PD-AAW-708

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 8/28/87

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 #4

Attachment

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PROGRESS REPORT NO. 4

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

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THAILAND

Rec'd in SCI. AUG 28 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

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Authorized Officer : Dr. Natth Bhamarapravati, M.D., Ph.D.
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Total Project Budget: US$ 147,740.00 (฿ 3,988,980.00)

Project Duration : 3 years

Reporting Period : January 1, 1987 - June 30, 1987

Budget Allocation for This Period : US$ 19,446.00
฿ 525,046.00

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.

OBJECTIVES OF THIS PERIOD

I. To develop and produce monoclonal antibodies to *Salmonella paratyphi A*, *S.paratyphi B* and *S.paratyphi C*.

II. To identify molecular components of *S.paratyphi A*, *S. paratyphi B* and *S.paratyphi C* which are specific to established mAbs.

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

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

<u>OBJECTIVE I</u> : To develop and produce monoclonal antibodies to *S.paratyphi A*, *S.paratyphi B* and *S.paratyphi C*.

<u>OBJECTIVE II</u>: To identify molecular components of *S.paratyphi A*, *S.paratyphi B* and *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 A* or *S.paaratyphi B* or *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. Around 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 A*, *S.paratyphi B* and *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.

Antibody production in vivo

To obtain high yields of antibodies, the hybridomas were grown as ascites tumours in pristane (2,6,10,14-tetramethylpentadecane, Aldrich chemical Co., Milwaukee, WI) primed BALB/cJ mice. Ascitic fluid developed within 2 weeks after hybrid cells injection intraperitoneally, and the total of 10-15 ml fluid per mouse could be withdrawn.

Immunoblot technique for mAb specificity testing and identification of molecular component of *S.paratyphi A*, *S.paratyphi B* and *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 Laemmli (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 B-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.

The molecular weight of protein bands of Salmonellae specific to mAbs on nitrocellulose filter membrane were determined by comparing their electrophoretic mobility with known protein markers as described by Weber and Osborn (12).

Modified double antibody sandwich ELISA for efficiency testing of mAbs

All monoclonal antibodies in the ascitic fluid were precipitated by the addition of saturated $(NH_4)_2SO_4$ to a final concentration of 50%. The precipitate was dialysed 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 was 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 corresponding Bp antigen from *S.paratyphi A* or *S.paratyphi B* at various concentrations 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 the second antibody which was rabbit anti-

veronal buffer extract of the corresponding Salmonellae 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 phosplate, 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 (Titertek Multiskan®, Flow Laboratory, GmbH, Bonn, West 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 rabbit anti-VBE Ig in the conjugate control and also for conjugate in the substrate blank. Several assays were performed with each mAb and the Student's t test was used for statistical analyses.

RESULTS

By indirect ELISA using a panel of 12 Bp antigens and dried cell of *C.jejuni*, one each of hybridoma specific to protein antigen of *S.paratyphi A* (SPA20) and *S.paratyphi B* (SPB20) (Table 1), were selected for further investigation. Their originality (lymph node or spleen) and isotypes were shown in Table 2. Unfortunately, we could not obtain

neither a hybridoma specific to only *S.paratyphi C* nor specific to common epitopes of *S.typhi*, *S.paratyphi A*, *S. paratyphi B* and *S.paratyph C*. Another fusions are being performed for the hope that the missing clones might be obtained.

Demonstration of specific protein bands by immunoblotting.

The separated protein bands 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* on nitrocellulose filter membrane were stained with immunoenzyme using mAbs SPA20 and SPB20 as the specific antibodies.

The SPA20 and SPB20 could react with several protein bands of their corresponding *S.paratyphi A* and *S.paratyphi B* (Fig.1). The range of molecular weight of protein bands specific to SPA20 was 30-58.0 Kd and for SPB20 was 30-56.0 Kd. Both SPA20 and SPB20 showed no cross reaction with other protein antigens in the panel (Fig.1,2,3,4).

Antigen detection by modified double antibody sandwich ELISA

Both mAbs were experimentally designed for detection of their corresponding antigens at the various concentrations. The results were shown in Fig.5 that both SPA20 and SPB20 could detect less than 0.1 μg/ml ($p<0.001$) of protein antigen of *S.paratyphi A* and *S.paratyphi B* respectively.

DISCUSSION

Two monoclonal antibodies (SPA20, SPB20) were established. By the immunoblotting technique, SPA20 and SPB20 were specific to 34-58.0 Kd protein bands of *S.paratyphi A* and 30-56.0 Kd protein bands of *S.paratyphi B* respectively. Both specific broad bands are rather unusual finding, one of the possible explaination might be that both SPA20 and SPB20 are specific to the epitopes located on the molecule of antigens with molecular weight range from 34-58.0 Kd and 34-56.0 Kd respectively.

To confirm the specificity of these protein specific mAbs, immunoblotting was performed with a panel antigens of 12 bacteria. The SPA20 ans SPB20 showed no cross reactivity with other protein ~~in~~ antigens in the panel.

In determining the ability of the established mAbs for Salmonellae antigen detection, modified double antibody sandwich ELISA was performed with various concentrations of antigen. They could detect very small amount of antigen.

In view of these results, the more sensitive and specific of the established mAbs over the conventional polyclonal antibodies make them considerable potential reagents for using in the test for detection of specific Salmonella antigens in the specimen of patients with enteric fever.

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Clone	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	C. jejuni	S. paratyphi A,B,C
SPA20	0.15	1.71	0.13	0.15	0.12	0.12	0.13	0.13	0.13	0.15	0.14	0.13	0.16	0.50
SPB20	0.11	0.13	1.11	0.13	0.11	0.13	0.12	0.13	0.12	0.14	0.14	0.13	0.14	0.63

Table 1. Reactivity of monoclonal antibodies to a panel antigen.

Clone	Isotype	Specificity	Source of immune cells
SPA20	IgG1K	protein	spleen
SPB20	IgG1K	protein	spleen

Table 2. Characteristic of established monoclonal antibodies

Fig. 1. Immunoblot analysis and immunoenzyme staining patterns of Bp antigen from *S.typhi* (1), *S.paratyphi A* (2), *S.paratyphi B* (3) and *S.paratyphi C* (4). The membranes were developed with the homologous antisera (A), SPA20 (B) and SPB20 (C).

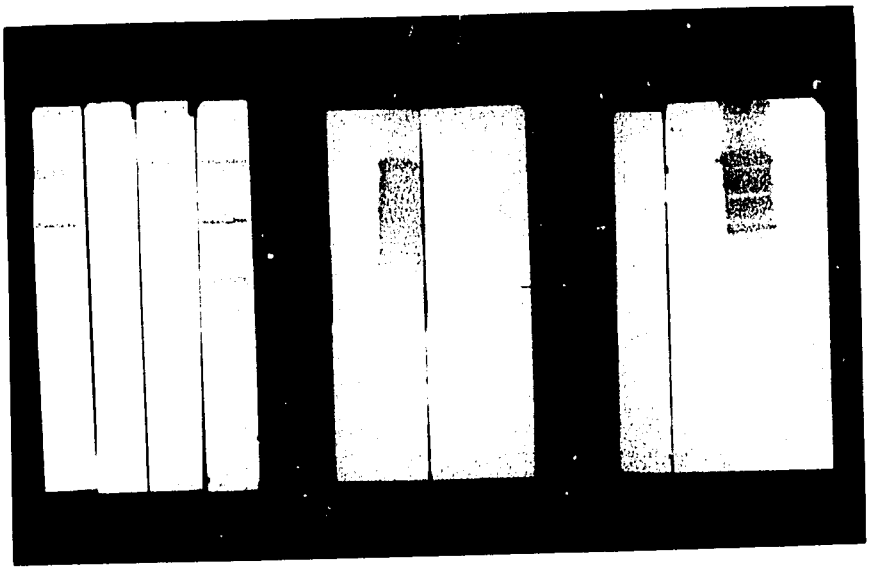


Fig. 2. Immunoblot analysis of Bp antigen from *S.typhi* (1), *S.cholerasuis* (2), *S.enteritidis* (3), *S.krefeld* (4), *S.panama* (5), *S.typhimurium* (6), *E.coli* (7), *Ps.pseudomallei* (8) and *Y.enterocolitica* (9). Each antigen (50 or 100 μg per lane) was run in an SDS-PAGE and transferred to nitrocellulose membrane and reacted with their homologous antisera produced in mice. The alkaline phosphatase-labeled goat anti-mouse Ig was used as a second antibody.

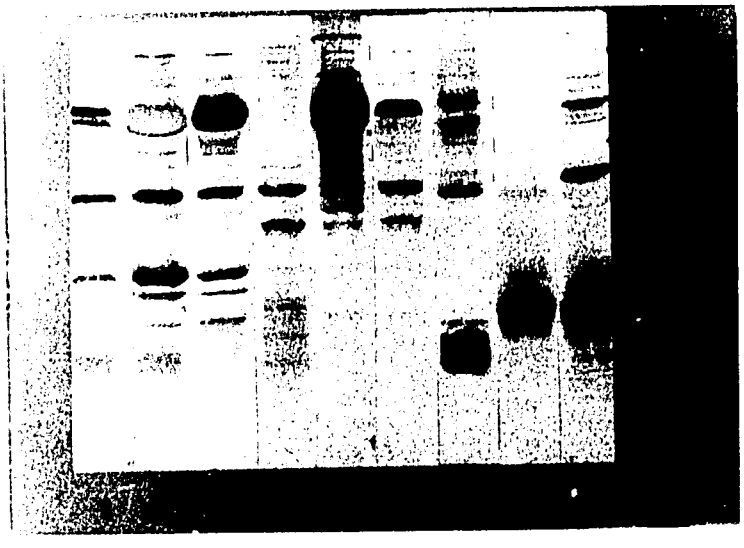


Fig. 3. Immunoblot analysis and immunoenzyme staining patterns of the same antigens shown in Fig. 2, except *S.paratyphi A* was replaced for *S.typhi* in lane 1. All antigens were reacted with mAb SPA20.

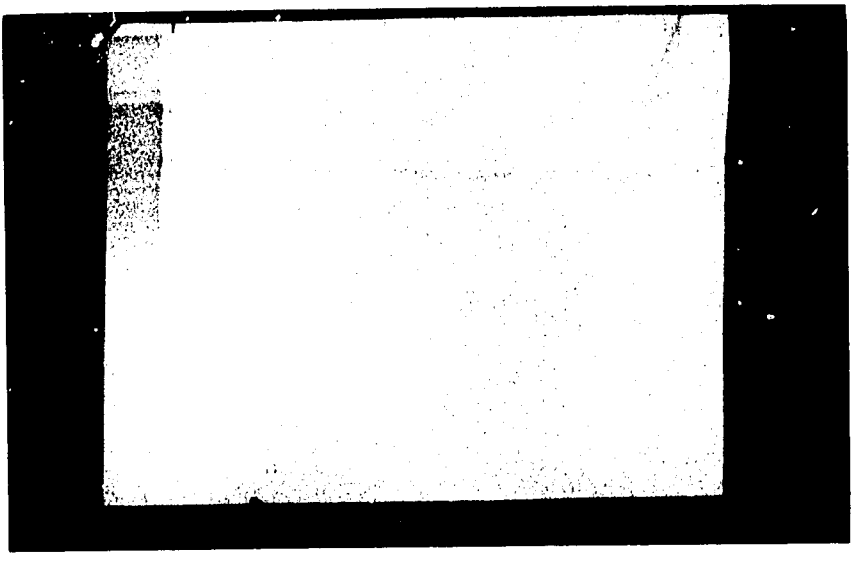


Fig. 4. Immunoblot analysis and immunoenzyme staining patterns of the same antigens shown in Fig. 2, except *S.paratyphi B* was replaced for *S.typhi* in lane 1. All antigens were reacted with mAb SPB20.

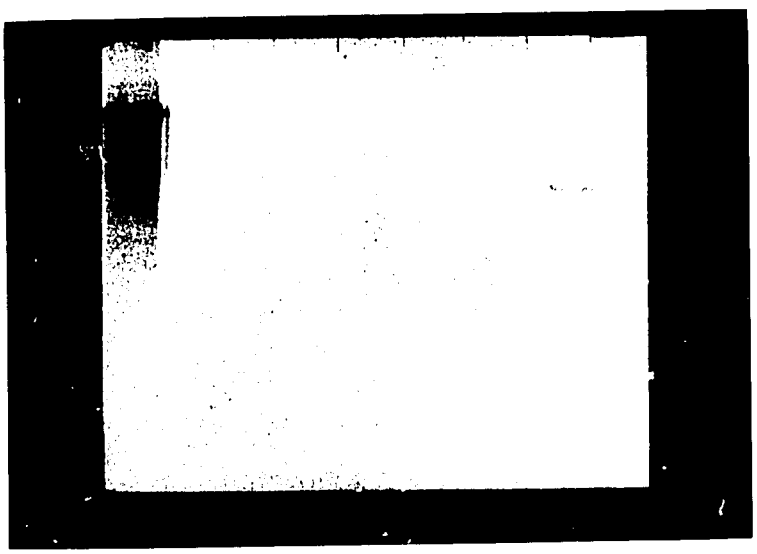
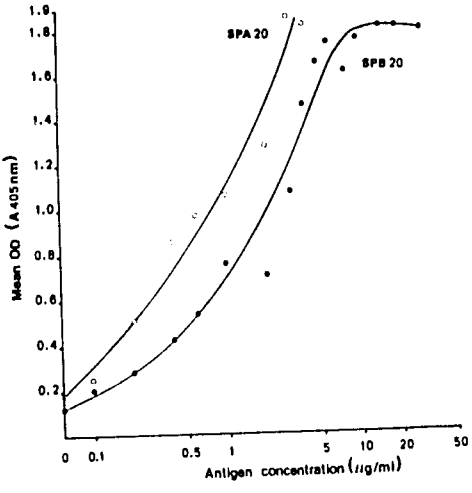


Fig. 5. Detection of Bp antigen of S.paratyphi A by SPA20 (o) and S.paratyphi B by SPB20 (●) using modified double antibody sandwich ELISA. y axis : various concentrations of Bp antigen of S.paratyphi A or S. paratyphi B in μg per ml. x axis : the mean of optical density (OD) at 405 nm.



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

At the present, we have acute sera and urine specimens from hemoculture positive patients as follows, 20 *S.typhi* positive cases, 29 *S.paratyphi A* positive cases, 23 *Salmonella gr. B* positive cases, 14 *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 still perform this activity at the present, until we can obtain sufficient number of the specimens.

<u>OBJECTIVE IV</u>: 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. These clinical diagnostic trials are now in progress in our laboratory.

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.).