# IMMUNITY IN TAENIASIS-CYSTICERCOSIS

## I. Vaccination against *Taenia taeniaeformis* in Rats using Purified Antigen*

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Species of tapeworms belonging to the family *Taenidae* are among the most important helminth parasites of man and domestic animals. Taeniasis and cysticercosis are serious problems of public health and economy in developing countries. The ultimate control of these diseases depends on the development of effective prophylactic vaccines.

Although vaccines against bacterial and viral infections are commonplace, no effective vaccine is yet available against many helminth infections. Among the cestodes, studies on antigen purification and characterization have largely been limited to use for immunodiagnosis (1–11). One of the problems is the difficulty in obtaining suitable experimental models for vaccination trials. *Taenia taeniaeformis,* which has a life cycle involving only two hosts, viz. cats and rodents, is an ideal experimental model.

The role of metabolites secreted by tissue cestodes in stimulating a host immune response has been suggested by Smyth (12), and Gemmel and MacNamara (13). The effectiveness of these excretory and secretory antigens (ES-Ag)[1] has been demonstrated previously in other helminths (14–16). In cestodes, several workers have studied artificial immunization against *T. ovis* (17), *Mesocestoides corti* (18), and *T. pisiformis* (19) using ES-Ag collected from in vitro culture media, and *T. taeniaeformis* using the in vivo method of implanting living larvae enclosed within diffusion chambers into the host (20).

In the present study, the humoral and cell-mediated immunities induced by ES-Ag and somatic antigen (Som-Ag), obtained from whole larval homogenate, were studied in detail. Es-Ag and Som-Ag were found to be highly effective in protecting the rat against challenge infection. Furthermore, an antigen fraction purified from both the Es-Ag and Som-Ag was also highly effective, when injected in small quantities together with complete Freund's adjuvant (CFA), in protecting rats against challenge infections of *T. taeniaeformis*.

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[1] *Abbreviations used in this paper*: DID, double immunodiffusion; DTH, delayed-type hypersensitivity; ES-Ag, excretory and secretory antigen; CFA, complete Freund's adjuvant; ITH, immediate-type hypersensitivity; 2ME, 2-mercaptoethanol; PBS, phosphate-buffered saline; Som-Ag, somatic antigen, whole larval homogenate.

## Materials and Methods

*Experimental Animals.* Outbred female albino rats (3- to 4-wk old) from the Central Animal Facility, Faculty of Medicine, University of Malaya, were used in all experiments except the cell transfer experiment, in which case an inbred strain of AS rats maintained in our laboratory was used.

*T. taeniaeformis.* An infection of *T. taeniaeformis* was maintained in the laboratory by oral innoculation of rats acting as reservoir hosts. A stomach tube was used after light anesthesia with ether. Strobilocerci were liberated after 8 wk and two each were fed to cats to obtain the adult worms and complete the cycle.

*Som-Ag.* Som-Ag was prepared from freshly liberated strobilocerci, washed several times in saline, and ground up with phosphate-buffered saline (PBS), pH 7.2, in an ice-cooled mortar and pestle. Extraction was achieved by continuous stirring at 4°C overnight. The homogenate was then centrifuged at 3,000 rpm for 1 h at 4°C and the supernate obtained. The protein concentration was determined (1.35 mg/ml) using Lowry's method and stored at −10°C until used.

*ES-Ag.* Freshly liberated strobilocerci were washed several times in saline and then twice in PBS. They were then placed in culture tubes containing Eagle's minimum essential medium (Spinner, Difco Laboratories, Detroit, Mich.) supplemented with glucose (1,000 mg/liter), penicillin (50 U/ml), and streptomycin (5 μg/ml) and incubated at 37°C for 24 h with gentle rotation (21). Contaminated cultures were discarded. The supernate was decanted and the protein concentration adjusted to 1.35 mg/ml using Lowry's method. This preparation was designated ES-Ag and was stored at −10°C until used.

*Fractionation of Antigens.* Ascending flow gel filtration chromatography was performed on a Pharmacia 2.6 × 84 cm column of Sephadex G-200-120 (Sigma Chemical Co., St. Louis, Mo.) equilibrated with PBS, pH 7.2. Flow rates of 18 ml/h were maintained. Som-Ag and ES-Ag samples (2.5 ml) were dialyzed against the equilibrating buffer before application, and eluted fractions were collected in 3-ml vol. Elution profiles of Som-Ag and ES-Ag were traced by measuring the optical density of each fraction at 280 nm in a Beckman Spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). All fractions were then tested for antigenicity against antiserum in a double-immunodiffusion test.

*Double Immunodiffusion (DID).* Double diffusion was performed using a micro-Ouchterlony method, with 1% Noble agar in Tris-barbital-sod barbital buffer at pH 8.8 (ionic strength 0.09) and 0.001% $NaN_3$ as preservative. Enhancement of precipitation bands was achieved by infusions of 15% polyethylene glycol 4000 (Sigma Chemical Co.) after the method described by Ceska (22).

*Polyacrylamide Gel Disc Electrophoresis.* The method used for the polyacrylamide gel disk electrophoresis is a slight modification of that of Davis (23). A sample gel was not used. Instead the sample was mixed with an equal volume of 40% sucrose to increase its density. The gel was run for 3 h at 100 V using the Vokam power pack.

The sample volumes used varied between 20 and 100 μl and samples consist of Som-Ag, ES-Ag, and their chromatography elution fractions which showed a strong positive with DID. After electrophoresis, all the gels were stained with amino black. The protein bands were visualized by a densitometer tracing.

*Antiserum.* Rats were infected with 1,000 eggs each and 8 wk later they were exsanguinated under ether anesthesia and the sera were collected, pooled, and stored at −10°C.

*Antibody Estimations.* Antibody titer was estimated by a passive hemagglutination technique based on that described by Jandl and Simmons (24). Briefly, sheep erythrocytes (SRBC) stored in Alsever's solution at 4°C were washed four times with saline and resuspended to 5% in saline. To 5 ml of 5% SRBC in saline was added 200 mg of Som-Ag and followed immediately by 2 ml of 0.01% $CrCl_3$ solution (in saline, pH 5.2). After stirring, the mixture was allowed to stand in room temperature for 5–10 min. The cells were washed three times with PBS and resuspended to 2% in PBS. Titration were done in V-bottom microtitration wells. PBS and 0.1 M 2-mercaptoethanol (2ME) in PBS were used as diluents in parallel titration.

*Hypersensitivity Measurements.* Groups of five rats were injected subcutaneously with 1 mg of Som-Ag or ES-Ag emulsified with CFA in the ratio of 1 CFA:3 antigens. Various times after the immunization, rats were elicited for hypersensitivity reaction by injecting 0.02 ml (25 μg) of Som-Ag into the left hind foot pad and the same volume of saline into the right hind foot pad as control.

Previous trials with ES-Ag for elicitation of foot pad swelling showed no significant difference compared to Som-Ag. Foot pad thickness was measured at 3, 6, 9, 16, 24, and 48 h after the injection, and specific foot pad swelling was determined by subtracting the thickness of the right hind foot pad from the left. Foot pad thickness was measured by a dial caliper (Mitutoyo no. 7309) which has a graduation of 0.01 mm. The accuracy of the method has been previously discussed (25).

*Transfer of Delayed-Type Hypersensitivity (DTH).* AS inbred rats were immunized with 1 mg of Som-Ag or ES-Ag in CFA. 5 days later, each rat was injected intraperitoneally with 5 ml of 0.001% (wt/vol) oyster glycogen and 2 days later peritoneal cells were obtained by washing out the peritoneal cavities with 10 ml of a solution of 5% normal rat serum in PBS containing 10 U/ml of preservative-free heparin. The peritoneal cells were collected in a chilled beaker, pooled, and washed four times with the same medium but lacking heparin. The average yield was $3 \times 10^7$ cells per rat. Each recipient rat was injected intravenously into a lateral tail vein with $3 \times 10^7$ peritoneal cells. Control rats were injected intravenously with the same number of peritoneal cells from unimmunized donors or with 2 ml of a hyperimmune serum (see section on antiserum). Immediately after the cell transfer, rats were challenged in the foot pads for DTH reaction.

*Vaccination against a Challenge Infection.* Groups of five rats were injected subcutaneously with 1 mg of Som-Ag, 1 mg of ES-Ag, or various doses (1–50 $\mu$g) of the fractionated antigen emulsified in CFA. Control rats were either injected with the same volume of saline emulsified in CFA or were uninjected. The injected rats were set aside for various times as indicated in the text. All rats were challenged at the same time with 1,000 oncospheres each and autopsy was performed 3 wk later and the number of cysts per rat was recorded.

*Histology of the Foot Pad at 24 h after Elicitation.* Sections of the left and right hind foot pads at 24 h after antigenic elicitation were stained with hematoxylin and eosin, and histological examinations were made.

*Statistical Analysis.* Standard errors of the mean were calculated and the Student's $t$ test was performed to analyze the statistical significance of the results.

## Results

Rats (five per group) were injected subcutaneously with 1 mg/rat of either Som-Ag or ES-Ag emulsified in CFA. 1 wk later, hypersensitivity reactions were elicited by the injection of 50 $\mu$g of Som-Ag into the foot pad. Fig. 1 depicts the time-course of the hypersensitive responses. Two distinct peaks appeared at 3 and 24 h after elicitation. The first peak was a typical immediate-type hypersensitivity (ITH) response and the second peak a DTH response. The ITH in this case may not be a specific response and may be due to contamination during elicitation, since control rats injected with saline-CFA gave a foot pad reaction only slightly weaker than that of primed animals at 3 h. Leid and Williams (26) have shown that the reagenic antibody which mediate ITH in *T. taeniaeformis* is detectable only by the 19th day after infection. A similar nonspecific ITH has been described previously in foot pad reactions using flagellin in rats (27).

In contrast, the DTH responses are highly significant compared to the control rats which were injected with saline-CFA ($P < 0.001$). It should be noted that no significant difference is apparent between the hypersensitive responses of rats primed with Som-Ag and ES-Ag, showing that for the same protein concentration, the ES-Ag is at least as effective as Som-Ag in inducing DTH response. In a separate experiment similar results were obtained when ES-Ag was used as the eliciting antigen.

*Cellular and Histological Nature of the DTH Induced by Som-Ag.* Experiments were performed to demonstrate that the DTH induced by the parasite antigens represent true delayed responsiveness. This entailed showing

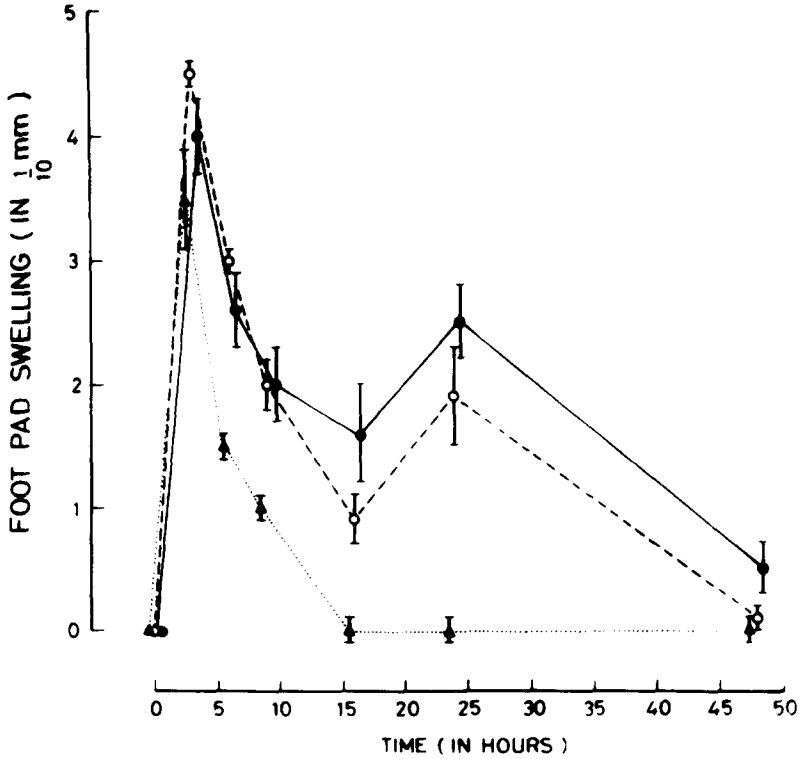


FIG. 1. 48-h hypersensitivity response, three groups of rats immunized with Som-Ag/CFA, ES-Ag/CFA, and saline/CFA, respectively, were elicited 7 days later with antigen injected into the foot pad. Foot pad measurements were taken at 3, 6, 9, 16, 24, and 48 h after elicitation. Vertical bars represent standard errors of the mean. (●—●), Som-Ag/CFA immunization; (O---O), ES-Ag/CFA immunization; (▲---▲), saline/CFA immunization.

that the delayed reactivity was transferable by cells and not by serum and had the characteristic histology of delayed hypersensitivity. 10 AS inbred strain of rats were immunized subcutaneously with 1 mg/rat of Som-Ag in CFA. 10 control rats were similarily injected with saline-CFA. 5 days later, each rat was injected intraperitoneally with 5 ml of a 0.001% of oyster glycogen solution. Peritoneal cells were harvested 2 days after the glycogen stimulation. Normal recipient rats were then injected intravenously with either the immune peritoneal cells, control peritoneal cells ($3 \times 10^7$ cells per rat, one donor to one recipient), immune serum, or control serum (2.5 ml/rat). Hypersensitivity reaction was elicited immediately by injecting 50 $\mu$g of Som-Ag in saline into the right hind foot pad. Foot pad swelling was determined 24 h after the eliciting injection. It was found that peritoneal cells from sensitized rats very effectively transfer DTH (Table I). In contrast, peritoneal cells and serum from control rats or serum from sensitized animals failed to transfer DTH. The histology of the DTH induced by Som-Ag was also examined in rats which had been sensitized and elicited as described above. A marked infiltration of mononuclear cells was observed at 24-h swelling (Fig. 2). The histology seen is characteristic of DTH (28).

TABLE I
*The Ability of Serum and Peritoneal Cells to Transfer the DTH Induced by Som-Ag*

| Transfer agent | Amount | Foot pad swelling (0.1 mm) 24 h after eliciting dose |
|---|---|---|
| Nil | – | 0.3 ± 0.2* |
| Normal peritoneal cells | $3 \times 10^7$ cells | 0.2 ± 0.1 |
| Immune peritoneal cells | $3 \times 10^7$ cells | 3.3 ± 0.3 (4.2)‡ |
| Normal serum | 2.5 ml | 0.1 ± 0.1 |
| Immune serum | 2.5 ml | 0.4 ± 0.2 |

* Standard error of mean (five rats per group).
‡ Value in parentheses represents the DTH response in sensitized rats which were used as cell and serum donors.

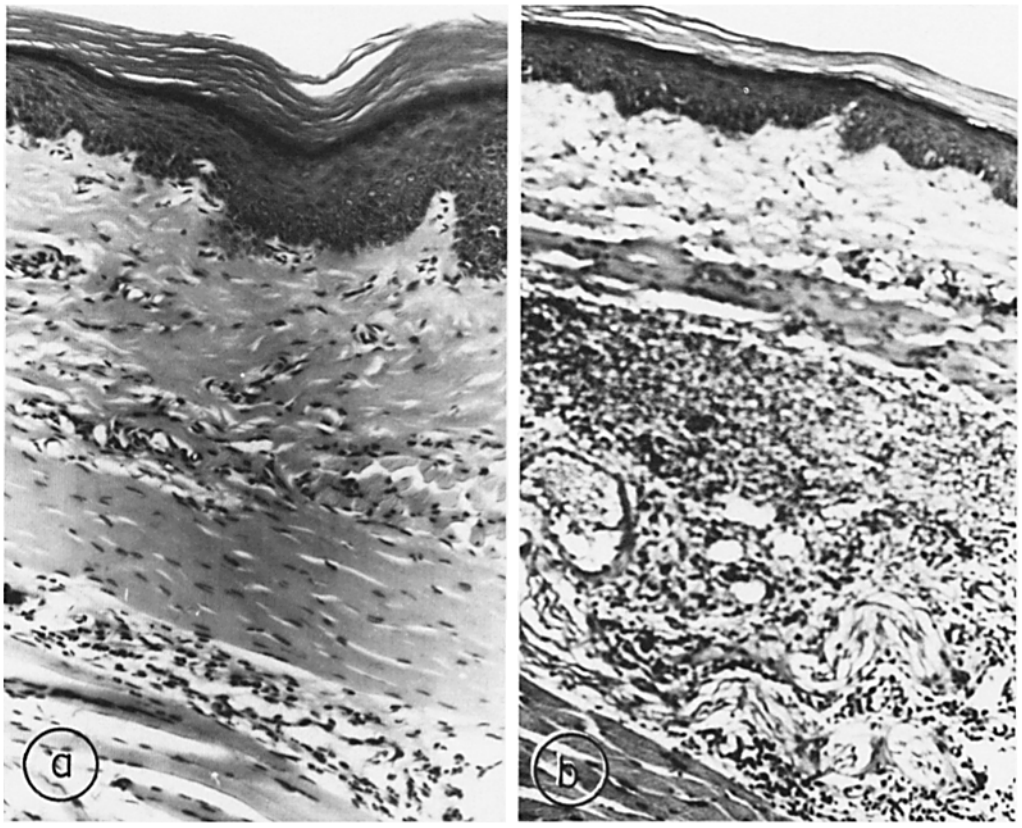


FIG. 2. Histology of DTH to Som-Ag in rats, 24 h after eliciting injection. Rats were injected subcutaneously with 1 mg Som-Ag or ES-Ag in CFA. 7 days later, 0.02 ml of Som-Ag was injected into the left hind foot pad and the same volume of saline injected into the right hind foot pad. (*a*) Transverse section of the hyperdermis of right hind foot pad. (*b*) Transverse section of the hyperdermis of left hind foot pad. Note the marked infiltration of mononuclear cells in (*b*). × 200. Sections stained with hematoxylin and eosin.

*Humoral Response Induced by Som-Ag and ES-Ag.* Experiments were carried out to determine the ability of Som-Ag and ES-Ag to induce antibody production. Groups of five rats were immunized subcutaneously with 1 mg/rat of Som-Ag or ES-Ag emulsified in CFA. Antibody titers against Som-Ag were measured weekly for 8 wk after the injection.

The time-course of antibody production in rats thus immunized are shown in Figs. 3 and 4. In both cases, antibody production was seen to rise only after the 1st wk, and rose to a peak around the 6th and 7th wk. The titers of 2ME-treated sera were only slightly lower than untreated sera. In general, the humoral responses induced by the two antigen preparations are remarkably similar.

*Protective Immunization with Som-Ag and ES-Ag against a Challenge Infection.* Rats were injected with either 1 mg of Som-Ag or ES-Ag in CFA and set aside for 2, 5, 8, 10, 14, 21, and 28 days, respectively. The control rats were injected with saline-CFA and set aside for 28 days. All rats were challenged at the same time with 1,000 oncospheres each and autopsy was performed 3 wk later, and the number of cysts per rat was recorded. The results are shown in Fig. 5. The average number of cysts per rat in the control group was $245.4 \pm 46.6$. Significant protection was recorded only 10 days after immunization with either the Som-Ag or the ES-Ag ($P < 0.01$) with little significant protection before that. The amount of protection also did not appear to increase significantly after 10 days, although the cyst number recorded was approximately 10% below control. In all cases, there was no significant difference between ES-Ag- and Som-Ag-primed animals ($P > 0.3$).

*Isolation and Characterization of the Effective Antigen.* Som-Ag and ES-Ag which induced strong protective immunity in rats against *T. taeniaeformis* infection were fractionated with Sephadex G-200 column chromatography. The protein elution profiles revealed two distinct peaks and several minor peaks in the Som-Ag (Fig. 6). ES-Ag revealed two peaks which coincide with the two main peaks in Som-Ag (Fig. 7). When the fractions were tested for antigenic activity against Som-Ag in the double-immunodiffusion technique, it was found that Som-Ag demonstrated antigenic activity from fractions 59 to 71 with a peak reaction at fractions 62 and 63 (Fig. 6). With the ES-Ag, antigenic activity was recorded with only fractions 61 and 62 (Fig. 7).

Since the strongest antigenic activity appeared around fractions 61, 62, and 63, these fractions were analyzed further with polyacrylamide gel disk electrophoresis. The patterns of the densitometer tracing are shown in Fig. 8. Only one band appeared for both fractions 62 and 63 of Som-Ag and this band coincides in position with the pooled 61 and 62 fraction of ES-Ag. This is out of a total of at least nine protein bands for Som-Ag and four protein bands for ES-Ag.

From the results of the fractionated antigens it appears then that the most antigenic fractions reside around fractions 62 and 63 of Som-Ag, which coincide with that of ES-Ag fractions 61 and 62. Thus a pooled and concentrated specimen of fractions 62 and 63 of Som-Ag was used for the vaccination trials to study its effectiveness in protecting against challenge fraction. The mol wt of this purified antigen was estimated to be approximately 140,000 daltons by comparing it with chromatographic run using standard proteins of known molecular weights.

*Vaccination of Rats with Purified Antigen.* Sephadex chromatography frac-

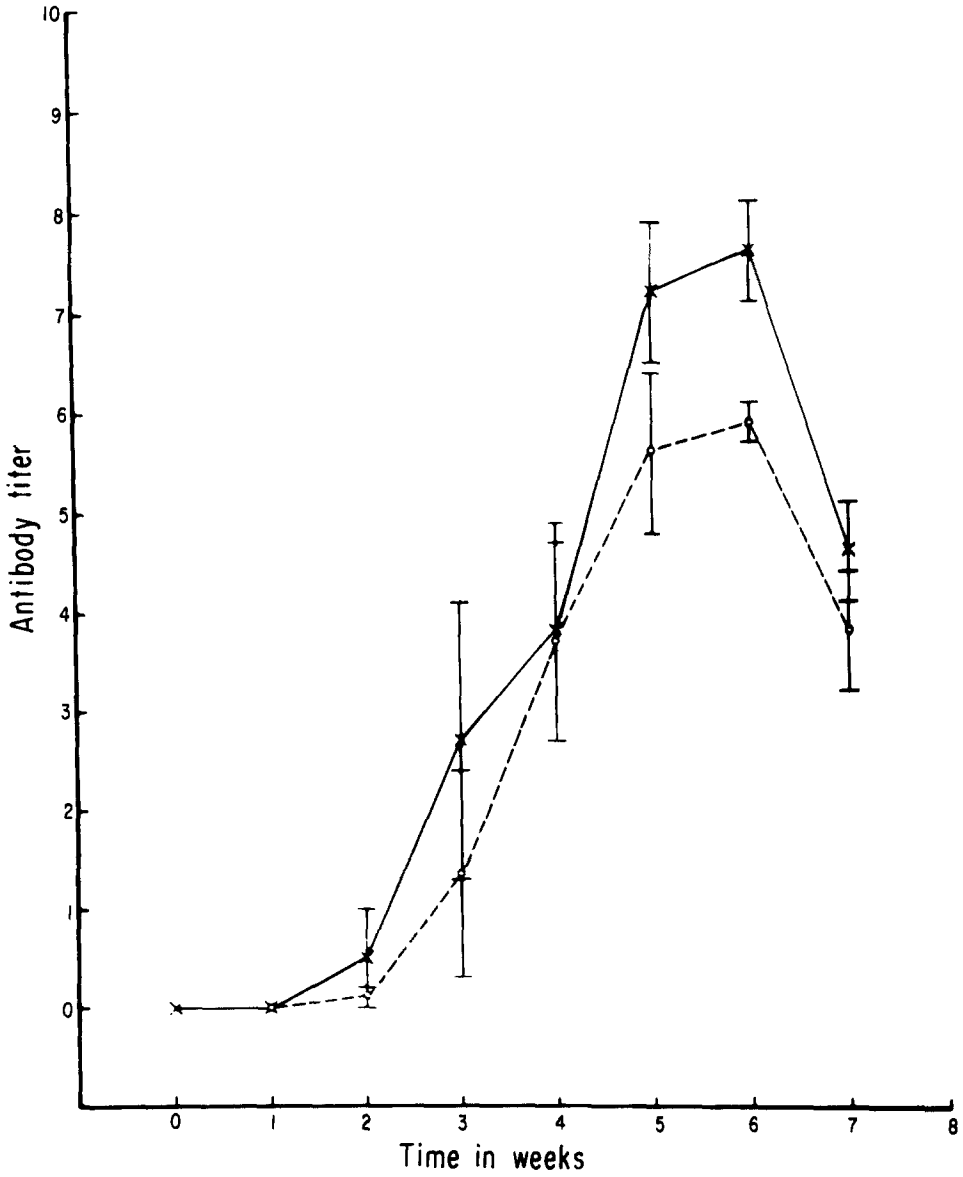


FIG. 3. Time-course of antibody production in rats immunized with Som-Ag/CFA. Solid line represents titer of untreated sera. Dotted lines represent titer of sera after 2ME treatment. Vertical bars represent standard errors of the mean. $Log_2$ titer (1 = 1/10).

tions 62 and 63 from Som-Ag were pooled and concentrated to 0.9 mg/ml protein concentration. Three groups of rats were immunized subcutaneously with 50, 10, or 1 $\mu$g/rat of the antigen in CFA. A fourth group of rats injected with saline-CFA acted as controls. 2 wk later all rats were challenged with 500 oncospheres each. Autopsy and cyst count were carried out 3 wk after challenge. Fig. 9 depicts the results of this vaccination trial. A dose of 50 $\mu$g/rat gave complete

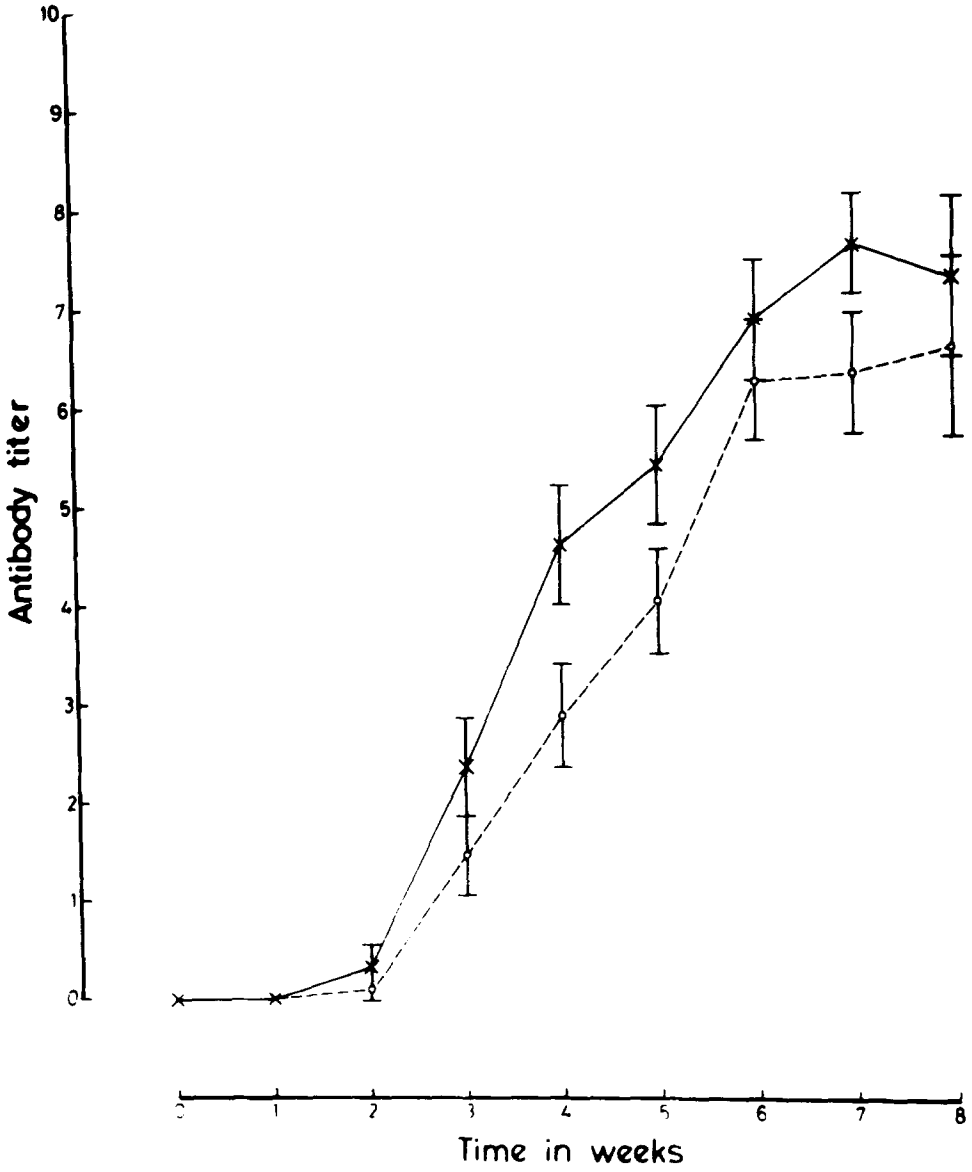


FIG. 4. Time-course of antibody production in rats immunized with ES-Ag/CFA. Solid line represents titer of untreated sera. Dotted lines represent titer of sera after 2ME treatment. Vertical bars represent standard errors of the mean. $Log_2$ titer (1 = 1/10).

protection, whereas 10 and 1 $\mu$g gave about 90% ($P < 0.01$) and 70% ($P < 0.05$), respectively.

## Discussion

Both ES-Ag and Som-Ag appear to be equally effective in stimulating humoral and cell-mediated immunities. The DTH reaction described here peaked

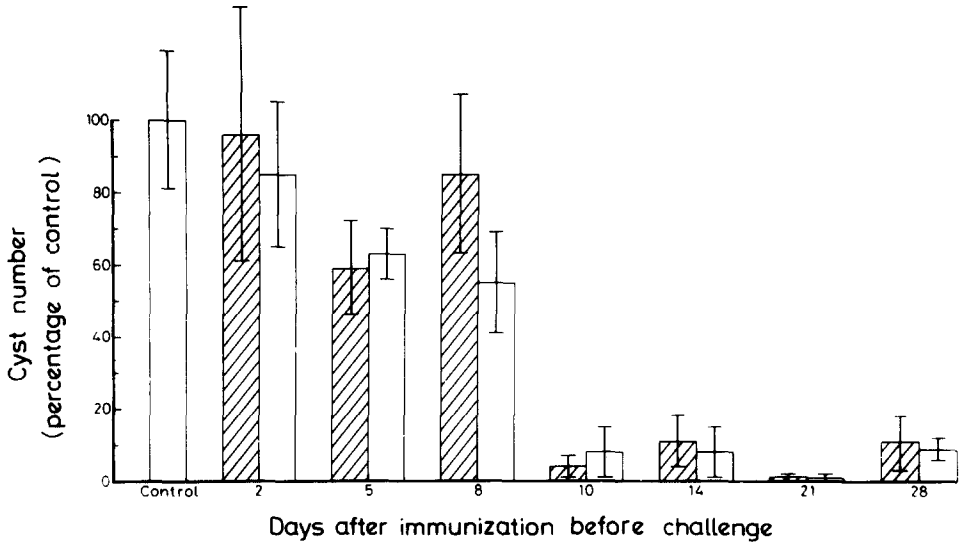


FIG. 5. Protection by immunization. Rats immunized with 1 mg of Som-Ag/CFA and ES-Ag/CFA were challenged with 1,000 oncospheres each after 2, 5, 8, 10, 14, 21, and 28 days. Cyst counts were expressed as percentage of control. Vertical bars represent standard errors of the mean. (▨), Som-Ag/CFA immunization; (□), ES-Ag/CFA immunization.

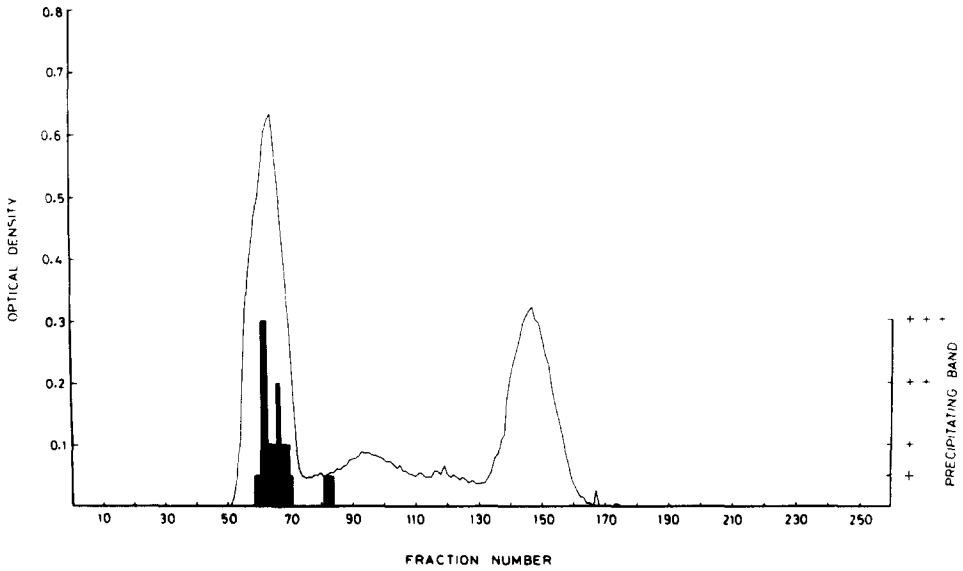


FIG. 6. Fractionation of Som-Ag using Sephadex G-200 chromatography. Graph shows protein fractions in Som-Ag. Dark columns represent positive precipitation reactions of the fractions using DID. Number of pluses (+) represent intensity of the precipitation reaction.

at 24 h and disappeared almost completely by 48 h. It is transferable by sensitized cells and not by immune serum. It also has the typical DTH histology. This report thus confirms and extends earlier work by others (19, 29) who reported cell-mediated immunity being induced by cestode infection.

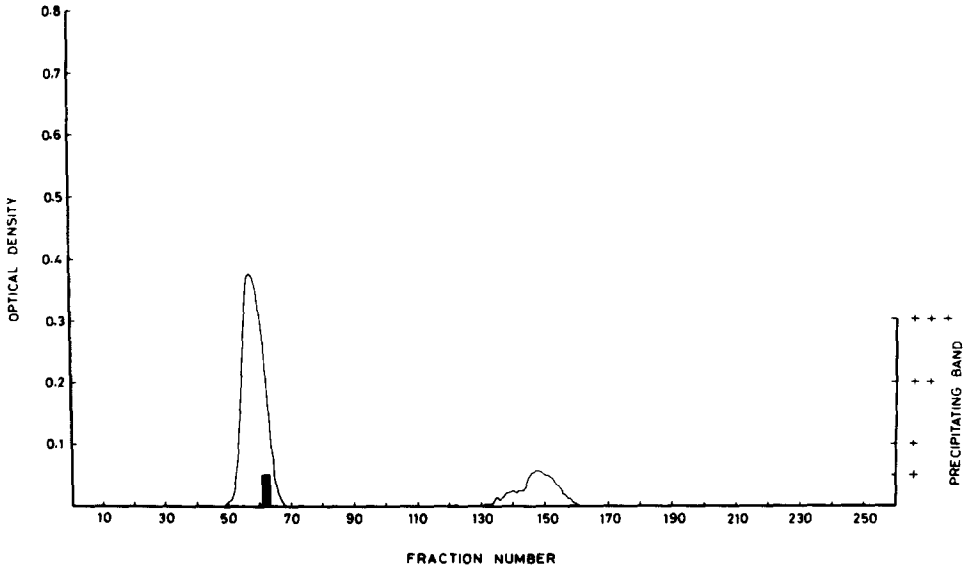


FIG. 7. Fractionation of ES-Ag using Sephadex G-200 chromatography. Graph shows protein fractions in ES-Ag. Dark columns represent positive precipitation reactions of the fractions using DID. Number of pluses (+) represent intensity of the precipitation reaction.

Detectable antibody appeared as early as the 2nd wk and a peak was reached around the 6th and 7th wk after immunization. Since treatment with 2ME showed only a slight drop in antibody titer, it appeared that IgM production was at a very low level compared to the much higher level of IgG. The validity of the 2ME method in destroying IgM activity was confirmed in a separate experiment using 7-day rat anti-SRBC serum. Furthermore, to test for the possibility of separate, early, and transient IgM production before 7 days, rats were immunized with Som-Ag/CFA or ES-Ag/CFA and bled daily from day 1 to day 7. The sera were titered and found to be negative. Thus the classic pattern of a sequential production of IgM and IgG does not appear to be present in this case. In fact, the characteristics of the immunoglobulin production described here, in which IgG appeared early and continuous at a high level with a concurrent appearance of low level of IgM, appeared to agree with observation of some other parasitic infections (30–32). In contrast, in live infection with *T. taeniaeformis* (33), IgM and IgG appeared sequentially and much later, antibody was detectable only 4 wk after infection. Thus it appears that the manner in which the parasitic antigens are presented and processed by the host immune system play a crucial role in its production of different classes of antibody. The early appearance of IgG is also consistent with the effectiveness of the antigen in protecting the host against challenge infection as early as 10 days after immunization. The protective roles of humoral and cell-mediated immunities are currently under investigation. In subsequent papers more comprehensive studies on the relationship between cell-mediated immunity and humoral antibody in protecting the host against *T. taeniaeformis* infection will be presented.

The results of the Sephadex chromatography and DID demonstrated that most of the antigenic activity occurred in the first peak. The precipitating

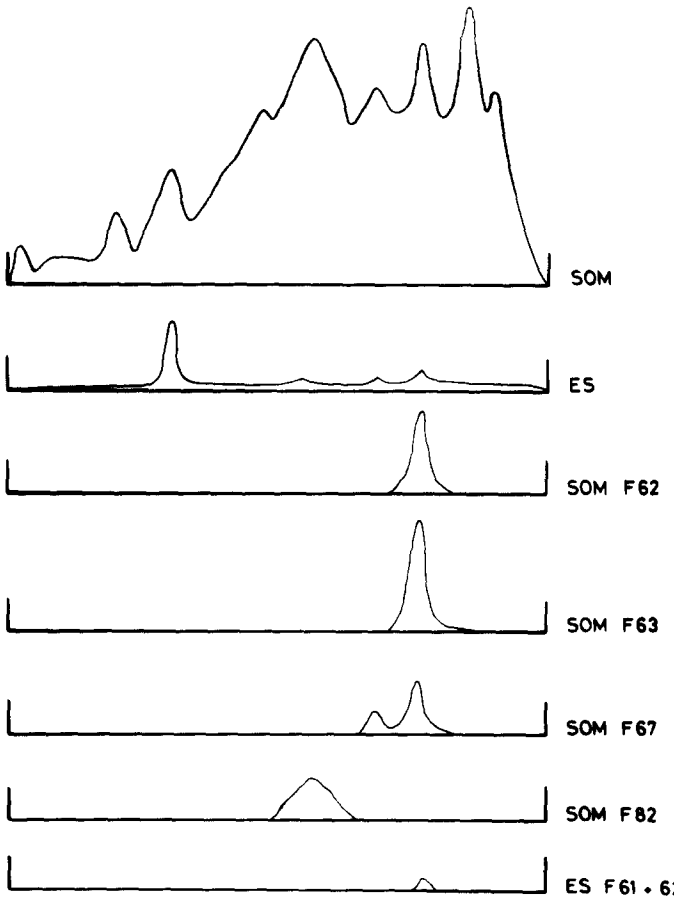


FIG. 8. Acrylamide gel electrophoresis of selected fractions of Som-Ag and ES-Ag. This figure shows the densitometer tracings of the disks. The first two tracings represent complete Som-Ag and ES-Ag, respectively. The next four tracings represent different fractions of Som-Ag. The last tracing represents combined ES-Ag fractions 61 and 62.

antigens were therefore composed predominantly of large macromolecules. Although rigorous methods for characterizing the purified antigen was not done, disk acrylamide gel electrophoresis revealed that the antigenic fraction was homogenous in that only one protein band appeared. The mol wt of this antigen was estimated to be approximately 140,000 daltons. Other studies on the purification and characterization of *Echinococcus granulosus* antigens in hydatid fluid had established that the mol wt of the important antigens were around 120,000 daltons (10, 11).

The results of the vaccination experiment confirmed that the purified fraction obtained contains a highly functional antigen. Dosages of 50, 10, and 1 $\mu$g/rat conferred 100, 90, and 70% protection, respectively, after 2 wk. A separate experiment had demonstrated that significant protection could be achieved after the 1st wk. Trials on the long-term effectiveness of the antigen could not be performed because of the phenomenon of age resistance in rats against infection by *T. taeniaeformis* (34).

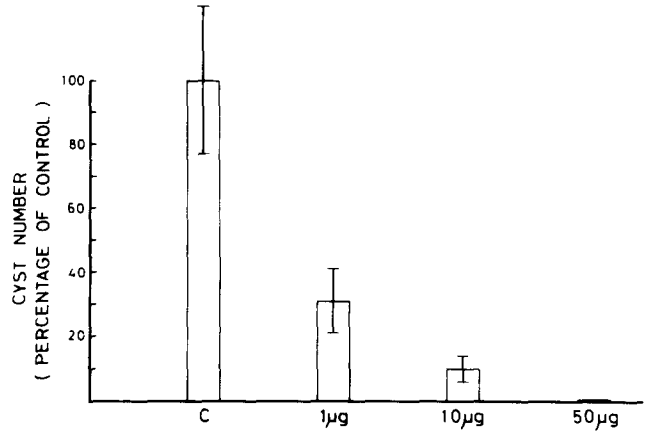


FIG. 9. Vaccination against *T. taeniaeformis* infection using different concentrations of the purified antigen. Groups of rats were injected subcutaneously with 50, 10, or 1 μg of the purified antigen emulsified with CFA. Control rats were injected with saline-CFA. All rats were challenged with 500 oncospheres each 2 wk later. Autopsy and cyst count were carried out 3 wk after challenge. A 50-μg dose confers complete protection with significant partial protection conferred by lower concentrations. Vertical bars represent standard errors of the mean. Five rats per group.

The general belief that ES-Ag are more effective than Som-Ag in stimulating protective immunity appears to be untrue, at least with *T. taeniaeformis*. In this study, we have shown that injection with the same dose (by weight) of Som-Ag and ES-Ag stimulated similar levels of protective immunity. Furthermore, a semiquantitative method (based on ratio of the optical densities of the fractions) was used to give an approximate estimate of the percentage of the functional antigenic fraction (fraction 62) in the total sample. The purified Som-Ag fraction is approximately 6.8% of the total Som-Ag sample and the purified ES-Ag fraction is approximately 8.1% of the total ES-Ag sample. Thus on a weight basis, Som-Ag and ES-Ag appear to have similar immunogenicity. Thus, the small amount of functional antigen available from ES-Ag would not justify the use of in vitro culture to collect and concentrate ES-Ag. It would be simpler and more efficient to obtain antigens for vaccination by using preparative chromatography to purify Som-Ag. We are now in the position to isolate and purify antigen and conduct vaccination trials for other related taeniasis-cysticerosis such as *T. solium* in swine and man, and *T. saginata* in cattle.

## Summary

Artificial immunization of rats against *Taenia taeniaeformis* was studied using somatic antigen (Som-Ag) and execretory-secretory antigen (ES-Ag). It was found that both Som-Ag and ES-Ag stimulated immediate-type hypersensitivity and delayed-type hypersensitivity reactions to similar levels. Antibody levels rose from the 2nd wk and peaked around the 6th and 7th wk. Both IgM and IgG were detectable from the 2nd wk onwards, with IgG at a considerably higher level compared to IgM. In terms of protection, 90–100% reduction in cyst counts were detected if the rats were challenged 10 days or more after immunization. In all cases, no significant difference was observed between immuniza-

tion with either Som-Ag or ES-Ag. Som-Ag and ES-Ag were purified and characterized using Sephadex G-200 chromatography, double immunodiffusion, and disk acrylamide gel electrophoresis. A purified antigen (mol wt, 140,000 daltons) was obtained, and highly significant protection against infection resulted with injections of 50, 10, or 1 $\mu$g doses of this antigen with complete Freund's adjuvant.

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