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PROGRESS REPORT NO. 4

DEVELOPMENT OF METHODS FOR IMMUNODIAGNOSIS OF HUMAN LIVER FLUKE INFECTION

A RESEARCH PROJECT

USAID/PSTC PROGRAM

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SUBMITTED BY

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PROJECT PROFILE

Country	Thailand
Grant No.	936-5542-G-00-6027-00
Program	Program on Science and Technology Cooporation
Project Title	Development of Methods for Immunodiagnosis of Human Liver fluke Infection
Project Leader	Dr. Stitaya Sirisinha
Organization	Mahidol University
Co-investigators	Dr. Peter B. Billings Dr. Skorn Mongkolsuk Dr. Araya Dharmkrong-at
Project Consultants	Dr. Sakol Panyim Dr. Pornchai Matangkasombut
Authorized Officer	Dr. Natth Bhamarapravati Rector, Mahidol University
Total Budget	US$150,000
Project Duration	3 years (May 1986 to December 1989)
Reporting Period	11/1987 -4/1988
Budget Allocation for this period	US$18,320.08 (Baht 466,690)

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1. Background

Liver fluke infection is a major health problem in Southeast Asia, the Far East, and Central and Eastern Europe, with at least 40 million people currently infected. In Thailand alone, it has been estimated that at least 7.5 million people (about 15% of the population) are infected with Opisthorchis viverrini and the prevalence in some districts is at least 90% or higher.

Attempts thus far to control the disease include interuption of life cycle, case detection and chemotherapy. These approaches with the exception of a trial mass chemotherapy with praziquantel in small villages are not satisfactory. Reinfection readily occurs after chemotherapy but can be reduced if such treatment is accompanied by health education aimed at changing food habits and improving sanitation and personal hygiene.

Currently the diagnosis of O. viverrini infection, based on the detection and identification of eggs in the feces, is tedious and time-consuming, and is reliable only under the supervision of experienced laboratory personel and therefore does not lend itself to wide application necessary for disease control programs in highly endemic areas where new cases alone overwhelm laboratory capacities. Moreover, cases with light infections and when there is biliary obstruction such as cholangiocarcinoma can result in false negative findings. The development of a simple, sensitive and specific test for antibodies to the parasites would provide a useful tool for epidemiological surveys, giving information on previous exposure to the parasite and, perhaps, some indication of pathological complications. The test can be

used to monitor the success of chemotherapy as well as the rate of reinfection. Serological tests for the detection of serum antibody including precipitation, agglutination and complement fixation have been used in the past. However, the results are not satisfactory as they lack sensitivity and specificity, and do not lend themselves readily to be used in small, poorly equipped laboratories. The development of enzyme-linked immunosorbent assay (ELISA) has reactivated the interest in developing serological methods for the diagnosis of liver fluke infections. In recent years, ELISA has been used in attempting to find suitable immunodiagnostic methods. However, due to the complexity of the antigens available, the test lacks specificity. An indirect immunofluorescent test has also been used but the technique cannot be readily performed in most laboratories in Thailand. The characterization and purification of parasite-specific antigens is hence a necessary starting point for establishing suitable systems with diagnotic potential. Moreover, some clues as to the possible immunological basis for protection may be obtained, thus allowing immunoprophylaxis or a way to enhance post-exposure immunity.

2. Objectives

The main objective of this project is to develop a simple, sensitve and specific immunoassay(s) for the diagnosis of human liver fluke infection caused by *Opisthorchis viverrini*. While the principal aim is to detect specific antibody in the serum and/or other body fluids (e.g., saliva and bile), a simultaneous

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development of a method to detect specific parasite antigen(s) will also be carried out. The immunoassays to be developed should:

a. Be able to establish diagnosis is suspects including those with low intensity of infection.

b. Identify O. viverrini infection in patients with biliary obstruction and other complications.

c. Be reliably and extensively performed by laboratory personnel with minimal laboratory training.

d. Be able to monitor the efficacy of individual and mass anthelmintic treatment as well as reinfection.

The approach is to characterize, identify and purify relevant antigenic components by appropriate physicochemical and immunological methods using a battery of sera from infected individuals, rabbit polyclonal and mouse monoclonal antibodies. Initial steps in the attempt at production of relevant antigens by recombinant E. coli carrying genomic O. viverrini DNA fragments will also be made. Polyclonal and monoclonal antibodies together with corresponding relevant antigens developed will be used in the formulation of immunoassays.

Research activity

Activity 1 : Preparation of antigens

(A) O. viverrini antigens. The various O. viverrini antigens, including somatic, surface tegument and metabolic products

(ES) of adult worms, were prepared as described in details in Progress Reports No. 1 and 2. Currently we have approximately 200 hamsers infected with 60 metacercariae each. These antigens were used for the various immunoassays as well as for screening of monoclonal antibodies and preparation of RNA an DNA.

(B) Schistosoma japonicum antigen. Crude somatic extract of S. mansoni was prepared from adult male and female worms obtained from albino Swiss mice infected with S. mansoni cercariae (kindly made availavble by Dr. E.S. Upatham, Department of Biology, Faculty of Science, Mahidol Unviersity). The adult worms were homogenized and sonicated essentially the same as done for O. viverrini adults. The antigen will be used for analyzing specificity of the immunoassay systems being developed as well as for specificity screening of monoclonal antibodies.

(C) Nematode antigens. These nematode antigens used for specificity study were prepared from Gnathostoma spinigerum and Angiostrongylus cantonensis as previously described in the Progress Report No. 3.

Activity 2: Collection of specimens from patients

We are now collecting serum, bile and urine from patients with positive O. viverrini eggs in the stool. These were patients with biliary obstruction coming in for surgery at Khon Kaen University Hospital. Whereas hepatic bile was previously obtained by cannulation as described in Progress Report No. 2, gall bladder bile was collected in this group of patients. In

some of these patients, saliva was also collected.

In addition to these opisthorchiasis patients, we also collected blood and sometimes urine and saliva from O. viverrini negative individuals who reside in the endemic area of O. viverrini infection, either with or without infections by other intestinal helminths. In order to locate these individuals, a large number of patients attending the Outpatient Clinic of the University Hospital were screened by fecal examination for O. viverrini eggs.

Activity 3 : Attempt at cloning O. viverrini genes

From previous report, we constructed the Opisthorchis viverrini DNA library in E. coli, using pUC 8, 9 and 12 as expression vectors. This library was screened with serum from rabbits immunized with Ov antigens and ^{125}I-prot A in colony radioimmuno assay. In the Progress Report No 3 we screened about 30,000 white colonies, and in the present report we secrened another 30,000. However, these clones also gave negative result.

The genomic DNA digested with Pst I restriction enzyme which has 6 bp recognition sequence gave an average fragment length of around 4 kb. O. viverrini is eukaryotic and therefore likely having introns which may prevent expression of antigen in the E. coli. To resolve the problem, the restriction enzyme MSp I and Taq I were used. These enzymes have 4 base pairs recognition sequence that gave average fragment lenght of around 500 bp. To clone MSp I and Taq I fragments, the plasmid blue script pks was used by digesting with Cla I restriction enzyme which has

compatible ends to MSp I and Taq I. The plasmid pks was ligated to MSp I and Taq I fragments by enzyme T4 ligase at 15°C overnight and transformed into E. coli JM107.

The screening method was the same as described in the recent report excepted the O. viverrini-infected human serum and a new lot of higher titered rabbit anti-somatic antigen were used.

The results from screening of 10,000 white colonies of library in pUC and about 20,000 white colonies library in pks gave 6 positive clones on the autoradiogram. These positive clones, five came from pks library and one came from pUC library. All of these positive clones were separately grown in L-broth with 100 μg/ml of ampicillin and induced with 10 mM IPTG (Inopropyl β-D-thiogalactopyranonide) at log phase. The cell pellets were dissolved in sterile distilled water and added equal volumn of E-buffer (2% SDS, 5% Mercaptoethanol, 10% glycerol). The cell suspensions after mixed with E-buffer were boiled for 5 minutes or until the suspension become clear. The supernatant of the positive clones lysate (around 50 μl) of each samples in comparison with the lysate from pUC and pks in E. coli JM107 were loaded into 12% SDS-PAGE. The SDS-PAGE was western blotted onto nitrocellulose paper. The nitrocellulose paper was blocked with 5% skim milk in PBS pH 7.4 and incubated with infected human serum and followed with ^{125}I-prot A. The autoradiogram of these clones showed three protein products of size smaller than lysozyme (which was the smallest marker on the gel), indicating molecular weight lower than 14,000.

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Activity 4 : Production of monoclonal antibodies

Additional successful fusions were made during this period to increase the number of hybridomas with antibody specificity against O. viverrini antigens. In particular, we are attempting to direct the response more toward antigens which appear highly immunogenic in infected animals and patients, and which are also parasite-specific. The most appropriate antigen identified thus far, is the 89 Kd protein, found in most extracts and antigen preparations, but especially abundant in the Excretory-Secretory fraction. This antigen fraction concentrated from in vitro maintenance cultures of parasites has been used in successful fusions. This antigenic polypeptide is also present in lower proportion in somatic extracts, and more random approach has also been used.

We have attempted to develop a method of immunizing with much smaller amounts of antigen, in anticipation of the need to immunize with selected polypeptide bands from polyacrylamide gels. Previous results indicated that the response to nitrocellulose-adsorbed somatic antigen was excellent even with extremely small amounts. Adsorption appears to enhance the immune response, but exactly how much is not quite clear. DMSO used as solvent to facilitate i.p. or s.c. injection rapidly diffuses out of the Freund's adjuvant-DMSO-nitrocellulose mixture resulting in re-solidification as large irregular masses. These probably limit direct access to antigen by Ag-presenting cells. Thus, a large part of the adsorbed antigen could be physically embedded, not contributing to the response, or it could slowly be

released. We do not know how porous these solid forms are; however quite good responses have been obtained with total somatic antigen. In the most recent extension of this approach, the immunization with adsorbed antigen was combined with SDS gel electrophoresis and electrophoretic transfer (western blotting) onto the nitrocellulose. The bands of interest as immunogen--a closely spaced doublet of M_r 89,000--were identified after transfer to introcellulose from a narrow preparative gel by reacting end strips with mouse antiserum to somatic antigen (i.e., by typical immunoblot reaction). The entire region of the nitrocellulose replica corresonding to 85-95 Kd was then excised and solubilized with DMSO. After being mixed with Freund's complete adjuvant, this material was used to prime BALB/c mice. Because of the generally poorer electrophoretic transfer of high MW polypeptide bands, a large proportion of the 89 Kd band was retained in he gel; each mouse probably received no more than 5 ug of this band. The remaining 89 Kd band, which failed to transfer from gel to nirocellulose, was stained with Coomassie Blue, cut out, crushed, mixed with FCA and also used to immunize (estimated by stain at about 10 ug/animal). By ELISA vs total somatic antigen, the resposne to the western blot 89 Kd region was good to excellent, while that to a higher amount of Coomassie-stained antigen embedded in acrylamide was only moderate. The best responder immunized with the blot 89 Kd band has been used in fusion after pre-fusion booster with crude total soluble somatic antigen. The expectation is that the primary response to the non-89 Kd "contaminants" will not involve

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sufficient cells within the 3 days before fusion to be recovered in hybridomas. Again, we should be able to do the first hybridoma screen also with total somatic antigen by ELISA. Remaining animals were boostered with similar amounts of the corresponding antigens.

From earlier fusions with animals immunized with nitrocellulose-adsorbed total somatic antigen or with the 89 Kd-enriched ES antigen, we now have a reasonably large back-log of monoclonal antibodies remaining to be screened by radioimmunoprecipitation for fine specifiities.

WORK PLAN FOR NEXT PERIOD

(1) Production of monoclonal antibodies

Existing monoclonal antibodies need to be characterized and sorted according to polypeptide specificities. This will be done by relatively straight-foreward immunoprecipitation of ^{125}I-iodinated somatic antigen using (rabbit) anti-mouse IgG prereacted with protein A-Sepharose. The gel dryer for analysis of radioimmunoprecipitates has recently been serviced to correct vacuum problems due to incomplete moisture entrapment, and a more effective dry-ice alcohol cold trap is being made loclly to prevent recurrence. Ambiguous cases of co-precipitation of antigen complexes may be resolved by western blotting, however this analysis used far too much precious antigen for general preliminary screening. The most promising monoclonals of each specificity will be used in immunofluorescence study for antigen localization on sectioned adult worms and on egg-enriched fractions to assess diagnostic utility.

(2) Gene cloning

The protein products shown to react with infected human serum will be used as an antigen in the ELISA to test for their sensitivity and specificity. The putative antigen clones will be used as DNA probe to search the O. viverrini cDNA library for a complete gene. This would permit a more definitive identification of the antigen.

PUBLICATION AND PRESENTATION OF THE RESULTS OBTAINED UP TO NOW INCLUDE

(1) Graduate students' theses

A portion of the research for this project has been conducted by a number of Ph.D. and M.Sc. candidates in the Department of Microbiology at the Faculty of Science, Mahidol University. These students include

1. Mr. Surasakdi Wongratanacheewin who has just received a Ph.D. degree and is now a lecturer at the School of Medicine, Khon Kaen University in Khon Kaen Province.

2. Miss Rasana Sermswan is a late-stage Ph.D. candidate and her work is concentrated on the gene cloning aspect of this project. She works primarily under the supervision of Drs. Skorn Mongkolsuk and Sakol Panyim.

3. Miss Suree Korbsrisate is an early Ph.D. candidate and will be working largely on O. viverrini genome under the supervision of Dr. Skorn Mongkolsuk.

4. Miss Sorujsiri Amornpunt is a second year M.Sc. student working on monoclonal antibodies specifically against the 89 kD

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component of O. viverrini. She works under the supervision of Dr. Suttipant Sarasombath of Siriraj School of Medicine (Mahidol Unversity) and Stitaya Sirisinha (Project leader).

5. Miss Nantana Utsakhit is a first year M.Sc. student working on monoclonal antibodies against various O. viverrini antigens. She works under the supervision of Dr. Peter B. Billings.

6. Miss Teeraporn Sureerug is a first M.Sc. student. She is characterizing biliary antibodies in patients with opisthorchiasis under Dr. Stitaya Sirisinha.

(2) Attending scientific meetings and presentation of papers

1. Paper entitled "Immunodiagnosis of human liver fluke infection caused by Opisthorchis viverrini" was presented at the conference on Medical Microbiology Towards 2000 in Hong Kong (29 May - 1 June, 1987).

2. Presenting a paper on "Approaches to develop immunodiagnostic methods for liver fluke infection caused by Opisthorchis viverrini" at the conference of USAID Science Research Award Grantees at Nakorn Pratom (24-26 July, 1987).

3. Participating in a sympocium on "Opisthorchiasis" organized by Ministry of Public Health at Petchburi (6 August 1987).

4. Presenting a paper on "Biotechnology Research in the Study of Opisthorchiidal in Thailand at the Thailand-U.S.-SEAMEO-TROPMED Conference on Applications of Biotechnology on the Study of Animal Parasites and Their Vectors in Bangkok (23-25 Nov., 1987).

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(3) Formal Publications

Articles that have been and/or will be shortly published since the initiation of this project are listed below.

1. Sirisinha, S. Immunodiagnosis of human liver fluke infections. Asian Pacific J. Allergy Immunology 4:81-88, 1986 (reprints were submitted with Progress Report No. 2).

2. Sirisinha, S. and Wongratanacheewin, S. Immunization of hamsters against Opisthorchis viverrini infection. Southeast Asian J. Trop. Med. Pub. Hlth. 17:567-573, 1986 (reprints were submitted with Progress Report No. 2).

3. Wongratanacheewin, S., Chawengkirttikul, R., Bunnag, D. and Sirisinha, S. Analysis of Opisthorchis viverrini antigens by immunoprecipitation and polyacrylamide gel electrophoresis. Parasitology 96:119-128, 1988 (2 copies of a galley proof of the paper were submitted with the Progress Report No. 3).

4. Wongratanacheewin, S., Bunnag, D., Vaeusorn, N. and Sirisinha, S. Characterization of humoral immune response in the serum and bile of patients with opisthorchiasis and its application in immunodiagnosis. Am. J. Trop. Med. Hyg. (In press) 1988.

5. Sirisinha, S. Approaches to develop immunodiagnostic methods for liver fluke infection caused by Opisthorchis viverrini. Proceedings of USAID Science Award Grantees. July 1987.

6. Wongratanacheewin, S., and Sirisinha, S. Analysis of Opisthorchis viverrini antigens: physicochemical characterization and antigen localization. Southeast Asian J. Trop. Med. Pub. Hlth. 18:511-520, 1988. (3 copies of the reprint are enclosed herewith).