IMMUNIZATION OF HAMSTERS AGAINST *OPISTHORCHIS VIVERRINI* INFECTION

S. SIRISINHA and S. WONGRATANACHEEWIN

Department of Microbiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.

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)

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.

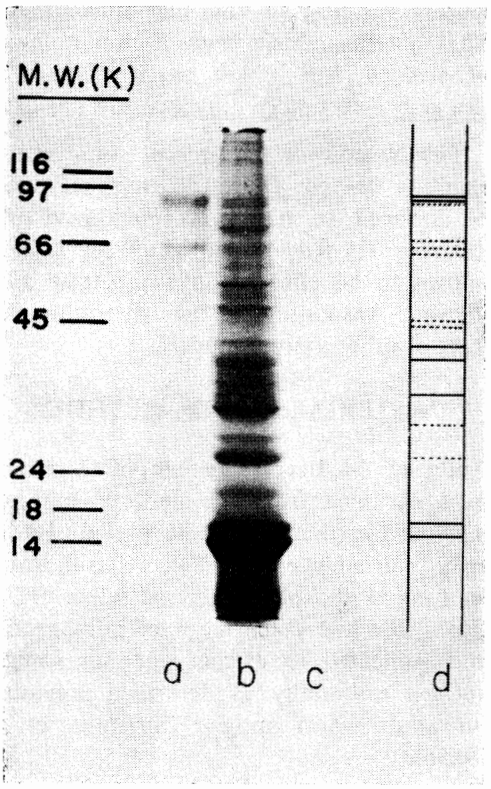

Fig. 1—SDS-PAGE Coomassie-blue stained profiles of ES (a), somatic (b) and metacercariae (c) of *O. viverrini*. The right hand diagram (d) represents that of the metacercariae which took up stain weakly. Molecular weight markers indicated on the left.

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 challenge 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

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

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-

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.

ACKNOWLEDGEMENTS

This work was supported by the Wellcome Trust, the U.S. Agency for International Development (Grant No. 936-5542-G-00-6027 -00) and Mahidol University. The authors are grateful to Dr. T.W. Flegel for his suggestions during the preparation of this manuscript.

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. *Ann. 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.