# HUMORAL IMMUNE RESPONSES IN HAMSTERS INFECTED WITH *OPISTHORCHIS VIVERRINI*

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## INTRODUCTION

Liver fluke infection caused by *Opisthorchis viverrini* is an important human disease found primarily in northeast Thailand and parts of Laos and Cambodia (Harinasuta, 1969). In some endemic areas in Thailand, more than 90% of the population may be infected by these parasites. The disease is chronic in nature and some patients may harbor thousands of flukes at any one time (Bunnag and Harinasuta, 1981), suggesting that the mechanism(s) for the elimination of parasites is not very effective and that reinfection is rather common in this disease. Flavell *et al.*, (1980a) failed to show by passive protection in hamsters receiving either serum or spleen cells, or both, from infected donors. Recently, we have shown that prior infection of animals did not confer significant protection against reinfection by the same parasites (Sirisinha *et al.*, 1983). These findings were unexpected in view of the fact that Flavell (1981a) recently demonstrated that in the presence of complement, sera from chronically infected hamsters could damage and kill both adult and juvenile flukes *in vitro*. Moreover, Bhamarapravati *et al.*, (1978) noted a small number of dying or dead worms in the bile ducts of experimentally infected hamsters and suggested the possible involvement of immune mechanisms in the *in vivo* elimination of the parasites. These investigators demonstrated further that tissue reactions were more severe from one month of infection owward, at a time when antibodies had been reported to be present in the circulation of these infected animals (Janechaiwat *et al.*, 1980). For these reasons, more detailed information on the host-parasite relationship is needed, if one is to fully understand the pathogenesis of this disease and to look for a better approach to its treatment and control. Moreover, a better understanding of the immune responses will lead to the development of more suitable immunological test for human opisthorchiasis in situations where patients develop biliary obstructions or in those with light infections. The purpose of this study was to examine the kinetics and to characterize the humoral immune responses in hamsters infected with various doses of *O. viverrini*.

## MATERIALS AND METHODS

Female golden Syrian hamsters weighing 90-110 g were infected with single or multiple doses of infective metacercariae (MC) as described previously (Tuti *et al.*, 1982; Sirisinha *et al.*, 1983). In most experiments, faecal egg counts were performed by a modified Kato's thick smear technique (Katz *et al.*, 1972) twice a week.

Antibody determination : Antibody levels were determined in the serum taken at various time intervals and at the time of killing of animals infected with various doses of metacercariae. Approximately 0.5 ml of blood was obtained from the ophthalmic plexus at 2, 4, 6, 8, 12 and 16 week of infection and at the time of killing (23 wk), and allowed to clot at room temperature. The serum was kept frozen at -20°C until analyzed for antibody activity. In some experiments, antibody was also determined from the bile

collected at the time of killing. Bile was collected directly from the gall bladder, using a 27-G needle attached to a small tuberculin syringe. Due to the fact that only a small volume of bile could be obtained from each animal by such a procedure, samples from the same experimental group were pooled for antibody determination.

Antibody to both somatic and ES antigens were determined by the passive haemagglutination technique using microtiter U plates (Falcon Plastics, Cockeysville, MD, U.S.A.). Sheep red blood cells (SRBC) were fixed with glutaraldehyde and sensitized with antigens essentially as described by Hirata and Brandiss (1968). In brief, one volume of washed, packed SRBC were suspended in 10 volumes of PBS and then 4 volumes of 2.5% glutaraldehyde was added slowly to the cell suspension. The suspension was stirred gently at room temperature for 2 hr and the cells were then washed thoroughly by centrifugation 3 times with cold saline. To one volume of a 1% glutaraldehyde-fixed SRBC (gSRBC) suspension in 0.1 M acetate buffer pH 4.0 was added 0.01 volume of antigens. Reaction was allowed to take place at 37°C for 30 min with gentle shaking at intervals. Then the cells were washed 3 times with PBS, and resuspended in PBS containing 0.5% bovine serum albumin (PBS-BSA) to a final concentration of 1%. The sensitized cell suspension was used within 24 hours. The optimal concentrations of antigens were predetermined by a checker-board titration of cells sensitized with various concentrations of antigens, using known positive antisera obtained from chronically infected hamsters (kindly donated by Dr. D.J. Flavell, London School of Hygiene and Tropical Medicine, UK).

Specimens to be titrated were preabsorbed with 9 volumes of gSRBC suspended in PBS-BSA prior to being diluted serially in 2 fold dilutions with the same diluent. Some serum specimens were also treated with 0.05 M mercaptoethanol for 30-60 min prior to being titrated. Equal volumes of sensitized cell suspension were then added and the reaction mixtures were kept at room temperature for 2 hours before results were recorded. Any specimen that was negative at 1:20 (the lowest dilution tested) was arbitrarily given a value of 1:10 for the purpose of calculating the geometric mean titres and 95% confidence limits.

Preparation of antigens : Adult flukes were collected from infected hamsters under sterile conditions (Tuti *et al.*, 1982). The worms were washed immediately a few times with Hank's balanced salt solution containing penicillin, streptomycin and fungizone at final concentrations of 100 I.U., 100 µg and 2 µg per ml, respectively. The worm were then cultured at 37°C in a 5% $CO_2$ atmosphere in a protein-free Eagle's basal medium (BME, GIBCO, Grand Island, N.Y., U.S.A.) containing the same concentrations of antibiotics. Generally, a ratio of 0.25 ml of the culture medium per worm was used and the medium was changed every 24-28 hours. Although practically all undamaged worms were alive and remained highly active under these conditions for at least 10-12 days (Tuti *et al.*, 1982), the culture was generally terminated within the first 7 days in order to minimize possible contamination of ES products with soluble somatic components that might be released from dying worms.

The culture fluids containing metabolic products (ES antigens) were pooled, centrifuged at 2500 rpm for 15 min to remove eggs and other debris, and the supernatant fluid was concentrated approximately 10 fold by ultrafiltration using a Diaflow membrane PM-10 (Amicon Corporation, Lexington, Mass., U.S.A.). The concentrated ES antigens were freed of the remaining culture medium by a few repeated additions of large

volumes of phosphate buffered saline pH 7.2 (PBS) and reconcentration by ultrafiltration.

The worms that had been used for the preparation of ES antigens were ground in a mortar in the presence of a small volume of PBS. The ground-up worms were homogenized in a glass tissue grinder and sonicated at 15-20 Kc/sec. Sonication was carried out for a total of 30-40 min, at which time most cells and eggs had been disintegrated judging from microscopic examinations. The suspension was then centrifuged at 15,000 rpm 30 min and the pellet was discarded. All these preparatory procedures were carried out at 4°C.

Both the ES and somatic antigens of adult flukes were dispensed in small aliquots and kept frozen at -20°C.

The protein concentration was determined by the Folin-Ciocalteau tyrosine method (Lowry *et al.*, 1951) using human serum albumin as the standard. Rabbit antiserum to hamster colostral IgA was kindly given by Drs. A.D. Befus and J. Bienenstock, McMaster University, Hamilton, Ontario, Canada. The antiserum was absorbed with either purified hamster IgG or IgA to make it specific for IgA or secretory component respectively.

The significance of differences between the geometric mean values of different experimental and control groups was determined by the Student's t-test (Sokal and Rohlf, 1969).

## RESULTS

### Serum antibody responses to somatic antigens

Results presented in Fig. 1 show the group means with the corresponding 95% confidence limits of serum antibody measured from the second week of infection onward. Low levels of antibody could be detected by this technique at the end of the second week in 25% of the animals infected once with between 25 MC and 100 MC. The peak response was attained toward the end of the second month of infection, at a time when faecal egg output was also near its maximal level. However, there was a significant decline in the mean antibody level during the later stage of infection. As shown in the figure, by the end of the fourth month the mean antibody titre of the whole group was already significantly below the peak value noted at the end of the second month ($p < 0.05$).

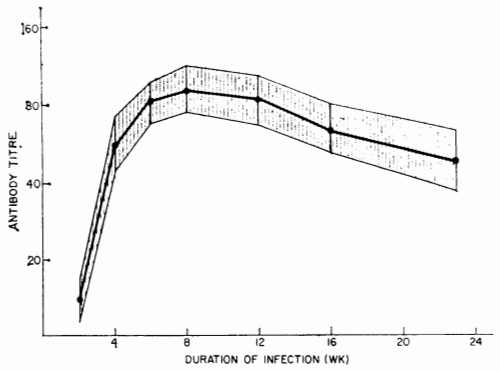


Fig. 1—Kinetics of humoral immune responses in hamsters infected with *O. viverini*. Antibody titres, expressed as geometric means and 95% confidence limits, were determined by haemagglutination technique using SRBC coated with somatic extract of adult flukes.

Specimens from animals with high antibody levels were also tested for their sensitivity to reduction by mercaptoethanol. A shown in Table 1 a significant portion of serum antibody produced throughout the course of infection was sensitive to mercaptoethanol. A similar degree of sensitivity was noted in all animals regardless of the degree and duration of infection.

There was no significant correlation between the antibody titres of the specimens taken at the time of sacrifice (Week 23) and the numbers of worms recovered or the terminal faecal egg output. However, when the data were analyzed according to the number

Table 1

Effect of mercaptoethanol treatment on hamster antibodies to somatic antigens.

| Duration of infection (Wk) | No. of specimens | Antibody titre | |
|---|---|---|---|
| | | Without mercaptoethanol | With mercaptoethanol |
| 2 - 4 | 13 | 123 (83 - 182)* | 20 (10 - 36)** |
| 6 - 8 | 14 | 195 (138 - 276) | 56 (44 - 71)** |
| 12 - 16 | 10 | 195 (115 - 331) | 43 (22 - 83)** |

* Mean and 95% confidence limits.
** Significantly different from the corresponding mean values ($p < 0.01$).

of metacercariae initially used to infect these animals some interesting points emerged (Fig. 2). For instance, the mean antibody titre determined during the first month of infection in the group infected with 100 MC was significantly higher than that of the 25-MC group (Table 2). When only the 2-week samples were analyzed, it was found that almost half of the animals infected with 100 MC had antibody detectable in their sera while only a few animals in the remaining 3 groups were positive in the same time interval. The more important finding however was that there was a large reduction in antibody titre toward the end of the observation period for the group infected with 100 MC (Table 2), moderate reduction for the group infected with 75 MC and almost no reduction for the group infected with 25 MC (Fig. 2).

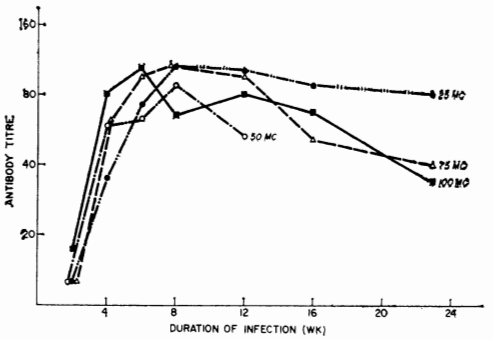


Fig. 2—Antibody patterns in the serum of hamsters infected with different doses of infective metacercariae (MC). Each curve represents geometric means calculated from 6 to 8 animals per group.

Table 2

Serum antibody responses to somatic antigens in hamsters infected with different doses of metacercariae.

| Duration of infection (Wk) | Antibody titre | |
|---|---|---|
| | 25-MC group | 100-MC group |
| 4 | 36 (18 - 74)* | 79 (42 - 152)** |
| 6 | 74 (35 - 159) | 105 (73 - 152) |
| 8 | 112 (49 - 251) | 67 (44 - 105) |
| 16 - 23 | 85 (66 - 110) | 46 (27 - 85)** |

* Mean and 95% confidence limits.
** Significantly different from the corresponding values of the 25-MC group ($p < 0.05$).

Table 3

Serum antibody titres* in hamsters with single or multiple infections with different doses of metacercariae.

| Experimental protocol | No. of animals | Time interval** | Antibody titres | |
|---|---|---|---|---|
| | | | Geometric means | Confidence limits 95% |
| Single infection with | | | | |
| 5 MC | 6 | 133 | 50 | 33 - 76 |
| 10 MC | 4 | 104 | 48 | 25 - 93 |
| 15 MC | 4 | 91 | 48 | 14 - 166 |
| 25 MC | 5 | 105 | 60 | 36 - 100 |
| 50 MC | 2 | 112 | 40 | **** |
| 75 MC | 7 | 112 | 54 | 31 - 93 |
| 80 MC | 12 | 81 | 38 | 30 - 49 |
| 100 MC | 6 | 112 | 71 | 33 - 151 |
| Multiple infection with*** | | | | |
| 5 MC+5 MC | 5 | 97 | 46 | 30 - 71 |
| 5 MC+5 MC+5 MC | 12 | 84 | 54 | 38 - 80 |
| 5 MC+5 MC+5 MC+5 MC+5 MC | 9 | 101 | 47 | 25 - 89 |
| 5 MC+80 MC | 6 | 93 | 56 | 30 - 107 |
| 5 MC+5 MC+80 MC | 4 | 61 | 56 | 16 - 195 |
| 5 MC+5 MC+5 MC+80 MC | 3 | 41 | 80 | * * * * |

* Determined at the time of killing.
** Interval between the last day of infection and the time of killing.
*** Intervals between infections varied between 1-2 months.
**** All animals in these experimental groups had the same antibody titre.

On the other hand, the mean antibody titres of hamsters infected with multiple doses of metacercariae were not noticeably different from those infected only once with identical numbers of metacercariae (Table 3).

### Serum antibody response to ES antigens

Antibodies reactive with ES antigens were measured in specimens from animals infected once with either 25 or 100 MC. In general, the patterns of antibody responses to ES antigens including the time and magnitude of the peak response (Fig. 3) were not different from those noted for somatic antigens. However with ES antigens, less than 10% of

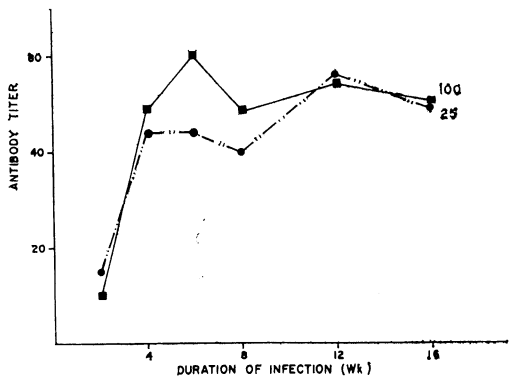


Fig. 3. Kinetics of humoral immune responses to ES antigens in hamsters infected with 25 and 100 infective metacercariae. Results represent mean values calculated from 7-8 animals per group.

the animals had detectable antibody at 2 weeks of infection. There was also a tendency for the antibody to decline during the later stage of infection. Unfortunately, due to an insufficient quantity of ES antigens, not all specimens could be tested, and therefore the data shown in Fig. 3 includes specimens up to 4 months of infection only.

**Biliary antibodies to somatic and ES antigens**

Because of the insufficient volume of bile from individual animal, samples in each infected group were pooled prior to being titrated. It was found that the antibody titres in the bile of animals infected only once with between 5 and 100 metacercariae or from those reinfected several times prior to being sacrificed were not noticeably different from one another. Regardless of the antigens used, the titres varied little among the different groups. In general, they varied from less than 1:10 (the lowest dilution tested) to 1:20. An attempt was also made to increase the sensitivity of the technique by adding appropriately diluted antisera specific for either hamster IgA or secretory component to those samples which gave negative readings. In most cases, the samples which were negative initially became positive at 1:10, indicating also that these antibodies were secretory IgA.

## DISCUSSION

The present study showed that antibodies specific for both somatic and ES antigens of adult liver flukes are detectable throughout the course of infection by these parasites. While only 25% of the experimental animals infected once with between 25 and 100 metacercariae had antibodies detectable in their sera during the early phase of infection when the parasites were still in the juvenile stage, i.e. 2 weeks after infection, sera from all animals were positive from one month of infection onward. The latter observation coincided with the maturation of the parasite in this animal host (Vichasri, 1981). Finding antibodies during the early stage of infection indicated that excysting metacercariae and developing juvenile flukes produced and released soluble metabolites that were antigenic in quantities sufficient for the stimulation of the host immune system, particularly in the group with heavy infection. Although in the present study no attempt was made to detect ES antigens in the bile of these animals. Sun and Gibson (1969) were able to detect these antigens in the bile of patients and of animals infected with *Clonorchis sinensis*. It is of interest to find out how these soluble antigens could reach the host immune machinery as it is generally agreed that these parasites to not penetrate the tissues during their development inside the host. Bhamarapravati *et al.*, (1978) reported necrosis of liver parenchyma, cellular infiltration and bile duct proliferation within 3 days of infection. It is possible that small foci of epithelial cell damage might be present and allow these soluble products to reach the host tissue. Sun and Gibson (1969) suggested that during the later stage of infection by *C. sinensis* the metabolic products may be absorbed with the bile regurgitated through damaged liver cells.

The observation that the antibody levels slowly declined with prolonged infection, particularly in the group with heavy infection (100 MC), was of interest. Limited data presented previously by Vongsangnak (1981) suggested that a certain degree of non-specific immunosuppression occurred in hamsters chronically infected by *O. viverrini*. This phenomenon should be investigated further and its mechanism elucidated. This point may be of practical importance as the chronic nature of the disease that occurs in man and the inefficient mechanism to eliminate either established or establishing parasites (Sirisinha *et al.*, 1983) may be associated with this phenomenon.

Although the immunoglobulin classes of serum antibodies were not determined, these antibodies were rather sensitive to reduction by mercaptoethanol treatment (Table 1). Whether this is typical for serum antibodies produced by hamsters infected by parasitic worms remains to be investigated. On the other hand, the limited data in this study indicated that at least some of the antibodies secreted in the bile were of the SIgA type. It is not known if these secretory antibodies were synthesized locally or derived by active transport of serum IgA (Andrew and Hall, 1982; Hall *et al.*, 1979). It is also not known if and how antibodies of the IgA class could adversely affect the worms. The parasiticidal effect of serum from chronically infected hamsters reported previously by Flavell (1981a) may have been associated with the IgM or IgG class but the exact nature of these serum component(s) remains to be investigated. On the other hand, it is not difficult to imagine how IgA antibodies may contribute to the pathogenesis of the disease process. Bhamarapravati *et al.*, (1978) suggested that eosinophilic material deposited in lesions might represent immune complexes. Moreover, it is also possible that these antibodies contribute to the formation of granulomas that develop in this experimental animal model (Bhamarapravati *et al.*, 1978; Flavell *et al.*, 1980b; Flavell, 1981b).

The finding that antibodies that developed in the infected animals reacted with ES antigens is encouraging, as it makes possible the development of a specific immunological test for this disease. Feldheim and Knobloch (1982) recently reported the use of enzyme immunoassay for the detection of antibodies to *O. viverrini* in refugees from Southeast Asia. However, low level of cross reactivity was noted with sera from patients having other parasitic infections. Although it was not the purpose of the present study to investigate this point, the problem is now being dealt with in our laboratory. Our preliminary analysis of the ES antigens used here showed one major component by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Progress is being made to isolate and to characterize this component and to look at the possibility for its future use in the immunodiagnosis of the disease in humans.

## SUMMARY

The kinetics and nature of humoral immune responses to somatic and excretory-secretory (ES) antigens were investigated in hamsters experimentally infected with different numbers of *Opisthorchis viverrini*. ES antigens were obtained from the *in vitro* culture of adult flukes and somatic antigens were aqueous extracts of adult flukes. Antibodies in the serum and bile of infected animals were determined by the microhaemagglutination technique, using glutaraldehyde fixed sheep red blood cells sensitized with these parasite antigens.

Antibody responses to both somatic and ES antigens were detected in the serum from the second week of infection onward. The peak response was noted at the end of the second month and declined slowly thereafter. Antibody levels in animals with heavy infections (100 metacercariae) appeared earlier but declined more rapidly than in animals with light infections (25 metacercariae). The serum antibodies were highly sensitive to mercaptoethanol throughout the course of infection (23 weeks). Antibodies also appeared in the bile obtained at the time of sacrifice but their titres were rather low compared with those in the serum. Like serum antibodies, biliary antibodies were reactive with both somatic and ES antigens. Biliary antibodies were of the secretory IgA type. These findings are discussed in relation to pathogenesis of the disease process and to the possible usefulness in immunodiagnosis.

## ACKNOWLEDGEMENTS

The authors are grateful to Drs. P. Matangkasombut and S. Upatham for their helpful discussion during the course of this study and to Dr. T.W. Flegel for his suggestions during the preparation of the manuscript. This work was supported by the Wellcome Trust, Rockefeller Foundation and Mahidol University.

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