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AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

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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 #6

Attachment

5.227

PROGRESS REPORT NO. 6

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. SEP 1 1988

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

Project Duration : 4 years (June 10,1985-October 31,1989)

Reporting Period : January 1, 1988 - June 30, 1988

Budget Allocation for This Period : US$ 11,716.-
฿ 296,243.-

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 which are specific to mAb produced in I.

OBJECTIVES OF THIS PERIOD

I. To develop and produce mouse monoclonal antibodies to *S.paratyphi C*, and to identify molecular components of *S.paratyphi C* which are specific to the established mAbs (cont.).

II. To develop and produce another mouse monoclonal antibodies to *S.typhi* and to identify molecular components of *S.typhi* which are opecific to the established mAbs.

III. Further recruit the subjects, collect blood and urine specimens for the clinical diagnostic trial of the ELISA test kits.

IV. To establish ELISA test kits (cont.).

<u>OBJECTIVE I</u> : To develop and produce mouse monoclonal antibodies to *S. paratyphi C*, and to identify molecular components of *S.paratyphi C* which are specific to established mAbs (cont.).

The attempt to complete this objective in this period was not successful. After another fusion, we could not find the hybrid clones which were specific only to *S.paratyphi C*. However, we have tried one more fusion and hope that the desired hybrid clones will be obtained.

OBJECTIVE II: To develop and produce another mouse monoclonal antibodies to *S.typhi* and to identify molecular components of *S.typhi* which are specific to the established mAbs.

MATERIALS AND METHODS

The procedures of hybridoma technology, screening, identification and isotypic determination of mAbs, immunoblot technique for mAbs specificity testing and identification of molecular components of *S.typhi*, were the same as previous report (progress report No. 3). Only difference from the previous report was that the purified protein antigen of *S.typhi* was used for immunization of BALB/c J mice in stead of the cluded one. This antigen was prepared as follows :

Briefly, 500 mg of cruded protein extracted of *S.typhi* (Barber protein, Bp) (1) in 7.3 ml of 25 mM Ammonium acetate buffer, pH 7.1 was passed through Sephadex G-200, G-100, and G-50 column. The fractions of protein eluted from the columns which had molecular weight approximately between 10-75 Kd were collected. These proteins were tested for specificity by ELISA with polyclonal rabbit anti-*S.typhi* Bp, anti-*S.paratyphi A* Bp, anti-*S.paratyphi B* Bp and anti-*S. paratyphi C* Bp. Two fractions which showed the best specific to anti-*S.typhi* Bp were selected for affinity chromatography. These fractions contained proteins with molecular weight between 13-64 Kd (demonstrated by SDS-PAGE).

Purified rabbit IgG, which is specific to *S.paratyphi A* Bp or *S.paratyphi B* Bp or *S.paratyphi C* Bp were prepared by using ammonium sulfate precipitation and DEAE-cellulose column chromatography. The specific IgG was coupled to

cyanogenbromide activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) and packed into 3 separate column. Each of 2 selected protein fractions mentioned above was passed through these 3 affinity columns successively. The effluent, which contained the antigens that were not adsorped to any affinity column, was collected. The specificity of antigens in each effluent were confirmed by ELISA and radioimmunoprecipitation against polyclonal rabbit anti-*S.typhi* Bp, anti-*S.paratyphi A* Bp, anti-*S. paratyphi B* Bp and anti-*S.paratyphi C* Bp. There was comparable specificity between the 2 effluents.

We then identified and concentrated the protein content of each effluent. Since we finally had only small amount of this purified antigen, we decided to use affinity chromatography for obtaining high yield of purified antigen. We immunized rabbits with this purified *S.typhi* antigen, obtained the polyclonal rabbit anti-*S.typhi* antigen, purified rabbit IgG and coupled to cyanogenbromide activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). The cruded Bp antigen of *S.typhi* was passed through this affinity column. The specific antigens adsorbed to the column were eluted out and were reconfirmed for *S.typhi* specificity by ELISA against polyclonal rabbit anti-purified *S.typhi* Bp, anti-purified *S.paratyphi A* Bp, anti-purified *S.paratyphi B* Bp and anti-purified *S.paratyphi C* Bp. The protein content in the supernatant was measured, lyophilyzed and kept at 4°C for immuniza ion of BALB/c J mice.

Results and discussion

In this fusion, 10 hybrid clones were selected. (Table 1). These clones can be divided into 4 groups according to their isotypes and molecular weight of their specific *S.typhi* protein antigens. All 10 mAbs are very specific to *S.typhi*, no cross reaction with other protein antigens in the panel by indirect ELISA (Table 1) and Immunoblotting (Fig. 1-4).

The molecular weight of *S.typhi* protein antigens which are specific to these mAbs are 25, 29, 40, 48, 52, >66 Kd (Fig. 1-4). The common and prominent bands among the 4 groups are the protein molecular weight of 48 and 52 Kd.

These mAbs will be used in clinical diagnostic trial of the patients with enteric fever in the next reporting period.

References

1. Barber C, Vladoianu JR, Dimache GH. Contributions to the study of Salmonella. Immunological specificity of proteins separated from *Salmonella typhi*. Immunology 11: 287-96.

Group	Clone name	Isotype	M.W. (Kd) of specific epitopes	O.D. against cruded protein antigen 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
I	13B1C3	IgG2bK	48,52,>66	≥2	0.153	0.170	0.181	0.163	0.134	0.154	0.131	0.161	0.170	0.152	0.140
	13B1B11			≥2	0.163	0.175	0.197	0.160	0.126	0.153	0.115	0.158	0.164	0.155	0.135
	13B1H3			≥2	0.153	0.159	0.165	0.143	0.146	0.161	0.158	0.144	0.158	0.163	0.152
	2B4D6			≥2	0.181	0.172	0.176	0.130	0.117	0.126	0.137	0.133	0.134	0.120	0.135
II	14B4A9	IgG1K	48,52	1.565	0.171	0.175	0.172	0.133	0.125	0.147	0.119	0.140	0.169	0.137	0.154
	14B4F2			1.327	0.159	0.164	0.168	0.122	0.124	0.137	0.114	0.120	0.158	0.118	0.156
III	14B2E3	IgG1K	25,29,48,52	≥2	0.159	0.176	0.190	0.125	0.159	0.149	0.152	0.123	0.139	0.130	0.138
IV	5B6E3	IgG1K	40,48,52,>66	≥2	0.166	0.179	0.183	0.129	0.161	0.122	0.161	0.148	0.144	0.128	0.136
	2C1E10			≥2	0.186	0.160	0.190	0.138	0.150	0.130	0.153	0.136	0.139	0.118	0.136
	2C1G11			≥2	0.181	0.163	0.177	0.139	0.167	0.131	0.151	0.130	0.141	0.116	0.149

Table 1. Four groups of 10 established monoclonal antibodies, demonstrate isotype, molecular weight of specific protein epitopes and reactivities to a panel of cruded protein extracted from 12 bacterial strains. The O.D. were measured at 405 nm.

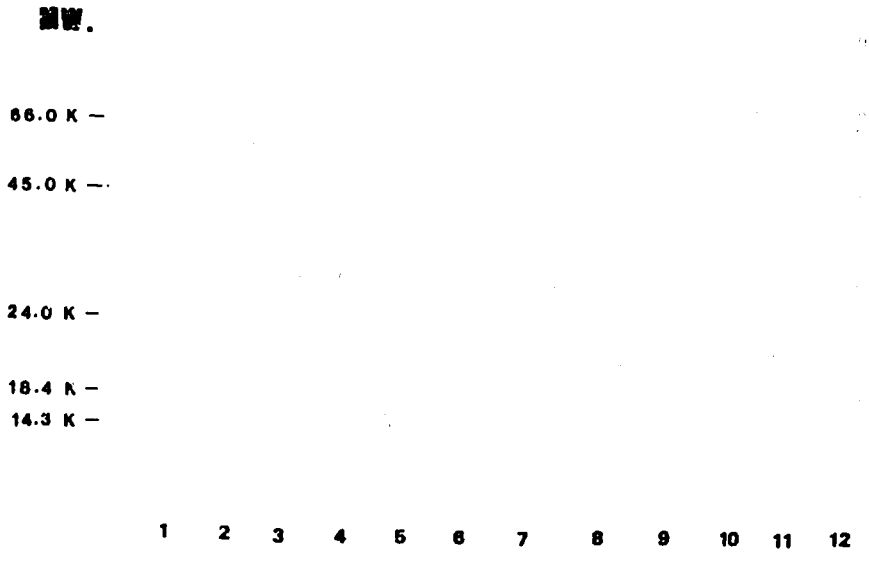

Fig. 1 Immunoblot analysis of the Bp antigen from *S.typhi* (lane 1), *S.paratyphi A* (lane 2), *S.paratyphi B* (lane 3), *S.paratyphi C* (lane 4), *S.cholerasuis* (lane 5), *S.enteritidis* (lane 6), *S.krefeld* (lane 9), *E.coli* (lane 10), *Ps.pseudomallei* (lane 11), and *Y. enterocolitica* (lane 12). Each antigen was run in SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with a mAb from gr. I. Alkaline phosphatase-labeled goat-anti mouse immunoglobulin was used as a second antibody.

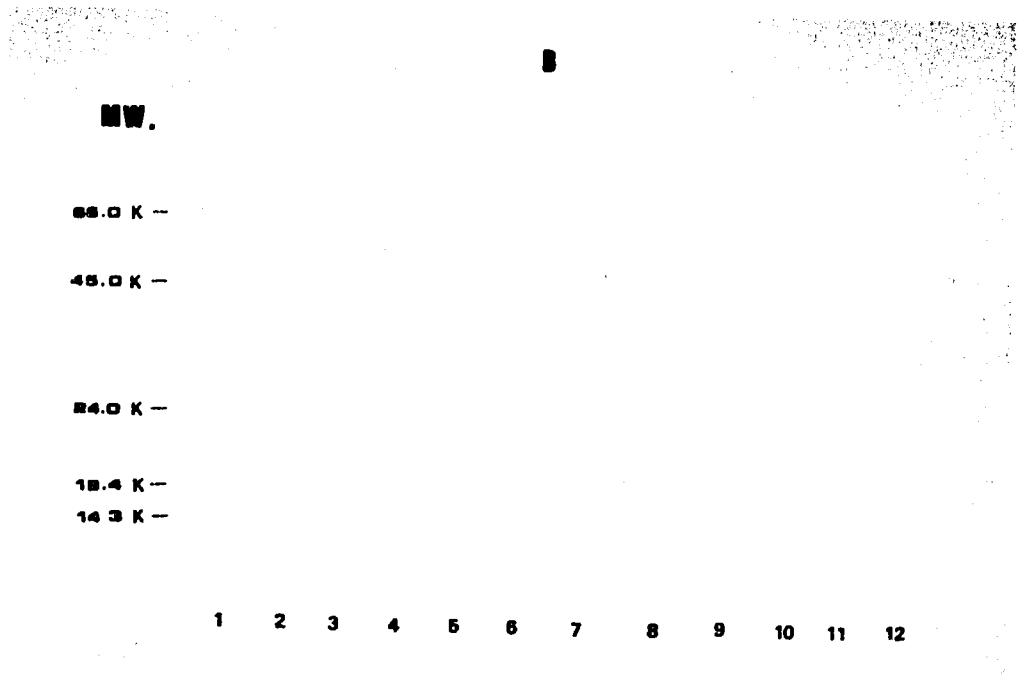

Fig. 2 Immunoblot analysis pattern of the antigens shown in Fig. 1. All antigens were reacted with a mAb from gr. II.

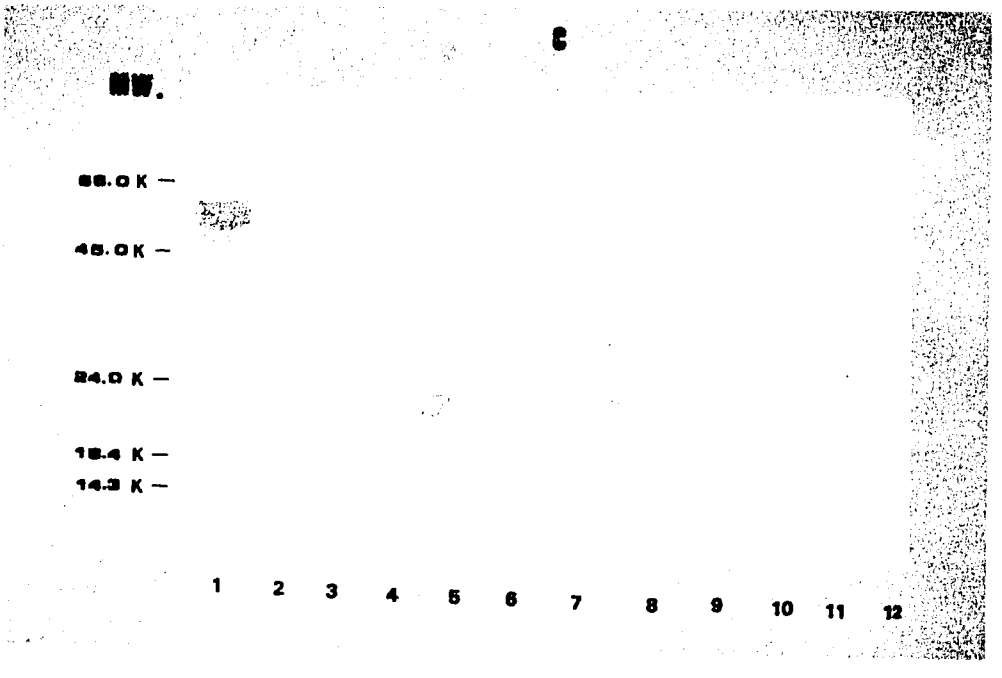

Fig. 3 Immunoblot analysis pattern of the antigens shown in Fig. 1. All antigens were reacted with a mAb from gr. III.

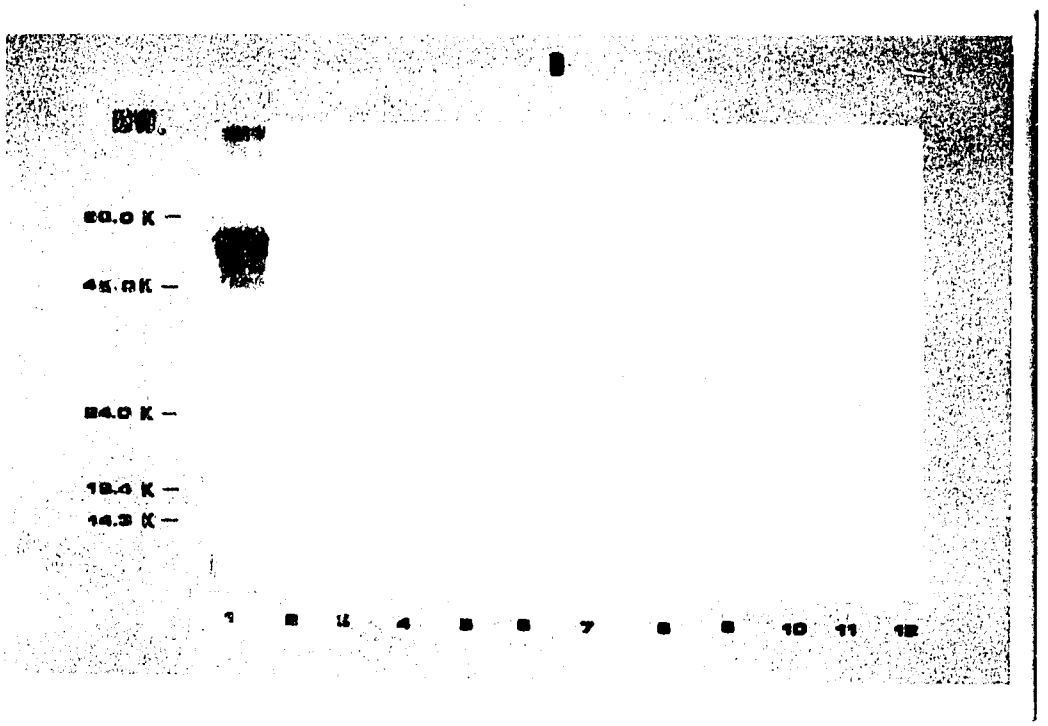

Fig. 4 Immunoblot analysis pattern of the antigens shown in Fig. 1. All antigens were reacted with a mAb from gr. IV.

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

Only few specimens could be obtained in this period. However, we have solved the problem of specimen deficiency by collaboration with <u>Dr. M.M. Levine</u> to get the clinical specimens from Chile where the incidence rate of enteric fever is about 5 times higher than Thailand.

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

Our established modified double antibody sandwich ELISA which can detect microgram of antigen in the efficiency testing mentioned in the previous report was used in clinical trials of 3 culture supernatant mAbs (XIII : 2D1E6 and XIV : 1D6F7 are specific to 34 Kd protein antigen of *S.typhi*, XIV : 8A2E1 is specific to Vi antigen) with the clinical specimens from patients with typhoid fever. We had very disappointed results that we could not detect any antigen either in serum or urine specimens. This means either the assay technique was not sensitive enough or none of the 34 Kd protein and Vi epitopes of *S.typhi* was existed in the clinical specimens. However, we are solving this problem by setting up the more sensitive ELISA which we hope to detect nanogram or picogram of such antigen, and will use this technique with the old as well as newly established mAbs.

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.).