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PROGRESS REPORT NO. 2

DEVELOPMENT OF METHODS FOR IMMUNODIAGNOSIS OF HUMAN LIVER FLUKE INFECTION

A RESEARCH PROJECT
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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 Immuno-diagnosis of Human Liver Fluke Infection
Project Leader	Dr. Stitaya Sirisinha
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Project Consultants	Dr. Sakol Panyim Dr. Pornchai Matangkasombut
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Total Budget	US$150,000
Project Duration	3 years (May 1986 to December 1989)
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Budget Allocation for this period	US$25029.81 (Baht 650,700)

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 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 specific antigens

(A) Adult worm antigens. This is a continuation of the work already described in Progress Report No. 1. The parasite cycle is being maintained continuously for both somatic extract and excretory-secretory product in hamsters kept in the Animal Center of the Faculty of Science. Currently there are about 100 hamsters infected with approximately 100 metacercariae each.

(B) Metacercarial antigens. Again this is a continuation of the work described in Progress Report No. 1. The combined material currently available is still insufficient to carry on any extensive work.

(C) Surface tegument. External surface of adult worms was extracted with 1% deoxycholate for 60 min at 4°C, using 100 adult worms in 3 ml of the detergent solution containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl and 10 mM ethylenediamine-tetraacetic acid. The extracted material was centrifuged at

25000 rpm for 15 min and then dialyzed in saline to remove the detergent. The dialyzed extract was kept in small alliquots at -20°C.

(D) Soluble egg antigens. Adult worms were homogenized in a ground-glass homogenizer as previously described in Progress Report No. 1. Eggs, largely intact at this time, were separated by gravity, washed several times in saline and allowed to sediment a few more times to minimize cellular debris. The suspension was finally homogenized, this time very extensively, in order to break the egg shell. The procedure was carried on until a majority of eggs were broken, and their content released, as observed by light microscope. The suspension was then centrifuged at 3000 rpm for 30 min and the protein content of the supernatant fluid was determined by the Folin method.

Activity 2: Production of polyclonal rabbit antisera

Rabbits were immunized with surface tegument essentially as described in Progress Report No. 1. Antiserum to egg antigens was prepared by immunizing the animals with intact eggs. The material was prepared by mixing 1.5×10^6 eggs suspended in 0.1 ml of saline with an equal volume of complete Freund's adjuvant. The animals were boostered 2 months later with a total of 1.5×10^6 eggs suspended in saline and bled 1 week thereafter.

These antisera, together with those produced earlier in the previous report, were to be used subsequently for the identification of parasite antigens.

Activity 3: Collection of specimens from patients

Up to now sufficient numbers of serum samples have been collected from patients with proven opisthorchiasis (as shown by the presence of O. viverrini eggs in stools) and without any other clinical complications. These patients attended the

Outpatient Clinic of the Hospital for Tropical Diseases (Faculty of Tropical Medicine). However, at this time we only have a few samples from patients with other parasitic infections, and these included a few cases with *Paragonimus heterotremus*, *Angiostrongylus cantonensis*, *Gnathostoma spinigerum*, *Plasmodium falciparum* and hookworm infections. There was also one pooled serum sample from a group of individuals infected with *Schistosoma mansoni* (made available from Dr. A. Dharmkrong-at).

Another group of patients suspected of having been infected with *O. viverrini* (*Ov*) were those admitted to Siriraj Hospital with biliary obstruction. The cannulated bile was centrifuged and the pellet was examined for *Ov* eggs. These patients were then divided into 2 groups, Ov^+ and Ov^-. The Ov^+ patients were then subdivided into those with cholangiocarcinoma (CCA) and those without CCA. The diagnosis of CCA was based on the pathological examination of biopsy materials available during the time of surgery as well as on clinial impressions and the characteristics of bile secretions. The Ov^- patients were those with CCA, carcinoma of common bile duct, hepatic and head of the pancreas, stones in the common bile duct, endometriosis or chronic cholangitis. However, we do not have a sufficient number of patients in this category yet.

We are fortunate to receive serum samples from Dr. J.W. Mak (Medical Research Institute, Kuala Lumpur, Malaysia). These samples were supposed to be from native Malaysians who never would have been exposed to *O. viverrini*. These included several samples from healthy Malaysians as well as those having toxoplasmosis, filariasis and various types of malaria.

We also have a sufficient number of samples from normal healthy Thais who have never eaten raw or poorly cooked fish nor resided in the endemic area.

Activity 4: Selection of optimal conditions for ELISA

The optimal conditions were determined by a checkerboard titration, using adult somatic extract, E-S antigen and surface tegumental extract to coat the microtiter plates. Suitable concentration for all 3 antigens were found to be 5-10 μg/ml. Specific horseradish peroxidase conjugates were purchased from Dakopatts (Copenhagen, Denmark). Conditions required for the quantitation of specific Qv antibody associated with IgG, IgA, IgE or IgM isotypes in the serum and bile are now being determined.

Our preliminary results showed that it was possible to select an assay condition which could clearly distinguish the patients from normal healthy controls. A determination of serum IgG antibody titer appeared to be superior to that of other antibody isotypes.

Activity 5: Quantitation of total immunoglobulins

The total levels of IgG in both serum and bile were determined by radial immunodiffusion technique. ELISA was used for the quantitation of IgA and IgE as well as of secretory-component associated IgA in the bile of these patients. A significant increase of both serum and biliary IgE was noted in Qv^+ patients. About 50% of patients had secretory IgA antibody in their bile secretions.

Activity 6: Production of monoclonal antibodies against O. viverrini antigens.

During this reporting period, a number of fusions were made in an attempt to produce mouse hypridomas secreting monoclonal antibodies against O. viverrini antigens. Two antigen preparations were used for immunization of BALB/c mice, a conventional adult worm somatic saline extract and an egg-enriched insoluble preparation from disrupted adults. Immunizations were made either i.p. (somatic) or s.c. (egg)in Freund's complete adjuvant, with boosters i.p. in incomplete

adjuvant. Animals primed and boosted with egg antigens were switched to the somatic extract for prefusion boosters for convenience, insofar as previous studies indicated that antisera against both preparations immunoprecipitated a presumably identical antigen of M_r approximately 89 kd which appears to be parasite-specific, and the somatic extract lends itself better to an ELISA hybridoma screening assay. Reasonable responses were obtained and improvement with boosters in all but a couple mice as assayed by ELISA against somatic Ag.

Several attempts were made between fusions with *in vivo* immunized splenocytes, to immunize spleen cells from non-immune mice *in vitro*. The justification for this attempt is that exceedingly small amounts of antigen (e.g., highly purified and consequently highly precious parasite-specific antigen) are required, the immunization period is short, and the procedure can be applied to partially denatured antigen such as that eluted from SDS gels. As we narrow down our list of antigens to those which appear to have better diagnostic value or utility for various experimental applications (e.g., an antigen to be enriched/ purified for use as immunogen), we would like to focus the response (or more specifically, to restrict our monoclonal antibody recovery) more to just these antigens. One advantage of the hybridoma approach is that purified antigen is not required, however, if crude antigen mixtures are used in immunization and screening, many, if not most of the recovered McAb may be redundant, being directed against the same few antigens of limited value. If McAb recovered are to be confined to single components then, purified antigen is required at at least one of the steps: the priming and boosters, the pre-fusion booster (day-4 or -3), or the initial screening (or very soon thereafter). Sorting out and selecting desired specificities at a late stage necessitates considerable effort/expense in maintaining what will ultimately prove unncessary clones. Thus, the most

economical and practical application of precious purified antigen would be to use it early, and preferably in a way that very little is needed such as *in vitro* immunization. This approach is being used in parallel with conventional immunization at present, as we have but limited knowledge of desired antigen and appropriate methods of purifying them.

The basic procedures used for *in vitro* immunization have been taken predominantly from those described by Reading (*J. Immunol. Methods* 53. 261, 1983) and Van Ness, Laemmli and Pettijohn (*Proc. Natl. Acad. Sci. USA* 81: 7897, 1984). Somatic antigen adsorbed to fumed SiO_2 was incubated for 5-6 days with normal BALB/c spelenocytes with- or without additional peritoneal macrophages in the presence of newborn mouse thymocyte-conditioned medium. An "induction period" of 6-8 hour in the absence of serum was used in an attempt to minimize competition by serum protein.

Several individuals involved in this Project have made fusions using basically the same procedure, however, with either no, or only temporary growth. Usually, fusions started well, with hybridoma growth in a high percentage of wells, however, colony growth almost invariably ceased and cells began dying within a few days or up to the first feeding. Several others gave no hybrid growth beyond the 4-8 cell stage. Since many of the fusion were either made or tended by persons in "training status", a common explanation was not sought for some time, however, repeated failure forced a more systematic investigation of the problem. There appeared to be no problems with the basic media, including the water ; none of the additives in HAT selection media (pyruvate, non-essential amino acids, antibiotics, etc.) showed inhibition, either tested individually or sequentiallly added. The complete HAT media fully supported growth of established hybrids and complete HT media supported cloning of the myeloma line 653. Nonetheless, the puzzling observation was that complete HAT media used in an unsuccessful fusion was toxic to HAT-

resistant hybridomas under both cloning and mass culture conditions. Working back from this observation, and using the hybridoma as assay for this strange toxicity, we eventually found that the myeloma cells or their 0.45μ filtered (but not 56°- inactivated or 0.1μ filtered) supernatant rendered HAT toxic, suggesting microbial contamination--likely mycoplasma. Finally, the 0.1μ filter-sterilized myeloma supernatant originally containing HT or simply T could not support hybridoma growth in the presence of A or HA unless supplemented with additional thymidine. The presumed mechanism of death then, is aminopterin-toxicity, as a result of high thymidine-(or pyrimidine-nucleoside-) phosphorylase activity of mycoplasmal origin, which effectively depletes HAT medium of T. We do not know whether such high activity is characteristic of all or most mycoplasmas, or is strain or species specific, however, a potential replacement myeloma obtained from another laboratory and maintained separately from all other cell lines in our laboratory has also failed this simple bioassay. We have obtained the myeloma 653 and Sp2/0 from 2 other laboratories and are testing them by this crude assay as well as their fusability, etc., and are at the same time attempting to "cure" another line through antibiotic treatment and ascites passage, despite the fact that cured lines may differ in many important ways from parent stocks.

Like many laboratories we have completely overlooked the potential problem of mycoplasma until compelled to confront it. We were aware that undetected mycoplasma can result in slower growth, altered morphology, or even cell death, and in the case of hybridomas, reduced fusability, but in the absence of any of these symptoms, we were not convinced that mycoplasmal contamination could present difficulties. Clearly, because of this biochemical effect, it can result in almost total failure of the system, with only a very few hybridomas surviving to the assay stage (we still do not know how these few managed to grow). This has temporarily setback the

hybridoma aspect of the project, however, knowing exactly what the problem was, we should be able to avoid repetition by routine screening. We hope that our frustration is now over and that we shall have several hybridomas to begin characterizing during the next period.

Activity 7: Attempt at cloning O. viverrini genes

(A) Isolation of O. viverrini from infected hamster and DNA preparation

Three to six month O. viverrini-infected hamsters were killed and the adult worms were taken from the biliary tract and gall bladder. These fresh worms were used for preparation of DNA. Briefly, 100-150 worms were disrupted by grinding in glass tissue grinder soaked in ice until a fine homogenate was obtained. The cold homogenate was centrifuged. The supernatant was discarded and the pellet was dissolved in 2x pronase buffer (10 mM Tris-10mM EDTA, 150 mM NaCl, pH 8). After mixing, an equal volume of extraction buffer containing 2% sodium dodecyl sulfate (SDS) together with 1 mg/ml of pronase was added and incubated for 3-4 hours at 37°C on rotator shaker (flip-flop). The lysate was extracted 5-6 times with water-saturated phenol followed by chloroform and ether. RNase A was added to a final concentration of 100 µg/ml and incubated at 37°C for 1 hour. The resulting solution was re-extracted with saturated phenol, chloroform and ether. This solution was finally adjusted to 0.3 M sodium acetate and 2 volumes of cold absolute ethanol was added. The mixture was kept at -20°C for at least 1 hour and centrifuged to collect DNA. The precipitated DNA was washed with 70% ethanol 3-5 times to remove salts, then dissolved in 500 µl TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). The purified DNA obtained from 100-150 worms was only about 15-20 µg.

(B). Plasmid preparation

E. coli JM 107 harboring pUC12 was grown in LB-broth containing 100 µg/ml of ampicillin at 37°C on a shaker until log phase. The plasmid was amplified by addition of chloramphenicol to a final concentration of 170 µg/ml and further incubated overnight. Culture broth was centrifuged to obtain a cell pellet. The pellet was suspended in 5 ml of 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8 containing 2 mg/ml lysozyme and incubated on ice for 30 minutes. Two volumes of freshly prepared 0.2 N NaOH- 1% SDS was added, gently mixed and further stood on ice for 30 minutes. The mixture was neutralized by 3-5 ml of 3 M sodium acetate pH 4.8 and centrifuged after keeping for one hour on ice. The supernatant was precipitated with two volumes of absolute ethanol and kept overnight at -20°C. DNA was pelleted by centrifugation, washed with 70% ethanol and dissolved in TE.

The plasmid solution was further purified by CsCl ultracentrifugation at 45,000 rpm for 18-20 hours at 20°C. The plasmid band was collected, washed, precipitated and then dissolved in TE.

(C) Construction of recombinant plasmids and transformation into *E. coli* JM 107

DNA from adult *O. viverrini* was partially cut by using therestriction enzyme Sau 3A to get small fragments about the size of 1 Kb. The DNA fragments were ligated at the Bam HI site of the bacterial plasmid pUC12 by using T4 DNA ligase. These recombinant plasmids were transformed into *E. coli* JM 107 using the DMSO method (Hanahan D. J. Mol. Biol. 166: 557, 1983) and identified for recombinant colonies by spreading on LB-Agar plate containing 100 ug/ml ampicillin together with 200 mM Isopropyl β-D-thiogalactopyranoside (IPTG) and 2% 5-bromo-4-chloro-3-idolyl β-D-galactopyranoside (x-Gal).

Recombinant plasmid construction was done using

plasmid pUC12 and parasite DNA at a ratio of 1:4. The concentration of plasmid was about 30 ng/µl. The results of the transformation experiment are shown in Table 1.

Table 1. Transformation of *E. coli* JM 107

Plasmid	Plasmid concentration	Efficiency (colonies/µg DNA)
pUC12	100 pg	1.59×10^{8}
recombinant plasmid	50 ng	8.42×10^{5}
	25 ng	2.52×10^{6}
	4 ng	4.10×10^{6}

Only 20% of the total transformants on IPTG + x-Gal plates showed white color. To check the average size of inserted DNA, white colonies were randomly picked for plasmid extraction and cut with EcoRI. By comparing restriction patterns with pUC12 in 0.7% agarose gel electrophoresis, the size of inserts was determined and found to vary from 0.1 - 0.7 kilobases or even only a few bases. Some colonies with white color but which did not have any insert resulted from vector deletion at the lac Z gene.

(D) Colony radioimmunoassay

White colonies from IPTG + x-Gal plates which contained recombinant plasmids (with parasite DNA inserts), were spotted in duplicate onto nitrocellulose paper (NC) and grown at 37°C overnight. Each NC was placed on 1% SDS-soaked filter paper for 15 minutes and then put in a chamber saturated with chloroform vapor for another 15 minutes to lyse

colonies. Cell debris was then removed by washing with phosphate-buffered saline (PBS) pH 7.4. All NC sheets were blocked by blotto solution (PBS + 5% skim milk pH 7.4) at room temperature for 2 hours to overnight on a shaker. The filters were then incubated with rabbit-anti-somatic antigen (dilution 1:200) for 1 hour, washed with blotto solution and incubated with iodinated protein A (^{125}I-protein A) for 1 hour with occasional shaking. After a final wash with blotto solution, NC sheets were dried and autoradiographed.

None of transformants gave positive results even though about 9,000 white colonies were screened.

E) Southern blotting and DNA hybridization

Each colony was grown in LB-broth containing 100 µg/ml of ampicillin at 37°C overnight and the plasmid was extracted using the rapid alkaline method (Nuc. Acid Res. 7:1513, 1979). Each ample was cut with the restriction enzyme EcoRI, electrophoresed in 0.7% agarose gel and Southern transfered to NC.

Genomic DNA of Q. viverrini to be used as a probe was labeled by nick-translation replacing dATP with 20 µCi/µl of ^{32}P dATP. Incorporation of ^{32}P dATP was about 40%. The probe was hybridized with DNA on NC at 42°C overnight and washed two times with 3 x SSC at room-temprature, 3 times with 0.1 x SSC - 0.1% SDS at 65°C then blot dried and autoradiographed.

Southern blot hybridization of randomly selected transformed clones using genomic DNA as a probe was performed in order to demonstrate whether Q. viverrini DNA had been inserted into the vector even though they did not express any product that could be detected by selected antiserum. It was found that some of them hybridized with the genomic DNA probe.

Problems

There are a few problems that we have encountered during this period. The major ones include:-

1. Uncertainty regarding naturally infected fish. We mentioned earlier in our Progress Report No. 1 that currently the source of parasite used in this project comes from naturally infected cyprinoid fish caught in the endemic areas. Fish bought from local market in Khon Kaen Province have given poor results. Only a low degree of infection was noted in these fish as a proportion of them may be caught from resevoirs which have very little parasites. We have to go to villages known to have high degrees of infection. Again this is unreliable as sometimes we get good results but not at other times. We are thinking of maintaining the parasites in the laboratory but this is not very practical as there are 2 intermediate hosts for this fluke and keeping both of them (snails and fish) in the laborary condition may have a lot of complications.

2. Monoclonal Antibodies. We have recently discovered that the myeloma line which we have been using in our fusions is contaminated with a mycoplasma strain which very rapidly converts thymidine to thymine. With this line, almost all hybrid cells have died shortly after fusion, the variability probably depending upon the recent history of the myeloma culture (lenght of time in culture since thawing or cloning where antibiotics were used) and our use of between 10-20% myeloma-conditioned media to support clonal growth, either collected under sterile conditions or 0.45/0.22 um filter-sterilized. We are at present attempting to overcome this obstacle by several methods in order of decreasing preference: to locate a mycoplasma-free myeloma from another lab, to attempt a "cure" using animal passage or anti-mycoplasmal drugs, or as last resort, to find some combination of drugs that will reduce contamination prior to fusion sufficient that gentamicin (or the extra antibiotics) hold down mycoplasmsa sufficient to allow hybride to grow the required short interval in the HAT/HT media. We would prefer to avoid this latter alternative, as our policy has been to use no

antibiotics in mass cultures of established hybridoma or myeloma lines. This prevents use of less careful technique through reliance on antibiotics, avoids eventual emergence of resistant strains of microorganisms, and largely excludes introduction of new mycoplasma of human origin, but obviously will not prevent culture-to-culture spread. Another disadvantage of continual use of antibiotics is that gentamicin, perhaps the most effective of these, is known to cause chromosomal damage to murine cells, but even a "cured" line may have sustained irreversible damage or altered properties from mycoplasma. We have obtained 3 myeloma lines from other laboratories for testing, however, with the limited number of local researchers using these lines, their unknown, but probable common origin, and the common practice of extensive antibiotic use without mycoplasma screening, perhaps attempting cures may be our most viable temporary alternative.

3. Cloning Qv Genes and Gene Fragments. Several problems have been encountered recently. These, and our approaches to overcome them are discussed in the following section under "Workplan for the next period".

Workplan for the next period

All activities initiated during the last period as well as this period will be continued. Particular effort will be placed on the production of monoclonal antibodies and on the recombinant DNA work. The latter include:-

1. Improvement of cell lysis method

The use of 1% SDS has been found to reduce the positive signal between antigens and antibodies. An alternative method of lysing the cells will be tested, for example, using lysozyme and DNase in the absence of or at the lowest amount of detergent.

2. Different reading frame cloning

The plasmids pUC8, pUC9 and pUC12 give 3 different reading frames when cut with Pst I. Cloning of the same DNA fragments in these 3 vectors should give a higher probability of expression for inserted pieces.

3. Cloning of larger DNA fragment

In our experiment the average size of inserts was about 500-800 bp. Such small fragment may not be enough to encode for any polypeptide chain or epitope that could react with our antibodies. Larger DNA pieces will be used for insertion in the Pst I sites of the pUC plasmid series. In addition, the cloning of larger DNA fragment using the expression vector, λgt 11, will be tested. This method will enable us to clone large fragments (about 8.0 Kb.) with high efficiency.

4. Problems with the amount and quality of worm DNA

DNA must be prepared from the very freshly isolated living worms picked from infected hamster, since frozen worms or the dead worms isolated from patients always gave very low yield and often smeared DNA. This result indicates that DNA is distroyed very quickly and efficiently by endogenous DNase soon after death of the worms. In order to obtain more DNA we needed to recover more worms from infected harmsters.

At present, we are preparing DNA from 100-200 worms at one time using the phenol extraction method ; the amount of DNA obtained is not sufficient for purification by CsCl gradient centrifugation. Our results in the cloning experiments may to some extent reflect the quality or purity of DNA prepared by our present procedure, since UV scans of extracted DNA always showed extrapeaks of unidentified material. This could contribute to the low transformation efficiency of our recombinant DNA, which is upto 2 orders of

magnitude lower than the pUC12 vector. In order to get better DNA we plan to obtain more adult fresh worms to isolate enough DNA for purification by ultracentrifugation.

Publications

Articles published since the initiation of this project are listed below. Six copies of each are enclosed herewith.

1. Sirisinha, S. Immunodiagnosis of human liver fluke infections: Asian-Pacific J. Allergy Immunol. 4:81-88, 1986.

2. Sirisinha, S. and Wongratanacheewin, S. Immunization of hamsters against Opisthorchis viverrini infection. Southeast Asian J. Trop. Med. Pub. Hlth. 17:567-573, 1986.

6. 392

Asian Pacific Journal of Allergy and Immunology (1986) 4 : 81-88

EDITORIAL

Immunodiagnosis of Human Liver Fluke Infections

Liver fluke infection is still an important endemic disease in many parts of the world. It is not unrealistic to estimate that at least 40-50 million people are currently being infected, and in some areas, the incidence is increasing. The three important parasites causing the disease are *Clonorchis sinensis*, *Opisthorchis felineus* and *Opisthorchis viverrini*.[1,2] Clonorchiasis is prevalent in China, Hong Kong, Korea, Taiwan, North Vietnam and Japan. It was estimated that more than 19 million people are infected with *C. sinensis*. Opisthorchiasis caused by *O. felineus* has been reported from the U.S.S.R. and central and eastern Europe. There are probably several million cases of *O. felineus* infection with a prevalence rate as high as 85% in some areas. On the other hand, *O. viverrini* infection in humans is found mainly in the northeastern and northern parts of Thailand, and in Laos, Cambodia and parts of Vietnam. Opisthorchiasis caused by *O. viverrini* is one of the diseases of public health importance in Thailand, as it has been estimated that at the present time there are at least 7 million people infected by this parasite, representing approximately 15% of the total population. The prevalence of *O. viverrini* infection among the population in northestern Thailand has risen from 3.5 million cases in 1965 to 5.4 million cases in 1981.[3] In some villages in the endemic areas, a prevalence of more than 90% has been reported. All age groups including infants are known to be infected.[4]

In the past, both opisthorchiasis and clonorchiasis have been reported sporadically from non-endemic areas including North America and Western Europe but at the present time increasing number of cases have been diagnosed. One main reason is due to ever increasing number of immigrants and refugees from less-developed countries within the last few years.[5-8] Another possible reason is the importation of fish from the endemic areas. For example, the disease has been reported in native Hawaiians as a result of consumption of infected fish imported from endemic areas.[2]

Clinical manifestations of patients infected with liver flukes vary considerbly depending on the intensity and duration of infection.[9] The majority of cases are well tolerated and asymptomatic, and are diagnosed based on routine stool examination. In those with symptoms, there may be only intermittent dull pain with slight discomfort. However, in more severe and chronic infection, there can be biliary obstruction, cholangiocarcinoma and even death.

Adult flukes inhabit the biliary system of man. The early pathological changes consist of an acute inflammatory reaction of the bile duct and the portal connective tissues.[9] Periductal inflammation with mononuclear cell and lymphocyte infiltration has been observed. These pathological changes can be the result of worm movement, of toxic metabolites released from the flukes or of immunological response of the host. The latter has been amply demonstrated, particularly with regard to the humoral immune response. On the other hand, immunopathological changes similar to egg granulomas in schistosomiasis have never been well documented in the liver fluke infection.

Current methods for the diagnosis of human liver fluke infection are based on the demonstration of eggs in either stool, duodenal fluid or bile.[1,4,10] Although such an examination is reliable and permits the identification of species, it is unfortunate

that such a technique is useful only when the intensity of infection is high and is reliable only in the hands of experienced personnel. It is easy to diagnose cases with heavy infection when several thousand eggs per g of feces are present. However, the diagnosis of light infection based on stool examination is uncertain, can be easily missed and may vary considerably from one sample to another. Moreover, a decision is even more difficult to make when samples are from populations with mixed parasitic infections, *i.e.*, eggs from *O. viverrini* are difficult to distinguish from those, of small intestinal flukes, particularly by those who are rather ، experienced. These flukes include, for example, *Prosthodendrium molenkampi, Phaneropsolus bonnei, Haplorchis pumilio* and *H. taichui*.[11] Moreover, in infections sufficiently severe so as to result in biliary obstruction, eggs will not be recovered in the feces. In order to identify eggs in the latter situation, one has to examine for the presence of eggs in cannulated bile. However, such a technique is not practical and is not without danger. These drawbacks can be minimized if one is aware of the problem and when experienced clinicians and parasitologists are available. However, nowadays with mass migration of people from endemic areas (*e.g.*, immigrants and refugees from Far Eastern or Southeast Asian countries) to non-endemic areas such as North America and Europe, difficulty in establishing diagnosis based on morphological stool examination for fluke eggs can be a problem. Therefore, if techniques or reagents for identification of eggs, (as by immunofluorescent antibody specific for fluke eggs or ELISA for soluble parasite antigens) or immunodiagnosis based on detection of specific antibody in serum or secretions of suspected patients are available, the problem of diagnosis should be alleviated.

Immunodiagnosis of helminthic infections

To circumvent the tedious, time-consuming and frequently impractical microscopic examination, many parasitologists have now turned to immunodiagnostic procedures such as intradermal and serological tests as well as antigen detection for many helminthic infections. Intradermal tests of both immediate and delayed types have been used in several helminthic infections. Generally, these intradermal tests have rather poor specificity and thus have limited value for the diagnosis of individual cases although they may be of some value in epidemiological surveys. Moreover, standardization and interpretation of results are not yet satisfactory.

Unlike the intradermal tests, serological tests based on analysis of serum specimens are more popular, and have been widely used in parasitological research during the last 10-20 years. Within certain limitations, serological tests are invaluable aids to diagnosis of individual cases, for epidemiological surveys, and to aid in the evaluation of control measures. Up to now most of the standard serological procedures have been used in the diagnosis of helminthic infections.[12] These include complement fixation, flocculation, indirect hemagglutination, indirect immunofluorescence, immunodiffusion, immunoelectrophoresis, countercurrent electrophoresis, radio-immunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). In addition to these standard tests, some special tests have also been developed, *e.g.*, circumoval precipitation, circumsporozoite precipitation, " cercarienhullen " reaction, fluoroimmunoassay (FIAX) and miracidial immobilization. Newer procedures like the indium slide immunoassay have been modified for use in parasitic infections. Many of the tests mentioned above are now available commercially as preformed kits ready to be used. However, it is unlikely that others will be available in the near future as commercial enterprises are generally not too eager to develop them due to the low commercial benefit, as these diseases largely involve people of low economical status. Up until a few years ago, the complement fixation test was one of the most reliable and most widely used, for example, in trichinosis, filariasis and schistosomiasis. However, the test has to be performed under optimal conditions and requires experienced supervision. A further complication is that many antigens used, as well as a proportion of clinical specimens have anticomplementary activity. A large number of investigators have recently turned to ELISA as a possible new diagnostic tool for parasitic diseases, many of which have already been thoroughly evaluated and seem to give highly satisfactory results. Moreover, the two recent developments, namely hybridoma and recombinant DNA technique, will undoubtedly contribute to the future progress in this area of investigation.

The major problems about immunodiagnosis in parasitic diseases are their relatively poor sensitivity, low specificity and the non-availability of appropriate antigens. Nevertheless, it is to be expected that with new technology, these problems will soon be alleviated. Much attention has been given to the purification and production of sufficient quantities of antigens for the test, as both the nature and the source of these antigens contribute significantly to the success of these immunological proceduces. In the past, investiga-

tors had used the stage in the life cycle which can be most easily obtained in large quantities. However, this practice does not always provide the best antigen for immunodiagnosis. One of the main difficulties in developing reliable diagnostic tests in parasitic diseases lies in the complex nature of the antigens used for such a purpose. Complexity in this case involves not only the multicellular nature of the parasites themselves but also involves changes in the nature of antigens as the parasite develops from one stage to another in the same mammalian host. Therefore, when such a complex antigen is used for detection of antibody, phylogenetically related species, or sometimes even unrelated parasites, may give rise to cross-reacting antibodies. Attempts have been made with some success to prepare and purify antigens from phylogenetically related parasites of animals which may be obtained in much larger quantity. One should keep in mind however that the use of cross-reacting antigens may be inadequate for making reliable diagnosis.

Different parasite components have been use as antigens for immunodiagnosis of parasitic infections of man and animals. In general, these antigens are grouped into 3 main categories, namely somatic, excretory and secretory (ES or metabolic) including egg, and surface antigens.

Somatic antigens.

These antigens are highly complex. They are commonly prepared and used in the form of aqueous extracts from appropriate developmental stages that are available in sufficient quantity, *e.g.*, adult stages. After homogenization and brief sonication, the supernatant containing crude somatic conponents and stored metabolic products is dialyzed and lyophilized for storage. Some investigators have treated the worms with ether, prior to aqueous extraction, to eliminate lipids which may interfere with some serological procedures. However, these protocols sometime destroy labile parasite components. Various methods have been used to partially purify the crude somatic preparation and these include ion exchange chromatography, gel-filtration, electrophoresis, electrofocusing and alcohol precipitation. On a few occasions, affinity chromatography using enzyme inhibitors, lectins, and antibodies have also been employed to purify some special parasite components from the crude or partially purified preparations. Elimination of common or cross-reacting parasite antigens or host components has been achieved by affinity chromatography using polyclonal cross-reacting antibodies or anti-host components respectively. Recently however, opportunity to purify antigens using appropriate monoclonal antibodies is also available. The latter approach has very much simplified the purification of appropriate antigens which may be present only in trace quantity in the crude material. The availability of purified antigens has made immunodiagnosis of parasitic diseases as specific and sensitive as has been achieved with bacterial and viral diseases. Moreover, the identification of suitable parasite-specific antigens and the availability of monospecific antibodies, either monoclonal or polyclonal, open the possibility of producing diagnostic peptides containing important parasite epitopes in sufficient quantity, *e.g.*, by recombinant DNA technology.

Metabolic or excretory-secretory (ES) antigens.

As helminths develop from the infective larval stage to the adult stage, they have undergone metabolic changes during the course of infection, thus presenting different antigens to the host. It is reasonable to assume therefore, that the antibodies produced in response to these products are more specific and more closely related to active infection than those elicited by somatic antigens. The ES antigens have been used for the detection of antibodies by several standard serological procedures and for many of these infections they prove to be highly specific and are sensitive enough for immunodiagnostic purposes.[13] Chemically, they are in general less complex than somatic antigens but for those that have been well analyzed they are still quite antigenically complicated.[14] These components are largely enzymic in nature and are often the products of reproductive and digestive systems. They can be obtained in large quantity during the *in vitro* culture or maintenance of parasites in the laboratory. Some parasites produce and release sufficient amounts of ES into the culture fluid that they can be used without further concentration. One difficulty is that not all parasites can be cultured or maintained *in vitro* long enough to release sufficient amounts of ES antigens. In addition, for some parasites, protein supplement is required and may interfere with later steps of purification. These ES antgens can also be recovered in large quantity in the somatic extracts of these parasites.

In addition to these soluble metabolites, many parasites release eggs which are potent immunogens, *e.g.* those of Schistosomes. Both humoral and cell-mediated immune responses to egg antigens have been reported for several parasitic infections. These responses are also known to be associated with the immunopathology of seveal diseases, *e.g.*, egg granuloma in schistosomiasis. Moreover, the

21

antibodies to these antigens may be protective and are at times useful in immonodiagnosis. Antibodies to liver fluke eggs have not been successfully detected in experimentally infected rats, guinea pigs and rabbits nor in the serum of patients with clonorchiasis.[15-17] However, Flavell[18] and our group (unpublished) have shown that antibodies to egg antigens are present in the serum of infected hamsters. Because it was possible to raise antibodies to egg antigens by immunizing the animals with the egg extract, such antibodies may be of value from a diagnostic point of view, particularly if monoclonal antibodies to these antigens are available. Recently, sera from patients with opisthorchiasis were shown to react with *O. viverrini* eggs by an immunofluorescent technique.[19]

Surface antigens.

Teguments of many parasites are metabolically active. Surface protein serves as a dominant antigen and has a likelihood of stimulating the host immune system. Therefore, many investigators have used the external surface of parasites or external membranes of infected host cells for their studies. For some parasitic infections, these external surface components not only serve as host " protective " antigens but also are of value in immunodiagnosis. Various methods have been used to extract antigens from the surface of parasites or parasite-infected cells. Peripheral proteins can be solubilized with high ionic strength buffer or metal chelators. Integral membrane proteins, on the other hand, require more vigorous procedures. In general, this has been achieved with detergents, organic solvents or denaturing agents like urea or guanidine or a combination thereof. The use of non-ionic detergents is preferred by most investigators as they are mild enough not to denature these surface proteins. These extracts can be used as such or can be fractionated further via, for example, affinity chromatography, high performance liquid chromatography or isoelectrofocusing.

Although the above classification of parasite antigens may seem to be straightforward, a wide grey area exists. Common antigens have been repeatedly demonstrated among phylogenetically distinct species of parasites, among different developmental stages of the same or related parasites, and among different tissues in any one developmental stage.[20-24] The wide specificity that exists suggests the existance of common antigenic pools having small antigenic determinants with simple molecular configuration, as has been noted in malaria.

Assuming that reliable antigen is now available, the next step is to select an appropriate serological test. Unlike bacterial and viral infections, parasitic infections are more insidious and chronic in nature. Therefore, detection of IgM antibody is rarely needed and most tests involve the detection of IgG antibody. If the antigen is not pure enough, then cross-reaction with other parasitic infections can be a problem. The latter could however be minimized if other antibody isotypes are determined instead of IgG. For instance, for helminthic infections which generally have a potentiated IgE response, detection of specific IgE antibody may be more useful. For mucous surface membrane infections, the detection of IgA or IgE antibodies may be more specific than that of IgG antibody and is therefore more useful diagnostically. On occasion, however, the high concentration of IgG antibody present in the serum of these patients may interfere with the quantitation of IgA or IgE antibody. In such a situation, certain modifications have to be made to minimize this. When appropriate, one can use other fluids which have low concentration of IgG but higher concentration of other immunoglobulin classes. One possibility is, for these mucosal intections, to use external fluids instead of serum. Saliva can be a good candidate because it has a minimal amount of IgG and has a fairly high concentration of IgA.

Although detection of antibody is quite popular and is highly practical both for individual cases and for epidemiology surveys particularly when a paper-disc method for blood collection is used, it does not indicate active infection. All tests still give positive results soon after infection has been terminated, *i.e.*, following anthelmintic therapy. However, if a sufficient period of time is allowed before retesting, some tests will then give a negative result. For this purpose however, it is better to look for the presence of parasite antigen or immune complexes. These antigens can be detected in the circulation and urine, for example, in schistosomiasis. More frequently, they can be more easily detected in fluids within circumscribed spaces like cystic fluid or cerebrospinal fluid (*e.g.*, cysticercosis) In addition to these examples, parasite antigens have also been demonstrated in external secretions, *e.g.*, bile and intestinal secretions.[16] Many standard serological tests have been employed and these include immunodiffusion and ELISA. The concentration of antigen in these fluids depends primarily on worm burden. However, with the current serological methods like RIA or ELISA, antigens in nanogram or picogram amounts can still be detected, particularly when monoclonal antibodies to many of these antigens are or will be available in the near future.

22

Previous efforts to develop immunodiagnostic methods for liver fluke infections

It has been more than 20 years since investigators first attempted to develop immunodiagnostic methods for human liver fluke infections caused by *C. sinensis*, *O. felineus* and *O. viverrini*.[25,26] These early attempts made use of immunological procedures available at that time, *e.g.* intradermal skin tests, complement fixation, precipitation reaction, as well as various modifications of agglutination reactions. The species of parasite most intensively investigated during that early period was *C. sinensis*. Subsequently, different groups of Russian investigators have used these techniques to approach the problem caused by *O. felineus*.[27-30] On the other hand, investigation on *O. viverrini* has not started until quite recently.

The principal obstacles to progress in this area of investigation are due to insufficient knowledge concerning the immunology of these parasites, inadequate quantities of appropriate parasite antigens available for intensive investigation and strong immunological cross reaction between these flukes and other parasites, some of which are phylogenetically unrelated to them. The last problem can be automatically eliminated when the others have been solved. As can be seen, the antigens available during the early attempts were soluble aqueous crude extracts of adult worms which could be obtained readily in sufficient quantity either from the patients or from animals experimentally infected with these flukes. The early studies on *C. sinensis* therefore used these buffered saline crude somatic extracts for intradermal tests, complement fixation assays, and precipitation reactions. It was subsequently recognized that these aqueous extracts were not suitable for the complement fixation test as they often exhibit anticomplementary activity, and the extraction procedure was therefore modified. Such an interference could be largely eliminated by the use of fat-free antigens which could be readily obtained by ether extraction prior to the preparation of the crude somatic extract. Interference in the complement-fixation test could be further minimized by partial purification through the use of ion-exchange chromatography and gelfiltration.

Like other intradermal skin tests, skin testing in clonorchiasis suffers from the same reasons, including standardization of antigen and subjective interpretation of the results. Moreover, the test using the various crude somatic extracts gave a considerable degree of cross reaction with other parasites including paragonimiasis and schistosomiasis.[21,25] The test therefore has been abandoned by most recent investigators.

Most of the previous attempts on immunodiagnosis for *C. sinensis* infection have been concentrated largely on complement fixation reactions. Although difficulties arising from anti-complementary activity of the antigens have been satisfactorily overcome, the difficulty with inherent cross reaction among different parasite still exists. The latter makes it rather difficult to interpret the results, particularly when people in endemic areas are often infected simultaneously by other intestinal or tissue parasites. This situation is even worse when the previous history or fecal specimens are not available for confirmation. Sawada *et al*[31] attempted to purify the crude somatic extract for CF test by passing the delipidated aqueous and sonicated extract through a Sephadex G-100 column and then passing the void volume fraction through ion-exchange columns. The final preparation, more than 90% carbohydrate and later identified to be polyglucose, gave a satisfactory result in the CF test. However, the specificity of this antigen preparation was never rigorously analyzed. On top of this, complement-fixation reactions also suffer from the lability of the reagent, difficulties in standardization and the tedious protocols, thus making procedures based on CF rather unpopular for diagnostic laboratories, particularly when more simple and accurate techniques are now available.

The use of precipitation reactions as in the gel diffusion technique have been studied by a few laboratories. The partially purified CF antigen of Sawada *et al*[31] failed to give a precipitin reaction with patient sera. Subsequently, Sun and Gibson[16] showed strong precipitation reactions using concentrated metabolic products of adult worms. These metabolic products contained both carbohydrates and proteins. Enzyme activities associated with digestion including amylase, invertase, maltase, lactase and esterase have been detected. Attempts to use egg antigens have been reported by Sun,[15] and Sun and Gibson.[17] While these investigators could not demonstrate by precipitin reaction the presence of anti-egg antibodies in the serum of patients and experimentally infected animals, egg components were nevertheless antigenic, as shown by the presence of antibodies in animals immunized with egg extract. We have also observed that serum from infected hamsters and from rabbits immunized with either adult crude extract or metabolic products reacted with eggs released by the worms *in vitro* (Unpublished observations).

Attempt has also been made to use an indirect hemagglutination test for the diagnosis of clonorchiasis.[32] These investigators used triethanolamine buffered saline extract of delipidated crude somatic antigen to coat tanned sheep red blood cells and found it to be fairly sensitive, using sera from rabbits experimentally infected with *C. sinensis*. However, its value in clinical diagnosis has not yet been evaluated.

Passive cutaneous anaphylaxis for the detection of reaginic antibody in rabbits experimentally infected with *C. sinensis* has given encouraging results.[21] These investigations, using borate-buffered saline extract of adult worms, showed the assay to be fairly sensitive in experimental animals. A positive reaction, suggestive of the presence of reagin or IgE antibodies, was observed in some animals within 3 weeks of infection. However, this test is impractical for the diagnosis of the disease. If the appearance of IgE antibody is expected, its detection by other means such as ELISA or RIA will be more practical, and this point should be investigated.

Very recently the use of ELISA for the diagnosis of human clonorchiasis has been reported in Chinese literature.[33] These investigators compared the reactivity of various aqueous crude somatic extracts, *i.e.*, triethanolamine buffered saline, veronal buffered saline and phosphate buffered saline, and showed all 3 to be quite satisfactory in distinguishing patients from normal healthy controls. Moreover, a fairly good correlation between ELISA values and worm burdens expressed as eggs per gram of feces was noted. Although not yet vigorously investigated, the antigens used by these investigators seem to be specific when tested against a limited number of sera from patients with other parasitic infections. It could not be determined from the data available as to the composition or complexity of these crude antigens.

In contrast to clonorchiasis, investigation on opisthorchiasis, particularly that caused by *O. viverrini* is more scanty. What information regarding the immunodiagnosis of opisthorchiasis is available pertains largely to *O. felinous*.[27-30] The two techniques used were the indirect hemagglutination reaction and ELISA. All work employed crude somatic extracts of adult *O. felineus*. The reports dealing with indirect hemagglutination showed that it was possible to diagnose acute cases of infection by this technique.[27,28] Antibody was detected in 53 of 54 patients with acute infection but it was positive in less than 50% of patients with chronic infection. However, many healthy controls also gave low antibody titer with this antigen. On the other hand, Ponomareva,[29] and Ponomareva and Alekseeva[30] comparing intradermal tests, indirect hemagglutination and ELISA using crude somatic extract of adult *O. felineus*, found ELISA to be the best, and results seemed to correlate satisfactorily with the intensity of infection. Using an ELISA technique, these investigators were able to distinguish acute from chronic infections. Patients with acute infection had high IgM ELISA titers and relatively low IgG ELISA titers. A reversed situation was true for those with chronic infection. However, there was no information available regarding specificity of the test.

In contrast to opisthorchiasis caused by *O. felineus*, only limited information is currently available on attempts at immunodiagnosis of opisthorchiasis caused by *O. viverrini*. Janechaiwat *et al*[34] were able to detect many bands of precipitation in the sera of patients infected with *O. viverrini* by immunoelectrophoresis. Sera from some patients showed as many as 5 bands with this antigenic extract. However, the test lacked sensitivity as less than 80% of the patients whose stools were positive for *O. viverrini* eggs gave positive results. The proportion of positive specimens decreased with decreasing intensity of infection. More serious difficulty was the fact that some sera from patients with other parasitic diseases including gnathostomiasis and schistosomiasis also reacted positively in this technique. Using indirect hemagglutination, we were able to detect agglutinating antibody against crude somatic extract in the sera from all hamsters infected with *O. viverrini* 30 days beforehand.[35] However, a proportion of these infected hamsters, particularly those with high level of infection exhibited some antibody within 15 days of infection. Because the antibody titers obtained by this technique were not very high, we felt that the test was not sensitive enough to be of clinical usefulness and therefore needed to be improved. Feldheim and Knobloch reported satisfactory results using ELISA tchnique.[24] Although they were able to distinguish the patients form uninfected controls, a considerable degree of cross-reaction was detected, including patients infected with *F. hepatica*, *P. africanus*, *P. uterobilaberalis* and *Schistosome* species. Attempt to use this technique to monitor the success of anthelminthic treatment gave equivocal results, as in about 50% of the cases, no significant reduction in antibody titer was noted in speimens taken 12 months after treatment. Using ELISA technique, we recently observed that while it was possible to distinguish patients with positive fecal egg counts from normal, the technique used could not clearly

24

distinguish these patients from those with other parasitic infection.[36] However, it was not possible to rule out the possibility that the latter group might have had previous contact with these flukes. Using an appropriate animal model would give a more clear cut answer to this question. A similar conclusion was reached also by other groups using ELISA[37] and immunofluorescent techniques.[19]

Current approaches to develop immunodiagnostic methods for opisthorchiasis

It is clear form these studies that if one is to have a more specific serological test for the diagnosis of parasitic infections, including those caused by liver flukes, one needs a more refined antigen for whatever test system is used to detect the specific antibody. However, before this can become a reality, one has to know fairly extensively the antigenic mosiac of the parasite under study. This, in turn, is possible only when the host-parasite relationship is more fully understood. In attempting to do this, we have now begun to characterize the various antigens from *O. viverrini* and have preliminary data suggesting the presence of specific parasite antigens which may have serodiagnostic value.[38] Once a specific antigen (s) is identified and characterized, one has to think about mass production. The latter has to be cheap as the disease occurs largely in developing countries. In this regard, it is likely that the genetic engineering approach could be used, since it has made possible the availability of several other biological reagents for medical use.

Last but not least, it is also possible to develop an immunodiagnostic technique for the detection of *O. viverrini* antigens in those suspected of being infected with the parasite. The most likely possibility is a specific reagent for the detection of eggs or other soluble products in the feces of these people. Here monoclonal antibodies could play a significant role. These various approaches will be employed by our group in an attempt to develop suitable immunodiagnostic methods which would be specific for infection caused by *O. viverrini*. If our attempt is successful, then it should be possible for other investigators to use a similar approach for infections caused by other parasities, *i.e.*, *O. felineus* and *C. sinensis*.

Conclusion

Liver fluke infections caused by *Opisthorchis viverrini*, *O. felineus* and *Clonorchis sinensis* are still a major health problem in Southeast Asia, the Far East, and Central and Eastern Europe, with approximately 40-50 million people currently infected. Moreover, infections have been reported recently from several countries. Moreover, infections have been reported recently from several countries previously known to be free of the disease. The latter is attributed, at least in part, to ever increasing numbers of immigrants and refugees from countries in the endemic regions. Because of this increasing occurrence of the disease, clinicians and laboratory personnel in both endemic and non-endemic regions must be more aware of the possibility of opisthorchiasis and clonorchiasis in patients with gastrointestinal problems. It is for this reason that a simple, reliable, less-time consuming and more objective method for diagnosis is needed. Identification and large-scale production of specific antigen (s) should be initiated and used for the development of suitable immunodiagnostic methods for the detection of specific antibodies, either in the serum or other body fluids. The screening and production of monoclonal antibodies specific for parasite epitopes should be encouraged and made available for the detection of specific parasite antigen (s). If these various immunodiagnostic methods that are to be developed can be supplied in quantity cheaply, they would undoubtedly replace the more classical, time consuming and more subjective methods currently available. Such a development would also be an invaluable tool for epidemiology studies and for monitoring the outcome of anthelmintic treatment.

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REFERENCES

1. Komiya Y. *Clonorchis* and clonorchiasis. Adv Parasitol 1966 ; 4 : 53-106.
2. Markell EK, Voge M. Medical parasitology. 5th ed. Philadelphia : W.B. Saunders, 1981.
3. Preuksaraj S, Jeradit C, Sathitayatai A, Kijvanee S, Sridonrasmi T. Studies on prevalence and intensity of intestinal helminthic infection in rural population of Thailand 1980-1981. Commun Dis J 1982 ; 8 : 245-69.
4. Harinasuta C. Opisthorchiasis in Thailand : A review. In Harinasuta C, ed., Proceediing of the 4th Southeast Asian Seminar on Parasitology and Tropical Medicine, Schistosomiasis and Other Snail-transmitted Helminthiasis. 1969 ; 253-64.
5. Anderson JP, Moser RJ. Parasite screening and treatment among Indochinese refugees. Cost-benefit/utility and the general health policy model. JAMA 1985 ; 253 : 2229-35.
6. Barret-Connor E. Latent and chronic infections imported from Southeast Asia. JAMA 1978 ; 239 : 1901-6.
7. Catanzaro A, Moser RJ. Health status of refugees from Vietnam, Laos, and

25

Cambodia. JAMA 1982 ; 247 : 1303-8.
8. Hoffman SL, Barrett-Connor E, Norcross W, Nguyen D. Intestinal parasites in Indochinese immigrants. Am J Trop Med Hyg 1981 ; 30 : 340-3.
9. Proceedidngs of the International Symposium on Human Trematode Infections in Southeast and East Asia. Drug Res 1984 ; 34 : 1115-242.
10. Feldmeier H, Horstmann RD. Filtration of doudenal fluid for the diagnosis of opisthorchiasis. Ann Trop Med Parasitol 1981 ; 75 : 463-5.
11. Radomyos P, Bunnag D, Harinasuta T. Worms recovered in stools following praziquantel treatment. Drug Res 1984 ; 34 : 1214-7.
12. Cohen S, Sadun E. Immunology of parasitic infections. Oxford : Blackwell Scientific Pub. 1976.
13. van Knapen F, van Leusden J, Polderman AM, Franchimont JH. Visceral larva migrans : Examinations by means of enzyme-linked immunosorbent assay of human sera for antibodies to excretory-secretory antigens of the second-stage larvae of *Toxocara canis*. Z. Parasitenkd 1983 ; 69 : 113-8.
14. Pollard ZF, Jarrett WH, Hagler WS, Allain DS, Schantz PM. ELISA for diagnosis of ocular toxocariasis. Ophthalmology 1979 ; 86 : 743-52.
15. Sun T. The non-antigenicity of intact ova of *Clonorchis sinensis*. J Med Microbiol 1969 ; 2 : 358-60
16. Sun T, Gibson JB. Antigens of *Clonorchis sinensis* in experimental and human infections. An analysis by gel-diffusion technique. Am J Trop Med Hyg 1969 ; 18 : 241-52.
17. Sun T, Gibson JB. Metabolic products of adult *Clonorchis sinensis*. Their composition and antigenic potential. J Helminthol 1969 ; 43 : 395-402.
18. Flavell DJ. The *in vitro* effects of serum on the adults, metacercariae, and eggs of *Opisthorchis viverrini*. Trans R Soc Trop Med Hyg 1981 ; 75 : 825-31.
19. Boonpucknavig S, Kurathong S, Thamawit W. Detection of antibodies in sera from patients with opisthorchiasis. J Clin Lab Immunol 1986 ; 19 : 135-7.
20. Hillyer GV, Serrano AE. The antigens of *Paragonimus westermani*, *Schistosoma mansoni*, and *Fasciola hepatica* adult worms. Evidence for the presence of cross-reacive antigens and for cross-protection to *Schistosoma mansoni* infection using antigens of *Paragonimus westermani*. Am J Trop Med Hyg 1983 ; 32 : 350-8.
21. Kojima S, Yokogawa M, Tada T.. Production and properties of reaginic antibodies in rabbits infected with *Clonorchis sinensis* or *Schistosoma japonicum*. Exp Parasitol 1974 ; 35 : 141-9.
22. Maizels RM, Partono F, Oemijati S, Denham DA, Ogilvie BM. Cross reactive surface antigens on three stages of *Brugia malayi*, *B. pahangi* and *B. timori*. Parasitology 1983 ; 87 : 249-63.
23. Maizels RM, de Savigny D, Ogilvie BM. Characterization of surface and ES antigens of *Toxocara canis* infective larvae. Parasite Immunol 1984 ; 6 : 23-37.
24. Feldheim W, Knobloch J. Serodiagnosis of opisthorchis viverrini infestation by an enzyme immunoassay. Tropenmed Parasitol 1982 ; 33 : 8-10.
25. Sadun EH, Walton BC, Buck AA, Lee BK. The use of purified antigens in the diagnosis of *Clonorchis sinensis* by means of intradermal and complement fixation tests. J. Parasitol 1959 ; 45 : 129-35.
26. Kagan IG. Serodiagnosis of parasitic diseases. In Rose NR, Friedman H, eds, Manual of clinical immunology. Washington, D.C.: American Society for Microbiology 1980 : 578-604.
27. Klebanovskaya IA. IHAT in the diagnosis of the early phase of opisthorchiasis. 1. Techniques for preparing an erythrocyte diagnostic antigen and results of tests in experimental opisthorchiasis in golden hamsters. Medisins Parazitol i Parazita Bolezni 1981 ; 50 : 20-3.
28. Klebanovskaya IA, Gorchakov VV. IHAT in the diagnosis of the early phase of opisthorchiasis 2. Clinical investigations. Medisins Parazitol i Parazita Bolezni 1981 ; 50 : 34-6.
29. Ponomareva AM. ELISA in the diagnosis of parasitic diseases. 4. A test system for the diagnosis of opisthrochiasis. Medisins Parazitol i Parazita Bolezni 1981 ; 50 ; 28-30.
30. Ponomareva AM, Alekseeva MI. Compared efficacy of several immunological tests in diagnosis of acute and chronic opisthorchiasis. Helminthol Abstracts-series A 1984 ; 53 : 151.
31. Sawada T, Takei K, Williams JE, Moose JW. Isolation and purification of antigen from adult *Clonorchis sinensis* for complement fixation and precipitin tests. Exp parasitol 1965 ; 17 ; 240-9.
32. Pacheco G, Wykoff DE, Jung RC. Trial of an indirect hemagglutination test for the diagnosis of infections with *Clonorchis sinensis*. Am J Trop Med Hyg 1960 ; 9 : 367-70.
33. Yen CM, Chen ER, Hsieh HC. Immunodiagnosis of human clonorchiasis by a micro-enzyme-linked immunosorbent assay. Chinese J Microbiol Immunol 1984 ; 17 : 219-25.
34. Janechaiwat J, Tharavanij S, Vajrasathira S, Chaicumpa W. The immunological diagnosis of human opisthorchiasis and humoral immune response to opisthorchis infection in the hamster. J Med Assoc Thailand 1980 ; 63 : 439-47.
35. Sirisinha S, Tuti S, Vichasri S, Tawatsin A. Humoral immune responses in hamsters infected with *Opisthorchis viverrini*. Southeast Asian J Trop Med Pub Hlth 1983 ; 14 : 243-51.
36. Wongratanacheewin S, Bunnag D, Sirisinha S. Enzyme-linked immunosorvent assay for detection of antibodies to *Opisthorchis viverrini*. Asian Pacific J Allergy Immunol 1985 ; 3 : 114.
37. Srivatanakul P, Viyanant V, Kurathong S, Tiwaweeh D. Enzyme-linked imunosorbent assay for detection of *Opisthorchis viverrini* infection. Southeast Asian J Trop Med Pub Hlth 1985 ; 16 : 234-9.
38. Wongratanacheewin S, Sirisinha S. Analysis of *Opisthorchis viverrini* antigens by radioimmunoprecipitation. Asian Pacific J Allergy Immunol 1986 ; 4 : 62.

26

6. 392

IMMUNIZATION OF HAMSTERS AGAINST *OPISTHORCHIS VIVERRINI* INFECTION

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INTRODUCTION

Liver fluke infection is still a major health problem in Southeast Asia, the Far East, and Central and Eastern Europe, with at least 30-50 million people currently infected. In Thailand alone, it has been estimated that at least 7 million people are infected with *Opisthorchis viverrini* (Preuksaraj, 1984). Thus, human suffering and economic loss due to illness, decreased ability to earn a proper livelihood and cost of health care represent major hindrances and a challenge to national and regional development.

Infections with *O. viverrini* rarely result in acute clinical disease. Rather the infections tend to become chronic in nature and may persist for many years (Viranuvatti and Stitnimankarn, 1972; Harinasuta *et al.*, 1984). Such chronic, persistent infections can lead to cholangitis and in some cases to cholangiocarcinoma (Sonakul *et al.*, 1978; Flavell, 1981; Kurathong *et al.*, 1985). It has been noted that some patients from the endemic areas may harbor several thousand worms (Bunnag and Harinasuta, 1981), suggesting that reinfections do occur and that concurrent infection fails to prevent reinfection by the same parasite. Such a conclusion was subsequently confirmed in an experimental animal model (Sirisinha *et al.*, 1982; 1983a). Data from these experimental studies showed that prior infection of hamsters with *O. viverrini* did not induce significant protective immunity against reinfection by the same parasite, although under certain circumstances, worm burden due to the challenge infection in animals harboring prior infection was reduced up to 25% when they were compared with animals without preexposure (Flavell, 1982). It is possible that both systemic humoral and cell-mediated immune responses developed during the course of infection (Bhamarapravati *et al.*, 1978; Sirisinha *et al.*, 1982, 1983b; Boonpucknavig *et al.*, 1986) fail to damage or to eliminate worms residing in the bile duct system. That system could serve as an immunological priviledged site where systemic response can only marginally influence the parasites.

The purpose of the present study was to examine whether protective immunity could be induced in hamsters immunized with different *O. viverrini* antigens via a route known to be effective in stimulating local immune response in the gastrointestinal tract of other animal models.

MATERIALS AND METHODS

Antigen: Metacercariae (Mc) of *O. viverrini* were obtained from the flesh of naturally infected cyprinoid fishes as previously described (Sirisinha *et al.*, 1984). After thorough washing in phosphate-buffered saline pH 7.2 (PBS), the metacercariae were homogenized and sonicated to extract aqueous somatic antigen essentially as described previously for adult worm antigen (Sirisinha *et al.*, 1983b).

Adult worms were obtained from experimentally infected hamsters. They were maintained *in vitro* in protein-free medium for the production of excretory-secretory (ES)

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antigen as previously described (Tuti *et al.*, 1982). Aqueous somatic antigen was subsequently extracted from these cultured adult worms.

Protein contents of these antigens were determined by the Folin method (Lowry *et al.*, 1951) using bovine serum albumin as standard. The complexity of each preparation was analyzed by SDS-PAGE (Dharmkrong-at and Sirisinha, 1983) and is shown in Fig. 1. The somatic antigen was considerably more complex and heterogeneous comparing with the ES and metacercarial antigens. The main protein component (MW = 89,000 daltons) in the latter two antigenic preparations represented a minor component in the somatic extract.

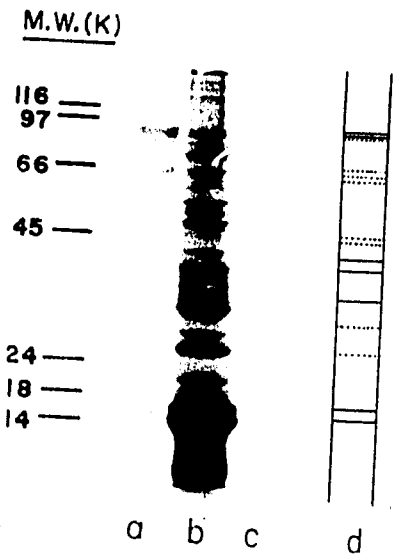

Fig. 1—SDS-PAGE Coomassie-blue stained profiles of ES (a), somatic (b) and metacercariae (c) of *O. viverrini*. The right hand diagram (d) represents that of the metacercariae which took up stain weakly. Molecular weight markers indicated on the left.

In one experiment, whole adult worm homogenate containing both soluble and insoluble materials was also used to immunize the animals. The insoluble materials included among other components eggs and tegument of the parasites.

Immunization of animals: Adult female Syrian hamsters weighing 100-120 g were used in this study. They were immunized by either the intraperitoneal or a combined intraperitoneal and oral route. The intraperitoneal route of immunization consisted of a single injection of antigen in complete Freund's adjuvant (CFA). For the intraperitoneal and oral route, the animals were first injected with antigen in CFA intraperitoneally. They were subsequently given a single oral feeding of antigen in 1.3% $NaHCO_3$ via a dosing needle and syringe, 2-3 weeks later. Unimmunized hamsters similarly treated using saline instead of antigen served as controls.

Experimental infection and assessment of protective immunity: Both immunized and unimmunized animals were challenged with 25 Mc by a dosing needle and syringe as described elsewhere. The development of protective immunity was assessed 2-3 months later by determining the magnitude of worm burdens and, in some experiments, also by fecal egg output (Sirisinha *et al.*, 1983a, b).

RESULTS

Effect of a single intraperitoneal injection of different *O. viverrini* antigens on a chellenge infection of hamsters with 25 metacercariae:

In this series of experiments, adult female hamsters were injected intraperitoneally with metabolic products (ES) of adult worms, adult somatic extract or adult worm homogenate mixed with an equal volume of complete Freund's adjuvant (CFA). The animals were challenged 2-3 wk later with 25 metacercariae (Mc) of *O. viverrini*. The animals

28

Table 1

Effect of a single intraperitoneal injection of antigens on a challenge infection with 25 metacercariae.

Antigens	Quantity of antigens	No. of hamsters	Worm recovery (mean ± SE)
–		12	14.58 ± 1.71
CFA		6	14.00 ± 1.67
ES + CFA	100 μg	5	10.20 ± 4.09
–		9	18.22 ± 0.91
CFA		9	18.22 ± 0.81
Adult homogenate + CFA	5 adult worm equivalent	8	17.75 ± 0.61
–		7	12.60 ± 1.42
Adult somatic + CFA	100 μg	8	15.38 ± 1.59

were sacrificed approximately 3 months after the challenge. Worm recovery from immunized hamsters were compared with those from unimmunized control groups, one of which received a similar injection of CFA in saline. The results are shown in Table 1. The two control groups from each set of experiments had similar numbers of worm recovery, varying between 40% and 70% of the challenging dose. Data summarized in the table failed to show statistically significant reduction of worm recovery in any of the 3 experimental (immunized) groups. The presence of insoluble material in worm homogenate which contained eggs, tegument and other cellular components did not enhance the protective potential of the soluble somatic antigen. In contrast to the results with these somatic antigens, there appeared to be a low degree of protection in the group immunized with ES antigen.

Effect of a combined intraperitoneal and oral route immunization on a challenge infection with 25 metacercariae:

In this series of experiments, the hamsters were first immunized by an intraperitoneal injection of antigen in CFA as previously described. They were then given an oral feeding of antigen 2 wk later. One week after the oral feeding, the animals were challenged with 25 metacercariae. Animals were sacrificed approximately 3 months thereafter and worm recoveries from all groups were determined and compared. The results presented in Table 2 showed again that with this protocol of immunization, neither adult worm somatic extract nor metacercarial somatic extract gave protection against a subsequent challenge. Thus, there is no addition advantage of a combined parenteral and local route of immunization over that of a parenteral route alone.

Effect of a combined preexposure and immunization on the induction of protective immunity:

Although previous experiments failed to demonstrate a significant protection in animals receiving several forms of antigen preparations by different protocols, it was still possible that a weak protection stimulated by natural infection could be enhanced via a subsequent immunization. To test this

Table 2

Effect of a combined intraperitoneal and oral route immunization on a challenge infection with 25 metacercariae.

Somatic antigens	Quantity of antigens		No. of hamsters	Worm recovery (Mean ± SE)
	Intraperitoneal	Oral		
Adult	CFA	–	8	15.63 ± 1.38
	200 μg + CFA	200 μg	7	15.00 ± 1.02
Metacercaria	–	–	10	8.20 ± 2.30
	CFA	–	6	7.83 ± 2.79
	20 μg* + CFA	30 μg*	8	8.13 ± 2.85

* Equivalent to 100 and 150 metacercariae, respectively.

Table 3

Effect of a combined pre-exposure to *O. viverrini* parasites and antigen immunization on a challenge infection with 25 metacercariae.

Pre-exposure	Antigens	Quantity of antigens	No. of hamsters	Worm recovery (Mean ± SE)
5 Mc	CFA	–	6	17.17 ± 1.48
5 Mc	Adult Somatic + CFA	200 μg	8	12.13 ± 1.17
5 Mc	CFA	–	4	17.75 ± 7.80
5 Mc	ES + CFA	100 μg	5	14.60 ± 1.82
5 Mc	CFA		10	7.20 ± 1.75
5 Mc	Metacercariae + CFA	50 μg*	9	6.38 ± 2.97

* Equivalent to 250 metacercariae.

possibility, experiments were initiated by first infecting all hamsters with 5 Mc. Then, 2-3 months later, they were immunized intraperitoneally with the antigens shown in Table 3. Using a similar protocol for challenge and assessment of protective immunity, it was found that a slight but significant enhancement of protective immunity ($p < 0.02$) could be induced when adult worm antigen was used as the immunizing antigen (Table 3). A small degree of enhancement of immunity induced by the other two antigens was not significantly different from the controls receiving only CFA.

DISCUSSION

The data presented in this paper demonstrates that immunization of hamsters with various *O. viverrini* antigens failed to protect against infection caused by this liver fluke in previously unexposed hamsters. This is not entirely unexpected, as we previously showed that a prior infection of hamsters with small

30

doses of *O. viverrini* did not confer significant protection against reinfection by the same parasites (Sirisinha *et al.*, 1982, 1983a). However, in view of an earlier report by Flavell (1982) showing a low degree of resistance in hamsters harboring a small number of worms from a previous infection, we attempted to potentiate acquired resistance in animals with prior infection by a subsequent immunization. Results presented in Table 3 showed that the immunization of animals harboring a low number of *O. viverrini* strengthened their acquired immunity to reinfection, particularly in the group receiving aqueous somatic extract of adult worms. However, even with this antigen only a 30% reduction of worm burden was noted.

Reports by Bunnag and Harinasuta (1981) on the recovery of several thousand worms from patients and by Upatham *et al.* (1982) on the peak intensity of infection at above age 40 provide circumstantial evidence for the occurrence of reinfection in people in endemic regions. More recently, Sornmani and his colleagues (1984) demonstrated that a large proportion of people who had been treated with praziquantel could be readily reinfected by *O. viverrini*. This finding confirmed our previous observation on reinfection in experimental animals (Sirisinha *et al*,. 1982). If one extrapolates the results from our present animal report to humans, it could be postulated that human vaccination in endemic areas would provide some beneficial effect against reinfection.

Our inability to further enhance the low degree of acquired immunity could have various explanations. Firstly, the procedures employed may not have been optimal for our hamster model. Hamsters are known to have immune responses somewhat different from most other common laboratory animals (Streilein *et al.*, 1981). Furthermore, chronically infected hamsters have been noted to be immunosuppressed and thus may not respond optimally to immunization (Wongratanacheewin and Sirisinha, manuscript in preparation). Secondly, antibodies and sensitized lymphocytes may not have reached the biliary tract or bile secretion in sufficient quantities. Several years previously Sun and Gibson (1969) detected antibodies reactive with *Clonorchis sinensis* antigens in the bile of patients as well as animals with clonorchiasis. We have also observed that antibodies can be detected in the bile of hamsters infected with *O. viverrini* within one month of infection (Unpublished). Bhamarapravati *et al.*, (1978) also reported the presence of mononuclear cells in the biliary wall of infected hamsters. Lastly, *O. viverrini* parasites themselves may be resistant to immune damage or be able to evade the defense system of the host. We recently demonstrated that *O. viverrini* were not killed by serum from infected animals or from patients with opisthorchiasis (Sirisinha *et al.*, 1986). In addition, the parasites were also found to be resistant to *in vitro* killing by splenic lymphocytes from infected donors, either in the presence or absence of antibody (Unpublished). Flavell and his associates (1980) reported that it was not possible to induce a significant level of immunity in hamsters by passive transfer of either serum or spleen cells from infected donors. Similarly, we were unable to induce protective immunity by oral feeding of recipient hamsters with pooled serum from infected animals prior to, together with or immediately after a metacercarial challenge (Unpublished). Resistance to immune damage can be related to tegumental shedding which had been observed previously (Sirisinha *et al.*, 1986).

Taken together, the results from experimental animal studies, from clinical findings in patients with opisthorchiasis and from epidemiological observations all suggest that only a low degree of protective immunity develops in opisthorchiasis and it appears un-

31

likely that acquired immunity to *O. viverrini* infection can be potentiated by immunization. Chemotherapy alone cannot effectively control infection in endemic regions, as people can be readily reinfected (Sornmani *et al.*, 1984). Therefore, the classical approach to control this infection ,by improvement in sanitation and health education aimed at changing food habits, in combination with effective chemotherapy, remains the method of choice.

SUMMARY

Attempts were made to induce acquired immunity against *Opisthorchis viverrini* infection in hamsters by immunizing them with aqueous somatic extract and metabolic products of adult worms, crude adult worm homogenates and metacercarial somatic extracts via either the intraperitoneal or combined intraperitoneal and oral routes. These procedures failed to stimulate significant protective response in animals that had never been exposed to *O. viverrini*. However, the protective response reached a significant level (30% worm reduction) in hamsters that had been infected with a small member of flukes prior to immunization with aqueous somatic extract of adult worms. Although these findings indicate that it may be possible to reduce reinfection in people in the endemic area by immunization, it appears that a better method currently available for the control of *O. viverrini* infection is health education aimed at changing food habits and improving sanitation and personal hygiene.

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REFERENCES

BHAMARAPRAVATI, N., THAMMAVIT, W. and VAJRASTHIRA, S., (1978). Liver changes in hamsters infected with a liver fluke of man, *Opisthorchis viverrini*. *Am. J. Trop. Med. Hyg.*, *27* : 787.

BOONPUCKNAVIG, S., KURATHONG, S. and THAMMAVIT, W., (1986). Detection of antibodies in sera from patients with opisthorchiasis. *J. Clin. Lab. Immunol.*, *19* : 135.

BUNNAG, D. and HARINASUTA, T., (1981). Study on the chemotherapy of human opisthorchiasis. II. Minimun effective dose of praziquantel. *Southeast Asian J. Trop. Med. Pub Hlth.*, *12* : 413.

DHARMKRONG-AT, A. and SIRISINHA, S., (1985). Induction of protective immunity against infection by *Angiostrongylus cantonensis* in rats. *Southeast Asian J. Trop. Med. Pub. Hlth.*, *16* : 31.

FLAVELL, D.J., (1981). Liver-fluke infection as an aetiological factor in bile-duct carcinoma of man. *Trans. Roy. Soc. Trop. Med. Hyg.* *75* : 814.

FLAVELL, D.J., (1982). Acquired resistance to *Opisthorchis viverrini* in the hamster. *Trans. Roy. Soc. Trop. Med. Hyg.* *76* : 800.

FLAVELL, D.J., PATTANAPANYASAT, K. and FLAVELL, S.U., (1980). *Opisthorchis viverrini* : partial success in adoptively transferring immunity with spleen cells and serum in the hamster. *J. Helminthol.*, *54* : 191.

HARINASUTA, T., RIGANTI, M. and BUNNAG, T., (1984). *Opisthorchis viverrini* infection : pathogenesis and clinical features. *Drug Res.*, *34* : 1167.

KURATHONG, S., LERDVERASIRIKUL, P., WONGPAITOON, V., PRAMOOLSINSAP, C., KANJANAPITAK, A., VARAVITHYA, W., PHUAPRADIT, P., BUNYARATVEJ, S., UPATHAM, E.S. and BROCKELMAN, W.Y., (1985).

Opisthorchis viverrini infection and cholangiocarcinoma. A prospective, case-controlled study. *Gastroenterology, 89*: 151.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.J. and RANDALL, R.J., (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem., 193* : 265.

PREUKSARAJ, S., (1984). Public health aspects of opisthorchiasis in Thailand. *Drug Res., 34* : 1119.

SIRISINHA, S., TUTI, S., TAWATSIN, A. and RATTANASIRIWILAI, W., (1982). Lack of evidence for protective immunity in hamsters infected with *Opisthorchis viverrini*. Proc. 25th SEAMEO-TROPMED Seminar, p. 91-100.

SIRISINHA, S., TUTI, S., TAWATSIN, A., VICHASRI, S., UPATHAM, E.S. and BUNNAG, D., (1983a). Attempts to induce protective immunity in hamsters against infection by a liver fluke of man *(Opithorchis viverrini). Parasitology, 86* : 127.

SIRISINHA, S., TUTI, S., VICHASRI, S. and TAWATSIN, A.,(1983b). Humoral immune responses in hamsters infected with *Opisthorchis viverrini. Southeast Asian J. Trop. Med. Pub. Hlth., 14* : 243.

SIRISINHA, S., PUENGTOMWATANAKUL, S., SOBHON, P., SAITONGDEE, P., WONGPAYABAL, P., MITRANONDE, V., RADOMYOS, P., BUNNAG, D. and HARINASUTA, T., (1984). Alterations of the surface tegument of *Opisthorchis viverrini* exposed to praziquantel *in vitro* and *in vivo. Southeast Asian J. Trop. Med. Pub. Hlth., 15* : 95

SIRISINHA, S., RATTANASIRIWILAI, W., PUENGTOMWATANAKUL, S., SOBHON, P., SAITONGDEE, P. and KOONCHORNBOON, T., (1986). Complement mediated killing of *Opisthorchis viverrini* via activation of the alternative pathway. *Int. J. Parasitol. 16* : 341.

SONAKUL, D., KOOMPIROCHANA, C., CHINDA, K. and STITNIMANKARN, T., (1978). Hepatic carcinoma with opisthorchiasis. *Southeast Asian J. Trop. Med. Pub. Hlth, 9* : 215.

SORNMANI, S., VIVATANASESTH, P., IMPAND, P., PHATIHATAKORN, W. and SITABUTRA, P., (1984). Infection and re-infection rates of opisthorchiasis in the water resource development area of Nam Pong Project, Khon Kaen Province, northeast Thailand. *Am. Trop. Med. Parasitol., 78* : 649.

STREILEIN, J.W., HART, D.A., STEIN-STREILEIN, J., DUNCAN, W.R. and BILLINGHAM, R.E., (1981). *Hamster Immune Responses in Infections and Oncologic Diseases.* Plenum Press, New York.

SUN, T. and GIBSON, J.B., (1969). Antigens of *Clonorchis sinensis* in experimental and human infections. An analysis by gel-diffusion technique. *Am. J. Trop. Med. Hyg., 18* : 241.

TUTI, S., VICHASRI, S. and SIRISINHA, S.,(1982) Effect of culture media on production of excretory-secrtory products and egg output of *Opisthorchis viverrini in vitro. J. Parasitol., 68* : 892.

UPATHAM, E.S., VIYANANT, V., KURATHONG, S., BROCKELMAN, W.Y., MENARUCHI, A., SAOWAKONTHA, S., INTARAKHAO, C., VAJRASTHIRA, S. and WARREN, K.S., (1982). Morbidity in relation to intensity of infection in opisthorchiasis viverrini: study of a community in Khon Kaen, Thailand. *Am. J. Trop. Med. Hyg., 31* : 1156.

VIRANUVATTI, V. and STITNIMANKARN, T., (1972). Liver fluke infection and infestation in Southeast Asia. *Prog. Liver Dis, 4* : 537.