total number of the specimens is still not sufficient. Therefore, we have extended this activity further to the end of this year and hope that the sufficient number of the specimens (patients and controls) will be obtained.

OBJECTIVE III : To identify molecular components of the antigenic structure of *S. typhi* which are specific to established mAb.

The immunoblotting experiment to identify the antigenic component(s) of *S. typhi* is now underinvestigation. The result is not obtainable yet.

WORKPLANS FOR THE NEXT PERIOD

I. Development and production of monoclonal antibodies to *Salmonella paratyphi A*, *Selmonella paratyphi B* and *Salmonella paratyphi C*.

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

III. Establishment of ELISA test kits if the desired monoclonal antibodies are achieved.

IV. Identification of molecular components of the antigenic structure of *S. typhi* (continued), *S. paratyphi A*, *S. paratyphi B* and *S. paratyphi C*.

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PROGRESS REPORT NO. 3

PRODUCTION OF MONOCLONAL ANTIBODIES TO SALMONELLAE THAT CAUSE ENTERIC FEVER

A RESEARCH PROJECT

USAID/PSTC PROGRAM

GRANT NO. : 936-5542-G-00-5059-00

SUBMITTED BY

DR. SUTTIPANT SARASOMBATH, M.D., F.C.A.P

DEPARTMENT OF MICROBIOLOGY

FACULTY OF MEDICINE SIRIRAJ HOSPITAL

MAHIDOL UNIVERSITY, BANGKOK 10700

THAILAND

Rec'd in SCI: FEB 19 1987

PROJECT PROFILE

Country : Thailand

Grant No. : 936-5542-G-00-5059-00

Program : Program on Science and Technology Cooperation

Project Title : Production of monoclonal antibodies to Salmonellae that cause enteric fever

Project Leader: Dr. Suttipant Sarasombath, M.D., F.C.A.P.

Organization : Mahidol University, Thailand

Co-investigators : 1. Dr. Napatawn Banchuin, M.D., Ph.D.
2. Mrs. Tassanee Sukosol, M.Sc.

Project Consultants: 1. Dr.Pornchai Matangkasombut,M.D.,Ph.D.
2. Dr. J.F. Kearney, D.D.S., Ph.D.

Authorized Officer : Dr. Natth Bhamarapravati, M.D., Ph.D.
Rector, Mahidol University

Total Project Budget: US$ 147,740.00 (B 3,988,980.00)

Project Duration : 3 years

Reporting Period : July 1, 1986 - December 31, 1986

Budget Allocation for This Period : US$ 17,488.00
฿ 472,169.00

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 difficulties 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.

Kanjanahareutai 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*.

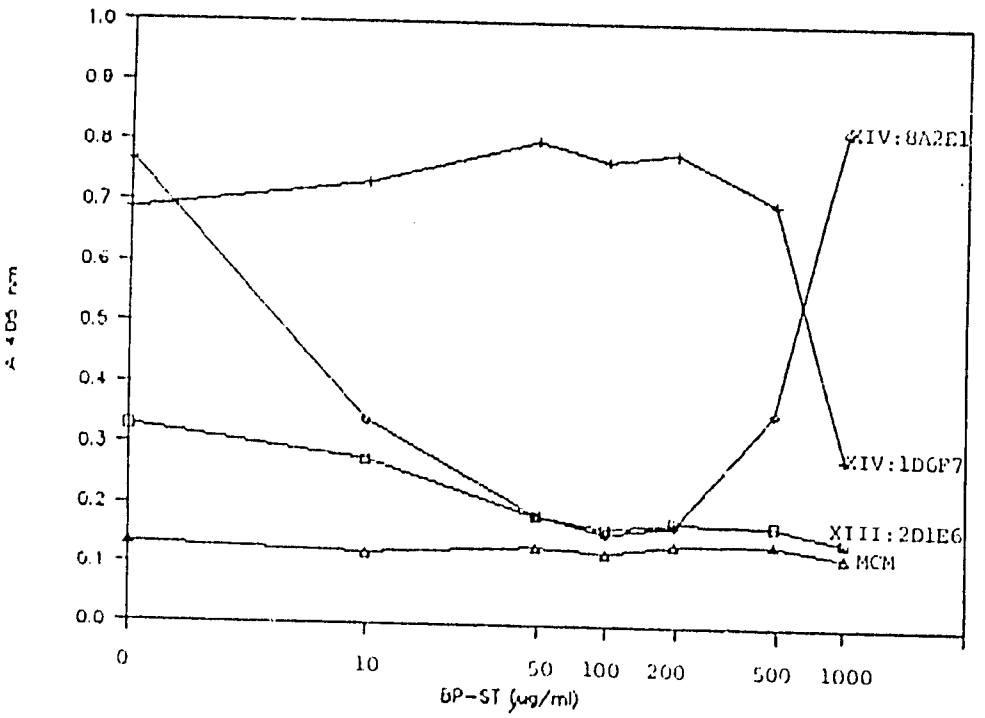

Figure 1: ELISA-inhibition of the three selected mAbs from culture supernatants of clone XIII:2D1E6, XIV:1D6F7 and XIV:8A2E1.
MCM = Myeloma culture medium

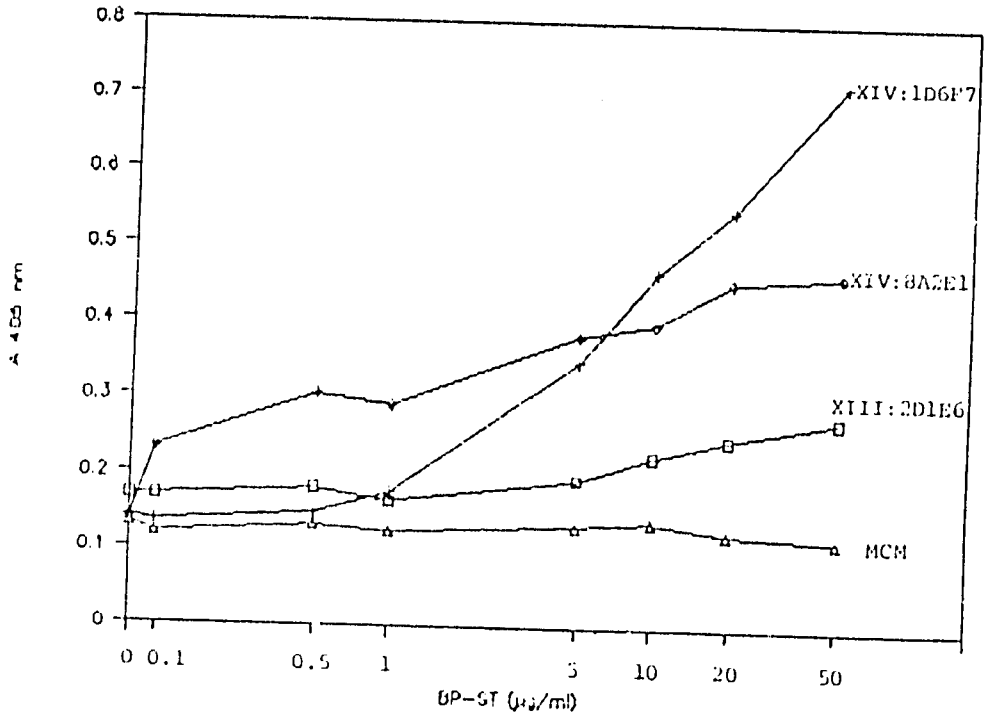

Figure 2: Double sandwich ELISA of the three selected mAbs for detection of BP-ST.
MCM = Myeloma culture medium

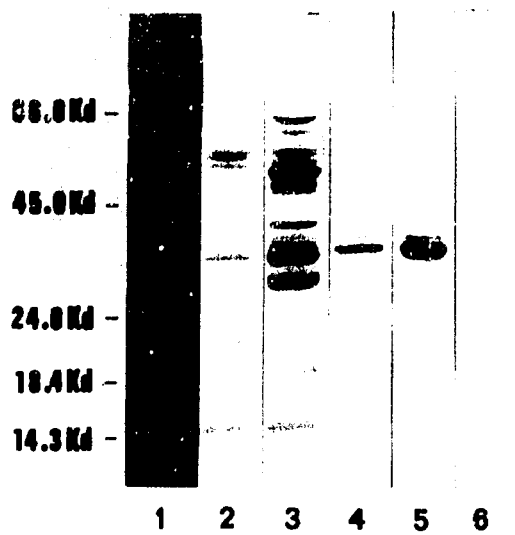

Figure 3 : Western blotting of protein antigen of S.typhi (50 μg/lane) stained or reacted with

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.

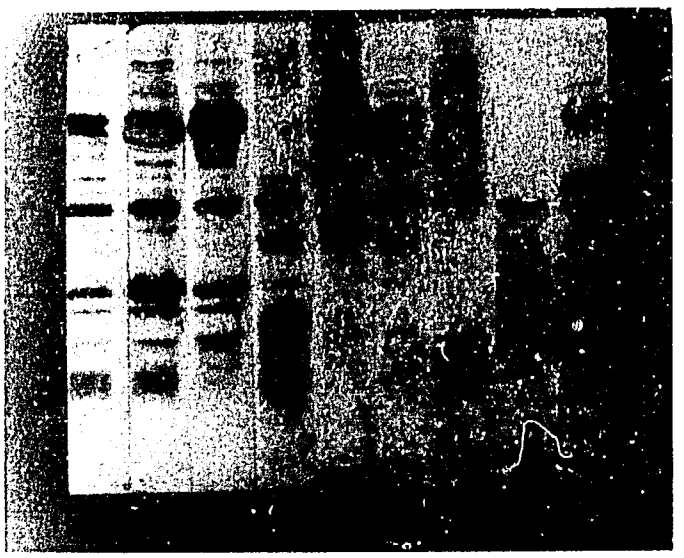

Figure 4 : Western blotting and immunoenzyme staining patterns of protein antigens of

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.

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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.

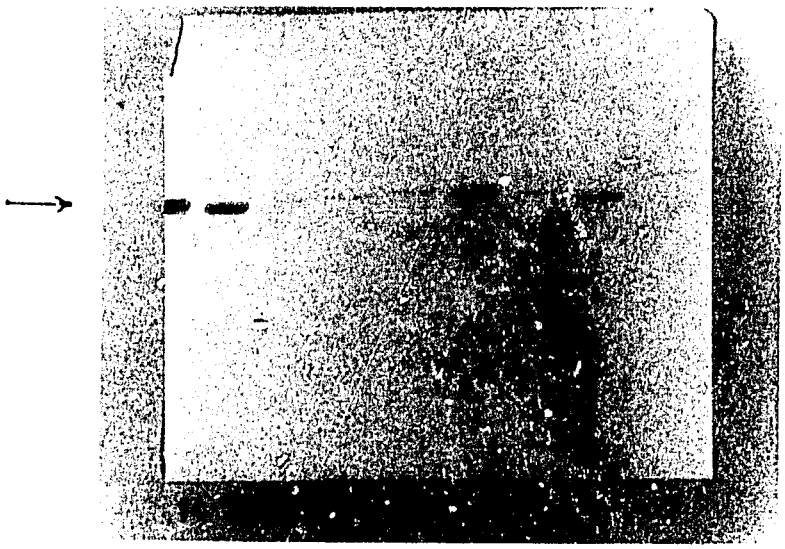

Figure 6 : Western blotting and immunoenzyme staining patterns of the same antigens as Fig. 4 reacted with mAb XIII:2D1E6 (1:200). Arrow indicates protein molecular weight of 34.0 Kd.

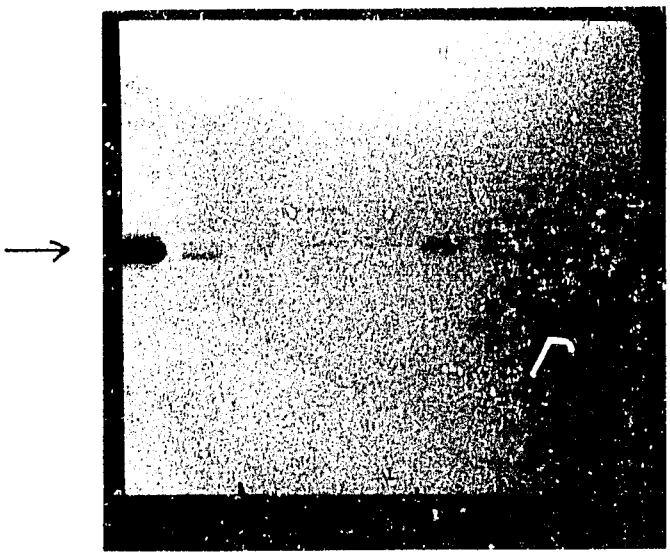

Figure 7 : Western blotting and immunoenzyme staining patterns of the same antigens as Fig. 4 reacted with mAb XIV:1D6F7 (1:400). Arrow indicates protein molecular weight of 34.0 Kd.

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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 405 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 1. 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.

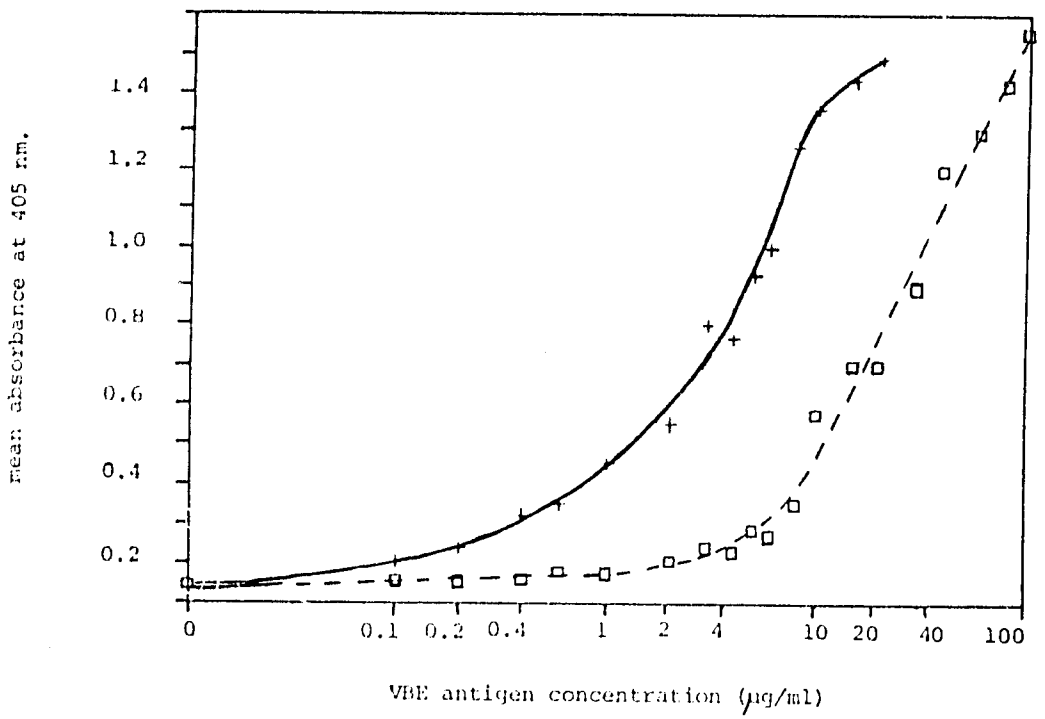

Figure 1 Dose response curves obtained from the assay of VBE antigen at various concentrations, with ELISA using either rabbit anti-VBE Ig as the immobilized first antibody and mAb XIV:8A2E1 Ig as the second antibody (□ - - □ - - □) or vice versa (+ —— + —— +).

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.

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<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.).

PN-ABA-540-B

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON D.C. 20523

DATE: 7/29/88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, Victoria Ose

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 5. 227
PR #5

Attachment

PN-AAB-593-B 5.227

PROGRESS REPORT NO. 5

PRODUCTION OF MONOCLONAL ANTIBODIES TO SALMONELLAE THAT CAUSE ENTERIC FEVER

A RESEARCH PROJECT

USAID/PSTC PROGRAM

GRANT NO. : 936-5542-G-00-5059-00

SUBMITTED BY

DR. SUTTIPANT SARASOMBATH, M.D., F.C.A.P.

DEPARTMENT OF MICROBIOLOGY

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THAILAND

PROJ. NO. 200 JUL 2 2 1988

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PROJECT PROFILE

Country : Thailand

Grant No. : 936-5542-G-00-5059-00

Program : Program on Science and Technology Cooperation

Project Title : Production of monoclonal antibodies to Salmonellae that cause enteric fever

Project Leader: Dr. Suttipant Sarasombath, M.D., F.C.A.P.

Organization : Mahidol University, Thailand

Co-investigators : 1. Dr. Napatawn Banchuin, M.D., Ph.D.
2. Mrs. Tassanee Sukosol, M.Sc.

Project Consultants: 1. Dr.Pornchai Matangkasombut,M.D.,Ph.D.
2. Dr. J.F. Kearney, D.D.S., Ph.D.

Authorized Officer : Dr. Natth Bhamarapravati, M.D., Ph.D.
Rector, Mahidol University

Total Project Budget: US$ 147,740.00 (฿ 3,988,980.00)

Project Duration : 3 years

Reporting Period : July 1, 1987 - December 31, 1987

Budget Allocation for This Period : US$ 16,662
฿ 416,566

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 difficulties 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 defined.

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 even in rural areas of LDC where scientific equipments are not readily available.

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

OBJECTIVES OF THIS PERIOD

I. Development and production of monoclonal antibodies to *S.paratyphi C*.

II. Identification of molecular components of *S.paratyphi C* which is specific to the established mAbs.

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.).

OBJECTIVE I : To develop and produce monoclonal antibodies to *S.paratyphi C*.

OBJECTIVE II: To identify molecular components of *S.paratyphi C* which are specific to established mAbs.

MATERIALS AND METHODS

Bacteria

All bacteria used in this study can cause enteric fever and enteric fever like illness. They were as follows, *S.typhi*, *S.paratyphi A*, *S.paratyphi B*, *S.paratyphi C*, *S. cholerasuis*, *S.enteritidis*, *S.krefeld*, *S.panama*, *S. typhimurium*, *Escherichia coli*, *Pseudomonas pseudomallei* and *Yersinia enterocolitica*.

Preparation of protein antigens

The protein (Bp) antigen from all bacteria except *C.jejuni* were prepared by the method originally described by Barber (5). Briefly, washed and acetone dried bacterial cells were extracted with veronal buffer pH 8.4 and the protein was precipitated out of the veronal buffer extract (VBE) by trichloroacetic acid. The precipitate was washed, resolubilized, lyophilized and stored at 4 °C until used. The protein content was assayed by the method described by Lowry and as modified by Hartree (6). This antigen had small amount of lipopolysaccharide contamination as confirmed by double immunodiffusion.

The *C.jejuni* was used as acetone dried cells antigen in this experiment.

Production of monoclonal antibodies

Female BALB/cJ mice 6 to 10 weeks of age [kindly provided by Division of Veterinary Medicine, Armed Force Research Institute of Medical Science (AFRIMS), Bangkok] were immunized with Bp antigen from *S.paratyphi C* once every 3 days for 15 days subcutaneously into 2 footpads, 2 axillary and 2 inguinal areas and also intravenously (7).

One day after the last administration of antigen, mice were sacrified by cervical dislocation. The axillary and inguinal lymph nodes and one spleen were aseptically removed for hybridization with P3x63-Ag8.653 mouse myeloma cells by using 42% polyethylene glycol 4000 (Accurate Chemical and Scientific Co., Westbury, NY.). The fused cells were dispended into 24 well tissue culture plates (Costar, Cambridge, Mass.) in the presence of syngeneic mouse peritoneal feeder cells and cultured at 37°C with 80% humidity and 7% CO_2. Hybridomas secreting specific antibodies were cloned by single dilution method in the presence of syngeneic mouse peritoneal feeder cells. About 7 days after cloning, the hybridoma could be seen by microscopy and each well was scored for monoclone.

Indirect ELISA for mAb screening and specificity testing

Hybridoma secreting specific antibodies against antigens of *S.paratyphi C* were detected by indirect enzyme-linked immunosorbent assay (ELISA) as described by Banchuin et al (8) using panel protein antigens of *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 dried whole cell of *C.jejuni*.

Determination of mAb isotypes

Culture supernatants of hybridomas secreting specific antibodies were characterized for their isotypes by indirect ELISA. The procedure was the same as in mAb screening and specificity testing except alkaline phosphatase-conjugated goat anti-mouse IgM, IgG1, IgG2a, IgG2b, IgG3, K and λ (Southern Biotechnology, Birmingham, Ala) were used instead of the anti-mouse polyvalent Ig.

Immunoblot technique for mAb specificity testing and identification of molecular component of *S.paratyphi C*

The protein antigens of 12 bacteria were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the method originally described by Laemmii (9), in LKB 2001 vertical electrophoresis unit (LKB-Produkter AB, Bromma, Sweden). After electrophoresis, the proteins were transferred to nitrocellulose by a Transblot apparatus with 15°C cooling system (LKB-Produkter AB, Bromma, Sweden) at 90 V overnight. The nitrocellulose membranes were washed with 1% (vol/vol) Tween 20-PBS (pH 7.2) at 37°C for 30 minutes and unoccupied binding site was blocked by incubation in 3% (w/v) bovine serum albumin in Tween-PBS at room temperature for 60 minutes on a rotated platform. MAbs diluted in Tween-PBS were added to the membranes,and they were incubated at room temperature for 2 hours. After the membranes were washed with Tween-PBS, alkaline phosphatase-conjugated goat anti-mouse polyvalent Ig (Dakopatts, Glostrup, Denmark) was added. The incubation was performed for 60 minutes at room temperature. Red purple color development was accomplished by incubating washed membranes in the mixture of 4 mg β-naphthyl phosphate (Sigma Chemical Co., St. Louis, MO) and 60 mg O-dianisidine tetrazotized (Sigma Chemical Co., St. Louis, MO) (10,11) for 5 minutes, then the filter was rinsed with tap water to stop the reaction.

RESULTS AND DISCUSSION

By indirect ELISA using a panel of 12 Bp antigens, dried cell of *C.jejuni*, LPS antigen and Vi polysaccharide antigen from *Citrobacter frundii*, 5 clones of hybridoma were selected for further investigation (Table 1). Their originality (lymph node or spleen) isotypes and specificity (protein or Vi antigen) are shown in Table 2. Four clones reacted with protein antigen of *S.paratyphi C* and *S.cholerasuis*, and one clone reacted with Vi polysaccharide of *S.typhi* and *S.paratyphi C*.

However, the result of immunoblotting using the four mAbs (clone 9B3D11, 4C4B5, 16A1G12, 16C4F11) which were specific to protein antigen revealed that these 4 mAbs had cross reaction with protein antigen of other bacteria in the panel besides *S.paratyphi C* and *S.cholerasuis*. Hence, the 4 selected mAbs would not be useful for further investigation and another fusion should be tried for the hope that the clone(s) specific to protein antigen of *S.paratyphi C* will be obtained.

The mAb specific to common Vi polysaccharide antigen of *S.typhi* and *S.paratyphi C* (clone 5C3C9) is being tested by modified double antibody sandwich ELISA for its ability to detect Vi antigen.

In vivo large scale production of mAbs

Five hybrid clones from the previous reports namely STP13 (specific to protein of *S.typhi*), STP14 (specific to protein of *S.typhi*), STVi14 (specific to Vi polysaccharide), SPA20 (specific to protein of *S.paratyphi A*) and SPB20 (specific to protein of *S.paratyphi B*) were injected intraperitoneally to BALB/cJ mice. The resulting ascitic fluid

was analysed by indirect ELISA for their specificity before further purification. The procedure used was the same as indirect ELISA for mAbs screening described above. The result is shown in Table 3. Sera of every healthy BALB/c mice already had high level of natural antibody to enterobacteriaceae including bacteria which caused enteric fever liked illness. These polyclonal antibodies could diffuse into peritoneal cavity where ascitic fluid was induced. Hence, contamination of mAb in the ascitic fluid with polyclonal antibodies from systemic circulation could not be avoided. We conclude that large scale production of mAb specific to any enterobacteriaceae can not be performed in vivo system unless the germ free mice are used. Since the germ free mice are too expensive and are not presently available in Thailand, the only way to produce large amount of mAb is to use the tissue culture system which provides very small amount of antibody. However, this work is now in progress in our laboratory.

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Clone	*S. typhi*	*S. paratyphi A*	*S. paratyphi B*	*S. paratyphi C*	*S. choleraesuis*	*S. enteritidis*	*S. kralefeld*	*S. panama*	*S. typhimurium*	*E. coli*	*Ps. pseudomallei*	*Y. enterocolitica*	*C. jejuni*	*Vi antigen*	*LPS*
9B3D11	0.134	0.144	0.133	>2.0	0.331	0.134	0.117	0.128	0.108	0.121	0.123	0.169	0.147	0.153	-
4C4B5	0.134	0.138	0.140	1.605	0.806	0.130	0.121	0.130	0.122	0.147	0.138	0.129	0.184	0.141	-
16A1G12	0.134	0.135	0.127	1.410	0.238	0.148	0.111	0.125	0.151	0.141	0.122	0.144	0.162	0.158	-
16C4F11	0.126	0.127	0.131	1.454	0.630	0.134	0.132	0.127	0.109	0.134	0.136	0.138	0.154	0.145	-
5C3C9	1.391	0.134	0.137	0.704	0.147	0.125	0.132	0.133	0.119	0.146	0.126	0.130	0.166	1.695	0.122

Table 1. Optical density (OD) value of monoclonal antibodies from culture supernatant against panel antigens. The cut off OD value was 0.207.

Clone	Isotype	Specificity	Source of immune cells
9B3D11	IgMK	protein	spleen
4C4B5	IgG3K	protein	spleen
16A1G12	IgG1K	protein	spleen
16C4F11	IgMK	protein	spleen
5C3C9	IgMK	Vi	spleen

Table 2. Characteristic of established monoclonal antibodies

Clone	S. typhi	S. paratyphi A	S. typhimurium	S. paratyphi C	E. coli	Ps. pseudomallei
STP13	1.529	0.803	1.548	≥2.0	≥2.0	0.802
STP14	0.586	0.287	0.312	0.554	0.836	1.213
STVi14	0.304	0.168	0.212	0.379	0.604	0.935
SPA20	0.825	1.845	0.611	0.899	0.960	1.087
SPB20	0.254	0.474	1.359	0.484	0.842	0.776
$\bar{X}$ sera control (30 mice)	0.805	0.679	0.642	1.045	≥1.451	≥1.481

Table 3. Optical density (OD) value of ascitic fluid against panel protein antigens. Mean OD of sera obtained from 30 healthy mice without hybrid cells injection are also shown. The cut off OD value was 0.207.

OBJECTIVE III: To recruit the subjects and collect the blood and urine specimens for the clinical diagnostic trial of the ELISA test kits (cont.)

Since the incidence of enteric fever in Bangkok is much reduce at present, we are now hardly obtain the sera and urine specimens from such patients. However, we still perform this activity and hope that we should obtain sufficient number of the specimens within the next few months.

OBJECTIVE IV: To establish an ELISA test kit for the diagnosis of enteric fever.

We have developed the modified double antibody sanwich ELISA as described in the previous progress report for using in clinical trials of established monoclonal antibodies with the clinical specimens. The clinical diagnostic trials using mAbs from culture supernatant should be completed in the next reporting peroid.

WORKPLANS FOR THE NEXT PERIOD

I. Development and production of monoclonal antibodies to *S.paratyphi C*.

II. Identification of molecular components of *S.paratyphi C* which is specific to the established mAbs.

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.).