FINAL REPORT

"PRODUCTION OF MONOCLONAL ANTIBODIES TO SALMONELLAE THAT CAUSE ENTERIC FEVER"

BY

DR. SUTTIPANT SARASOMBATH

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"PRODUCTION OF MONOCLONAL ANTIBODIES TO SALMONELLAE THAT CAUSE ENTERIC FEVER"

Principal Investigator: Dr. Suttipant Sarasombath, M.D., F.C.A.P.

Grantee Institution: Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Collaborators: 1. Dr. Napatawn Banchuin, M.D, Ph.D.
      Both are at Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
   3. Dr. Stitaya Sirisinha, D.D.S., Ph.D.
   4. Dr. Skorn Mongkolsuk, Ph.D.
      Both are at Faculty of Science, Mahidol University, Bangkok, Thailand

Consultants: 1. Dr. Pornchai Matangkasombut, M.D., Ph.D.
               Faculty of Science, Mahidol University, Bangkok, Thailand
   2. Dr. J.F. Kearney, D.D.S., Ph.D.
      University of Alabama at Birmingham, B'ham, Alabama, U.S.A.

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EXECUTIVE SUMMARY

The purpose of this project is to produce mouse monoclonal antibodies (MAbs) directed to species specific antigens of 4 Salmonellae that cause enteric fever, they are *Salmonella typhi*, *S.paratyphi A*, *S.paratyphi B* and *S.paratyphi C*. These MAbs will be used to develop immunological test(s) for diagnosis of enteric fever by detecting corresponding Salmonella antigens in clinical specimens (urine and serum) of the patients. Furthermore, these MAbs will be used for identification of molecular components of their specific Salmonellae.

In this study, 2 types of immunogen from the 4 Salmonellae were used in immunization of BALB/c J mice for hybridoma production. They were "crude" protein antigen (Bp) and "affinity purified" Bp. The latter was more purified and specific to the 4 Salmonellae than the former. Several fusions were performed and several MAbs specific to these Salmonellae were selected. When using "crude" Bp as immunogen, fewer clones, less stable and less specific MAbs to the 4 Salmonellae were obtained than using "affinity purified" Bp. Unfortunately, all hybridomas from the first few fusions which were the products of "crude" Bp immunogen were lost due to Mycoplasma contamination. However, several hybridomas producing highly specific MAbs to the 4 Salmonellae which were the products of "affinity purified" Bp immunogen still exist. They were 10 clones for *S.typhi*, 73 clones for *S.paratyphi A*, 10 clones for *S.paratyphi B* and 18 clones for *S.paratyphi C*. All MAbs did not cross-react with the antigen of other Enterobacteriaceae in the indirect ELISA.
and immunoblot analyses and were species-specific, only MAbs specific to *S. paratyphi C* had cross-reactivity with *S. choleraesuis*.

MAbs to *S. typhi*, *S. paratyphi A* and *S. paratyphi B* were specific to a 52 Kd protein component of their corresponding bacteria, while MAbs to *S. paratyphi C* were specific to a 61 Kd protein of this bacterium and a 59 Kd protein of *S. choleraesuis*.

Using a modified double antibody sandwich ELISA, these MAbs could detect a minute amount of their corresponding protein antigens, but they could not detect such a protein in the clinical specimens of patients. When the acute sera of the patients with typhoid, paratyphoid A and paratyphoid B infections were reacted with crude Bp antigens prepared from *S. typhi*, *S. paratyphi A* and *S. paratyphi B* respectively in the immunoblot, specific IgM to the 52 Kd band were demonstrated in all patients (the sera from *S. paratyphi C* infected patients were unobtainable due to unavailability of these patients). Thus, the specific protein antigen targets of these 4 bacteria are very interesting for immunodiagnosis of enteric fever. These findings encouraged us to set up a preliminary study combining recombinant DNA technology and MAbs. We have successfully established recombinant *E. coli* clones expressing specific genes and producing specific protein antigens of *S. typhi*. Upon testing these recombinant *E. coli* with the acute sera of 4 patients infected with *S. typhi*, 6 patients with hemoculture positive for other bacteria and 6 normal controls, IgM antibody to the specific protein band was demonstrated only in typhoid patients.
The specific protein antigen of *S.typhi*, *S.paratyphi* A, *S.paratyphi* B and *S.paratyphi* C should be of great value not only in immunodiagnosis of enteric fever, but also in epidemiological surveillance of this disease as well as in *Salmonella* taxonomy.

As the result of this project, we have developed a small number of scientists who have acquired expertise in hybridoma technology and immunoblotting techniques, and a well equipped hybridoma laboratory. One scientist also has had a chance to learn recombinant DNA technology. This project has strengthened not only our research capability but also research of the country as well, since many scientists from the other places in the country have been trained in hybridoma technology with us.
RESEARCH OBJECTIVES

Enteric fever (typhoid and paratyphoid fever) is caused by 4 species of Salmonellae, namely *Salmonella typhi*, *S. paratyphi A*, *S. paratyphi B* and *S. paratyphi C*. One of the diagnostic problems of enteric fever is to differentiate it from enteric fever-like illness caused by other Enterobacteriaceae. The most dependable way to establish a definitive diagnosis of this disease is by hemoculture. However, the laboratory diagnosis by hemoculture suffers from certain disadvantages. Many cultures are falsely negative due to prior antibiotic therapy. Even when appropriate hemocultures are taken, the presumptive bacteriological results require at least 48 hours or longer for confirmative results.

Serological diagnosis of enteric fever by the Widal test has been found by some investigators to be unreliable (1,7) and the confirmative-diagnosis of four-fold rising antibody titer requires both acute and convalescent sera which are rarely collected (7). Hence, the antibodies in a single serum specimen are not very useful for serodiagnosis, since they can not distinguish current from previous infection due to the non-specificity of antigen used in the test.

Therefore, there is a need for a test that will permit rapid laboratory diagnosis of enteric fever by a single specimen. That test should aim to detect specific Salmonella antigens in the biological fluids of the patient. Many investigators have established such tests by using conventional polyclonal antisera in their tests but they have
met the problems of cross reaction with other members of family Enterobacteriaceae (2, 5, 6, 10, 12). Attempts to reduce the cross reactivities by absorption have not been successful in eliminating all of the cross reactive antibodies (6). For overcoming this problem, the MAbs which are specific to antigens of the above-mentioned Salmonellae are required so that immunological tests for diagnosis of enteric fever can be established. Furthermore, the molecular components of the antigenic repertoire of these four species of Salmonellae which are specific to the corresponding established MAbs can also be defined.
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. choleraesuis*, *S. enteritidis*, *S. krefeld*, *S. panama*, *S. typhimurium*, *Escherichia coli*, *Pseudomonas pseudomallei*, and *Yersinia enterocolitica*.

Preparation of antigens: For ELISA and immunoblot experiments, the "crude" protein antigen was used. The preparation of this antigen was as previously described (3, 11).

In immunization of BALB/c J mice for production of monoclonal antibodies, the "crude" Bp (3, 11) and the "affinity purified" Bp were used (4). The later antigen was prepared by passing crude Bp of *S. typhi* or *S. paratyphi A* or *S. paratyphi B* or *S. paratyphi C* through an affinity column made from sepharose conjugated to IgG specific for partially purified Bp antigen of these 4 bacteria.

Production and characterization of MAbs: Female BALB/c J mice 6-8 weeks of age (kindly provided by the Division of Veterinary Medicine, Armed Force Research Institute of Medical Science, Bangkok, Thailand) were immunized with "affinity purified" Bp antigen of *S. typhi* or *S. paratyphi A* or *S. paratyphi B* or *S. paratyphi C*. Their spleen cells were taken for hybridization and production of MAbs.

The specificity of MAbs was monitored by indirect ELISA (1) using a panel of crude Bp antigens prepared from *S.*
typhi, S. paratyphi A, S. paratyphi B, S. paratyphi C, S. choleraesuis, S. enteritidis, S. krefeld, S. panama, S. typhimurium, E. coli, Ps. pseudomallei and Y. enterocolitica. The hybrids which produced antibody specific only to S. typhi or S. paratyphi A or S. paratyphi B, or S. paratyphi C, were identified (we couldn’t find hybrids which recognised the common epitopes among these 4 Salmonellae) and cloned 3 times by limiting dilution and were then expanded in 250 ml tissue culture flasks for bulk production. The isotypes of the MAbs were determined by indirect ELISA.

**Immunoblot techniques**: The immunoblot techniques using crude Bp of 12 enteric bacteria separated by SDS-PAGE were performed essentially as described in the previous report (11). However, in some experiments, crude bacterial homogenate (WC antigen) freshly prepared by mixing a loopful of viable bacteria grown on nutrient agar plate with 300 µl of sample buffer containing 0.0625 M Tris-HCl (pH 6.8), 1% SDS, 10% glycerol, 5% 2-mercaptoethanol, bromphenol blue, and heated for 2.5 min in boiling water was also used in order to rule out any possible proteolytic degradation that might have occurred during the preparation of crude Bp.

**Molecular weight determination**: The molecular weights of protein bands of S. typhi, S. paratyphi A, S. paratyphi B and S. paratyphi C specific to MAbs on nitrocellulose filter membrane were determined by comparing their electrophoretic mobility with known protein markers as described by Weber and Osborn (13).
Modified double antibody sandwich ELISA for detecting specific Salmonellae antigens: All monoclonal antibodies in the culture supernatant were precipitated by the addition of saturated (NH₄)₂SO₄ to a final concentration of 50%. The precipitate was dialyzed against PBS, pH 7.2 for 48 Hrs with 5 changes of buffer and then stored at -20°C until used in ELISA.

The purified MAbs were diluted in 0.05 M carbonate buffer, pH 9.8, containing 0.1% sodium azide, to obtain the optimum protein concentrations. One hundred microlitres of the solution were added into each well of Microelisa Immulon plates (Dynatech Laboratories, Inc., Alexandria, VA), 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 Bp antigen of S.typhi or S.paratyphi A or S.paratyphi B at various concentrations in PBST or the patients' urine or sera samples were 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 the second antibody which was rabbit anti-veronal buffer extract of the corresponding bacteria (anti-VBE) Ig diluted in PBST was added to each well. The plates were then incubated at 30°C for 2 hours. After washing, 100 μl of goat anti-rabbit Ig-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) diluted 1:2000 in PBST containing 1% BSA was added to each well and incubated at 4°C for overnight. The excess conjugate was washed out 3 times with PBST and 100 μl of substrate solution (p-nitrophenyl phosphate, Sigma chemical Co., St. Louis, MO) concentration 1 mg/ml 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 (Titretek Multiskan, Flow Laboratory, GmbH, Bonn, West Germany). In each assay, negative control, conjugate control and substrate blank were also included. One hundred microlitres of PBST were substituted for the antigen in the negative control, for both the antigen and rabbit anti-VBE Ig in the conjugate control and also for conjugate in the substrate blank.

Molecular cloning of antigen gene from *S.typhi*: We have cloned and expressed the *S.typhi* antigen gene in *E.coli* (9). Briefly, total chromosomal DNA was isolated from culture of *S.typhi* using the SDS/proteinase K method. Approximately 10 μg of high molecular weight chromosomal DNA was partially digested with Sau 3A under the conditions that most of the DNA fragments were in the range of 1-7 kb. The DNA fragments were separated in 1% agarose gel and the fragments ranging from 0.7-8 kb purified from the gel by the electroelution method. The Bam HI digested-dephosphorylated pUC19 was used as expression vector. The insert and vector DNAs were ligated at different ratios in the range of 3:1 to 1:1 at 15°C with 0.5 U of DNA ligase for 12 hours. The ligation was terminated by heating at 65°C for 15 min and diluted with 10 μl Tris-EDTA buffer.

The competent *E.coli* was prepared by the DMSO method (8). The ligated recombinant plasmids were transformed into competent *E.coli*. 5x1,000 cells of the transformed *E.coli*
were plated on LB/Ampicillin agar plate and cultured overnight at 37°C.

Screening of the recombinant clones expressing the antigen genes. The DNA libraries of transformed E.coli consisted of E.coli containing specific genes of S.typhi and E.coli containing other non-specific genes. The former was selected by simply screening the DNA libraries with specific monoclonal antibodies to S.typhi. Briefly, the nitrocellulose paper which had been precoated with Isopropyl-B-D-thiogalactoside was placed on an LB plate and incubated at 37°C for 3-4 hours. Then, the nitrocellulose filter was removed and placed in the chloroform saturated atmosphere for 3-5 min in order to lyse the bacterial cells. Subsequently, the filter was washed in PBS supplemented with 0.05% tween 20 for 10 min to remove any agar which may have stuck to the NC filter. The filter was incubated in blocking solution (5% milk) for 30 min. Meanwhile, MAb was suspended in 1% milk and preadsorbed with the E.coli lysate to eliminate non-specific MAb which might crossreact with the bacterial protein. After blocking, the NC filter was incubated with the preadsorbed MAb at room temperature for 4 hr. The filter was washed twice with PBS-Tween, followed by incubating in alkaline phosphatase conjugated rabbit anti-mouse Ig which had previously been preadsorbed with E.coli lysate for 1-2 hr. Identification of specific protein expressed by the transformed cells was performed by addition of alkaline phosphatase substrate. The red-purple colony was considered positive, while no color development was seen in the negative colony.
The positive colony was subcultured on LB/Ampicillin agar plates 2-3 times until the strongest colonies were obtained. The consistency of specific protein of *S.*typhi produced by the colonies in each subculture was confirmed by the immunoblot.
RESULTS

Monoclonal Antibodies

I. Using "crude" Bp as immunogen. We were able to obtain a few clones of hybrid cells specific to the 34 Kd protein component of *S. typhi* but which reacted weakly with 34 Kd protein bands of *S. paratyphi C, S. cholerasuis* and *S. typhimurium*. One clone each of MAbs highly specific to 34-58 Kd protein bands of *S. paratyphi A* and 30-56 Kd protein bands of *S. paratyphi B* were also obtained; they did not react with antigen of other bacteria in the family Enterobacteriaceae. These latter 2 MAbs were later confirmed to both be specific to 52 Kd proteins of *S. paratyphi A* or *S. paratyphi B*. The wide range of specific epitopes of *S. paratyphi A* and *S. paratyphi B* in protein bands at 34-58 Kd and 30-56 Kd respectively were due to degradation of the specific protein of 52 Kd during the "crude" Bp antigen preparation. Unfortunately we have lost all these above-mentioned MAbs during storage because of Mycoplasma contamination.

Using this crude immunogen, we were not be able to obtain MAbs which were specific to *S. paratyphi C*.

II. Using "affinity purified" Bp as immunogen. We were able to obtain several highly specific MAbs to protein antigens of *S. typhi* or *S. paratyphi A* or *S. paratyphi B* or *S. paratyphi C* as follows:

*S. typhi*: Ten hybrid clones highly specific to *S. typhi* were selected (IgG2bK 4 clones and IgG1K 6 clones). These 10 MAbs exhibited a diverse immunoblot pattern when tested
against crude *S. typhi* Bp. However, the major common component found to be reactive with these MAbs migrated at a 52 Kd position (Fig. 1).

It was rather unexpected to find that each of these MAbs reacted with more than one protein component present in the crude *S. typhi* Bp. In order to determine if these various components were degradation products produced during the preparation of crude Bp or were entirely different components possessing common epitopes, the same immunoblot experiment was repeated but using freshly prepared *S. typhi* WC antigen as described in the Materials and Methods. Using the latter as antigen, all 10 MAbs gave exactly the same immunoblot pattern and the only positive component was found at the 52 Kd position suggesting that the components with MW other than 52 Kd represented degradation products of the native 52 Kd component (Fig. 2). Our further experiment also clearly demonstrated that the 52 Kd component was not a glycoprotein but rather pure protein. Thus, when the crude Bp was electrophoresed, blotted onto a nitrocellulose membrane and stained simultaneously with amido black, MAb and concanavalin A, there was no concanavalin A staining at the 52 Kd position (Fig. 3).

To further explore the specificity of these MAbs, the WC antigens from 18 strains of *S. typhi* and 3 other group D Salmonellae (one strain of *S. eastbourne*, 2 strains each of *S. panama* and *S. enteritidis*, kindly provided by Dr. Dumrong Chiewsilp, National Institute of Health, Nonthaburi, Thailand) were electroblotted onto nitrocellulose membrane and allowed to react with one of these MAbs. Results showed that the 52 Kd protein from all 18 strains of *S. typhi* but not
the protein from other group D Salmonellae could be readily recognized, which demonstrated that the MAb is species-specific.

The significance of this 52 Kd protein could be further demonstrated using sera from 4 patients with acute typhoid infection (all had hemoculture positive for S.typhi). These sera were diluted 1:200 and tested against S.typhi WC antigen prepared from one of the local strains by immunoblot. The results clearly demonstrated the presence of specific IgM antibody to 52 Kd protein in all 4 patients (Fig.4).

However, when these 10 MAbs were used in a modified double antibody sandwich ELISA for detecting S.typhi antigen, they could detect as little as 0.6 μg/ml of the protein antigen prepared from S.typhi, but they could not detect such a protein in the urine and serum specimens of typhoid patients.

_S.paratyphi A_: Seventy-three clones highly specific to _S.paratyphi A_ were selected (IgG1K 58 clones, IgG2aK 9 clones, IgG2bK 4 clones and IgG3K 2 clones). These MAbs were proved as described with _S.typhi_ above to be specific to the 52 Kd protein antigen of _S.paratyphi A_ (Fig.5, Fig.2, and Fig.3) and all were species-specific. They could detect 0.1 μg/ml of the protein antigen prepared from _S.paratyphi A_ but could not detect this antigen in the clinical specimens of paratyphoid A patients.

The significance of this 52 Kd specific protein antigen of _S.paratyphi A_ was also demonstrated in two paratyphoid A patients. Both patients demonstrated strong
specific IgM antibody responses to the 52 Kd protein in their sera (Fig. 6).

*S. paratyphi B*: Ten clones highly specific to *S. paratyphi B* were selected and all were IgG1K. These MAbs were proved (as described in *S. typhi* section) to be specific to the 52 Kd protein antigen of *S. paratyphi B* (Fig. 7, Fig. 2 and Fig. 3) and all were species-specific. They could detect 0.1 μg/ml of the protein antigen prepared from *S. paratyphi B*.

We do not have paratyphoid B patients in Thailand but we have received some urine and sera of paratyphoid B patients from Chile (kindly provided by Dr. M. Levine, Center for Vaccine Development, Baltimore, M.D., USA). These MAbs could not detect antigens in the clinical specimens of paratyphoid B patients.

The significance of the 52 Kd specific protein antigen of *S. paratyphi B* was also demonstrated in 4 paratyphoid B patients. All patients demonstrated strong specific IgM response to this antigen in their sera (Fig. 8).

*S. paratyphi C*: Eighteen clones specific to *S. paratyphi C* were obtained (IgG1K 7 clones, IgG2bK 2 clones, IgG3K 2 clone and IgMk 7 clones). These MAbs were shown (as described in *S. typhi* section) to recognize a 61 Kd protein antigen of *S. paratyphi C* (Fig. 9, Fig. 10, Fig. 2 and Fig. 3) and a 59 Kd protein antigen of *S. choleraesuis*. These MAbs did not react with protein antigens from other group C Salmonellae (Fig. 11).

Since paratyphoid C patients have not been identified in Thailand (*S. paratyphi C* was never been isolated in
clinical specimen) for more than 20 years, we were unable to obtain the clinical specimens from such patients. Hence, the clinical testing of these MAbS with protein antigen of S. paratyphi C was not performed.

**Recombinant E.coli expressing the specific gene of S. typhi:**

We have successfully cloned and expressed the 52 Kd gene from *S. typhi* in *E.coli* (Fig.12). These recombinant *E.coli* could produce the specific protein antigen of *S. typhi* as confirmed by immunoblot using WC antigen of recombinant *E.coli* reacted with MAbS. Two groups of recombinant *E.coli* were obtained. The first group produced a large amount of 45 Kd specific protein of *S. typhi* and the second group produced small amount of 52 Kd specific protein of *S. typhi*. When the acute sera of 4 patients with hemoculture positive for *S. typhi*, 7 patients with hemoculture positive for other bacteria and 6 normal controls were tested against recombinant *E.coli* WC antigen of the first group in the immunoblot, three out of four typhoid patients showed the presence of specific IgM antibody to the 45 Kd protein (Fig.13) while such antibody was not observed in the other patients (Fig.14). This preliminary study revealed the significance of the 45 Kd specific protein antigen of *S. typhi* in the early serodiagnosis of typhoid fever by using a single acute serum specimen.
DISCUSSION

The success of obtaining hybridomas for production of any specific MAb depends to a large extent on the immunogen used for the immunization of an animal. Although purified immunogen is generally not required for such a procedure, the cleaner the immunogen is, the higher the chance of obtaining hybrids in sufficient quantity for screening would be. The production of MAbs specific to antigens of *S. typhi*, *S. paratyphi A*, *S. paratyphi B* and *S. paratyphi C* clearly demonstrates this point. In this study, the successful production of MAbs specific to bacteria of interest was accomplished by using the affinity purified Bp which contained only a few components that cross reacted with other Salmonellae.

All MAbs established were proved to be specific to a particular protein antigen which is very interesting, since there has been no previous report of the specific target epitope(s) of Salmonellae. Furthermore, we have found that the specific epitopes of *S. typhi*, *S. paratyphi A* and *S. paratyphi B* are on of similar molecular weight proteins (52 Kd), while *S. paratyphi C* and *S. choleraesuis* have common epitopes but these are in different molecular weight proteins (61 Kd and 59 Kd for *S. paratyphi C* and *S. choleraesuis* respectively). These findings might be of some benefit in Salmonellae taxonomy, although the specific epitopic sites of these antigens are not yet identified.

Although these MAbs could detect minute amounts of specific protein antigens, they could not detect such a protein in the urine and serum specimens of the patients. It
is possible that epitopes reactive with these MAbs are hidden in the native configuration of the proteins or are blocked in the form of immune complexes in clinical specimens, thus rendering them inaccessible to react with the MAbs.

However, our finding that most of the patients with enteric fever had specific IgM antibody responses to the specific protein of their corresponding causative agents, encouraged us to consider the usefulness of these specific antigens in the serodiagnosis of enteric fever. The only way to obtain these antigens in the purified form is to establish recombinant *E.coli* expressing the genes encoded for the specific protein antigens. With the combination of these highly specific MAbs and recombinant DNA technology, we have successfully established such *E.coli* expressing the specific gene of *S.typhi*. Our preliminary study of these recombinant *E.coli* suggested that they could produce the specific protein antigen of *S.typhi* and this antigen could be used for detecting specific IgM antibody in the acute serum of typhoid patients. Thus, in the future, overproduction of *S.typhi* antigen in *E.coli* should enable us to have purified and highly specific antigen for early and rapid serodiagnosis of typhoid fever.

The same approach can be performed with *S.paratyphi A*, *S.paratyphi B* and *S.paratyphi C*. Thus, in the future, a panel of purified protein antigens specific to 4 Salmonellae that cause enteric fever can be established. This panel of antigens will be useful for serodiagnosis of enteric fever as well as epidemiological surveillance of this disease.
IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

The findings in this project will be useful not only in developing countries but also developed countries as well, since this is the first time that specific protein target antigens of Salmonellae have been identified and this information might be useful for Salmonellae taxonomy. By using these established MAbs, the purified specific proteins of the 4 Salmonellae could be obtained through recombinant DNA technology and we have planned to use these MAbs for such approaches in the next project. Thus, in the future, a kit using a panel of protein antigens of Salmonellae will be developed for serodiagnosis of enteric fever.

As the result of this project, we have a well equipped hybridoma laboratory, a few scientists expert in hybridoma technology and immunoblotting techniques, and one scientist who knows recombinant DNA technology.
PROJECT ACTIVITIES/OUTPUTS

Meetings


Publications (Both are enclosed) Part of the works in this project which have been published are as follows:


PROJECT PRODUCTIVITY

The goals proposed

1. To produce mouse MAbs directed to species specific antigens of *S.typhi*, *S.paratyphi A*, *S.paratyphi B* and *S.paratyphi C*.

2. To develop an immunological test(s) detecting Salmonella antigens in urine and sera of the patients for the diagnosis of enteric fever and identification of the causative species for epidemiological monitoring.

3. To produce test kits that are simple and suitable for use even in rural areas of developing countries where scientific equipment is not readily available.

4. To identify molecular components of the antigen structure of Salmonellae which are specific to MAbs produced in 1.

The work performed in this project accomplished all of the above goals except goal No. 3 and part of No. 2. Mouse MAbs which are highly specific to *S.typhi*, *S.paratyphi A*, *S.paratyphi B* and *S.paratyphi C* are established. They are all species specific, except *S.paratyphi C* which is also react with *S.cholerasuis*. The molecular components of antigens specific to these 4 Salmonellae were identified to be pure proteins of 52 Kd molecular weight, except *S.paratyphi C* which is 61 Kd. Highly sensitive immunological tests using the established MAbs for detecting Salmonella antigens in simulated clinical specimens have been sucessfully developed, but they fail to detect such antigens in the clinical specimens of the patients. Thus, the proposed test kits can not be accomplished.
However, we have performed work in addition to that originally proposed. That is cloning recombinant *E.coli* expressing the specific gene of *S.typhi* by using our MAbs and recombinant DNA technology. We have successfully established such clones and have been able to obtain specific *S.typhi* protein which can be used in detecting specific IgM in the acute sera of patients for early diagnosis of typhoid fever.
FUTURE WORK

The result of this project has led us to consider future work. That is overproduction and purification of specific protein antigens of all 4 Salmonellae by recombinant DNA techniques mentioned earlier. These purified antigens will be used in a panel for serodiagnosis of enteric fever. Furthermore, the specific genes of these Salmonellae will be used as DNA probes for detection of complementary DNA in feces and peripheral blood buffy coat of the patients.
LITERATURE CITED


Fig. 1 Immunoblot analysis of MAb specific to *S. typhi* reacted with panel crude Bp antigens from *S. typhi* (1), *S. paratyphi A* (2), *S. paratyphi B* (3), *S. paratyphi C* (4), *S. choleraesuis* (5), *S. enteritidis* (6), *S. krefeld* (7), *S. panama* (8), *S. typhimurium* (9), *E. coli* (10), *Ps. pseudomallei* (11) and *Y. enterocolitica* (12). The major component of *S. typhi* specific antigen was at 52 Kd molecular weight.
Fig. 2 Comparative electrophoretic blotting between crude Bp antigen (1,3,5,7) and WC antigen (2,4,6,8) from S. typhi (1,2), S. paratyphi A (3,4), S. paratyphi B (5,6) and S. paratyphi C (7,8), reacted with their corresponding MAbs. The specific component of S. typhi, S. paratyphi A and S. paratyphi B were at 52 Kd molecular weight, while that of S. paratyphi C were at 61 Kd molecular weight.
Electrophoretic blotting of crude Bp antigen from *S. typhi* (2,3,4), *S. paratyphi A* (5,6,7), *S. paratyphi B* (8,9,10), and *S. paratyphi C* (11,12,13); stained with amido black (2,5,8,11), MAb specific to *S. typhi* (3), *S. paratyphi A* (6), *S. paratyphi B* (9), *S. paratyphi C* (12), and concanavalin A (4,7,10,13). Ovalbumin (1) stained with concanavalin-A served as glycoprotein control. No concanavalin-A staining at 52 Kd position of *S. typhi*, *S. paratyphi A*, *S. paratyphi B* and at 61 Kd position of *S. paratyphi C*, but these bands were clearly seen with MAb and amido black.
Fig. 4 Immunoblot analysis of WC antigen of *S. typhi* (3-6) reacted with acute sera of 4 typhoid patients. Specific IgM band at 52 Kd molecular weight was detected in all patients (these bands were detected by using alkaline phosphatase conjugated goat anti-human IgM as second antibody).

Crude Bp (1) and WC antigen (2) of *S. typhi* were reacted with their specific MAb for comparison.
Fig. 5  Immunoblot analysis of MAb specific to *S. paratyphi* A reacted with panel crude Bp antigens from *S. paratyphi* A (1), *S. typhi* (2), *S. paratyphi* B (3), *S. paratyphi* C (4), *S. choleraesuis* (5), *S. enteritidis* (6), *S. krefeld* (7), *S. panama* (8), *S. typhimurium* (9), *E. coli* (10), *Ps. pseudomallei* (11) and *Y. enterocolitica* (12). With crude Bp antigen, the specific component of *S. paratyphi* A consisted of wide range molecular weight protein.
Fig. 6 Immunoblot analysis of WC antigen of *S. paratyphi* A (3,4) reacted with acute sera of 2 paratyphoid A patients. Specific IgM band at 52 Kd molecular weight was detected in both patients. This band was identified by using alkaline phosphatase conjugate goat anti-human IgM as second antibody.

Crude Bp (1) and WC antigen (2) of *S. paratyphi* A were reacted with their specific MAb for comparison.
Fig. 7 Immunoblot analysis of MAb specific to *S. paratyphi* B reacted with panel crude Bp antigens from *S. paratyphi* B (1), *S. typhi* (2), *S. paratyphi* A (3), *S. paratyphi* C (4), *S. choleraesuis* (5), *S. enteritidis* (6), *S. krefeld* (7), *S. panama* (8), *S. typhimurium* (9), *E. coli* (10), *Ps. pseudomallei* (11) and *Y. enterocolitica* (12). With crude Bp antigen, the specific component of *S. paratyphi* B consisted of wide range molecular weight protein.
**Fig. 8** Immunoblot analysis of WC antigen of *S. paratyphi B* (3-6) reacted with acute sera of 4 paratyphoid B patients. Specific IgM band at 52 Kd molecular weight was detected in all patients. This band was identified by using alkaline phosphatase conjugated goat anti-human IgM as second antibody.

Crude Bp (1) and WC antigen (2) of *S. paratyphi B* were reacted with their specific MAb for comparison.
Fig. 9 Immunoblot analysis of MAb specific to *S. paratyphi* C reacted with panel crude Bp antigens from *S. paratyphi* C (1), *S. typhi* (2), *S. paratyphi* A (3), *S. paratyphi* B (4), *S. choleraesuis* (5), *S. enteritidis* (6), *S. krefeld* (7), *S. panama* (8), *S. typhimurium* (9), *E. coli* (10), *Ps. pseudomallei* (11) and *Y. enterocolitica* (12). The MAb reacted with antigen of *S. paratyphi* C and *S. choleraesuis*. 
Fig. 10 Immunoblot analysis of MAb specific to *S. paratyphi* C reacted with WC antigens prepared from a mixture of *S. paratyphi* C and *S. choleraesuis* (1), only *S. paratyphi* C (2) and only *S. choleraesuis* (3). The MAb reacted with 61 Kd component of *S. paratyphi* C and 59 Kd component of *S. choleraesuis*.
Fig. 11 Immunoblot analysis of MAb specific to *S. paratyphi* C reacted with WC antigens from group C Salmonellae which were *S. paratyphi* C (1), *S. blockley* (2), *S. brunei* (3), *S. choleraesuis* (4), *S. emek* (5), *S. hardar* (6), *S. infantis* (7), *S. montevideo* (8), *S. potsdam* (9) and *S. virchow* (10). The MAb reacted only with *S. paratyphi* C and *S. choleraesuis.*
Fig. 12 DNA library, demonstrated 3 recombinant *E. coli* colonies expressing the specific gene and antigen of *S. typhi* (arrow).
Immunoblot analysis of WC antigen from recombinant *E. coli* which expressed specific 45 Kd protein of *S. typhi* (1A, 1B, 1C, 1D) and plasmid control (2A, 2B, 2C, 2D) reacted with acute sera of 4 typhoid patients (A, B, C, D). The specific IgM band at 45 Kd was detected in patients A, B and C (1A, 1B, 1C), while the corresponding plasmid control was negative (2A, 2B, 2C). The second antibody used in this study was alkaline phosphatase conjugated goat anti-human IgM.
Immunoblot analysis of WC antigen from recombinant *E. coli* which expressed specific 45 Kd protein of *S. typhi* (1A, 1B, 1C, 1D, 1E, 1F, 1G) and plasmid control (2A,2B,2C,2D,2E, 2F,2G), reacted with acute sera of the patients infected with *S. paratyphi* A(A), Salmonella gr. B (B), Salmonella gr. C (C), Salmonella gr. D (D), *E. coli* (E), *K. pneumoniae* (F) and *C. diversus* (G). All patients had no specific IgM band at 45 Kd. The second antibody used in this study was alkaline phosphatase conjugated goat anti-human IgM.