PRACTICABLE IFAT SLIDE ANTIGEN PREPARATIONS FOR DETECTION OF *GNATHOSTOMA SPINIGERUM* ANTIBODIES IN RABBITS

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Abstract. This study was designed to determine which stage of *Gnathostoma spinigerum* and which method of the preparation of test antigens are the most suitable for the detection of antibodies in serum of rabbits infected with advanced third stage larvae (AL3) of *G. spinigerum* by the indirect fluorescent antibody test (IFAT). Antigens from parasite ova and first stage larvae (L1) were obtained from freshly preserved specimens and affixed to glass slides with egg albumin. AL3 antigens consisted of paraffin sections, cryostat sections and pellets of crude worm soluble extract. Slides of adult male and female worms were prepared in cryostat sections. Pellets of crude worm soluble extract (AL3) smeared onto slides gave the best positive reaction followed by AL3 cryostat sections and L1.

INTRODUCTION

The application of the immunofluorescent antibody test (IFAT) as a diagnostic tool has been performed with satisfactory results in infections with *Brugia malayi* (Singh *et al*, 1980), *B. pahangi* (Au *et al*, 1982), *Schistosoma mansoni* (Azab *et al*, 1984), *S. japonicum* (Beisler *et al*, 1984), *Paragonimus ohirai* (Ohara *et al*, 1985). These studies showed that the detection of antibodies in infected animals or in patients' sera varied with the stages of parasites used and the methods of antigen preparation.

The objective of this study was to determine which parasitic stage and method of antigen preparation is most suitable for the detection of antibodies in gnathostomiasis in rabbits by indirect fluorescent antibody test.

MATERIALS AND METHOD

Animals

Two white rabbits, 2.0-2.5 kg body weight, of either sex were used in the experiment. One rabbit was infected orally 3 times at 2 weeks intervals with 5 *Gnathostoma spinigerum* advanced third stage larvae (GsAL3) recovered from experimentally infected mice. The other rabbit served as an uninfected control.

Sera

The animals were bled weekly for 20 weeks from marginal ear veins, the sera were collected and kept at -20°C until used. Ouchterlony gel diffusion tests were performed on these sera against GsAL3 crude soluble antigen. The serum with the strongest precipitin band was used for the IFAT.

Preparation of antigens

Seven batches of test antigens were prepared from 4 parasite stages.

Egg antigens. Adult female *G. spinigerum* obtained from tumor in a cat's stomach were kept in 0.15M NaCl and allowed to oviposit for 2-6 hours. The eggs were collected, washed several times with phosphate buffered saline (PBS) pH 7.2, then fixed with 5% formalin in PBS for 30 minutes. The eggs were washed once with distilled water (DW), mixed with 5% egg albumin in DW, then smeared on slides, adjusting numbers to ap-

proximately 40 eggs per slide. The smears were dried at 37°C for 30 minutes, kept at -20°C until used.

L1 antigens. First stage larvae (L1) were obtained from 10-day old cultures of eggs.

AL3 antigens. Experimentally infected mice were dissected for advanced third stage larvae (AL3), the larvae were washed several times with PBS, then divided into 3 lots as follows: a) Paraffin section antigens. These were larvae fixed in 5% buffered formalin, embedded in paraplast+, sectioned and mounted on slides previously smeared with 5% egg albumin then kept at -20°C until used. b) Cryosection antigens. Larvae were frozen at - 20° C, embedded in Tissue-Tek OTC compound (Miles Laboratory), sectioned at 8 μm thickness with a cold microtome (Sakura) and mounted on egg-albumin coated slides. These were kept at -20°C until used. c) Pellet antigens. After washing with PBS, the larvae were ground in a Potter-Elvehjem tissue grinder in an ice bath. The homogenates were centrifuged at 10,000*g* at 4°C for 30 minutes, then the supernates were collected as crude soluble antigen. The pellets were smeared on slides, dried and kept at -20°C until used.

Adult worm antigens. Male and female worms were obtained from stomach tumors of experimentally infected cats. The worms were washed 3 times with PBS, frozen at -20°C and used as AL3 cryosection antigens.

Immunofluorescence procedure

Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Sigma) was used. A checkerboard titration was performed to determine the optimal dilution of FITC conjugate by using positive and negative control sera. The working strength of the conjugate was 1:100 and the optimal serum dilution was 1:32. Meanwhile the frozen slide antigens were allowed to dry at room temperature after which a serum dilution of 1:32 in PBS was added to each antigen preparation. The slides were kept in a moist chamber for 30 minutes at room temperature. The sera were rinsed off and the slides were washed twice in PBS within 30 minute intervals. They were blotted dry then a drop (30 μl) of FITC conjugate was added onto each antigen. The slides were returned to the moist chamber for 30 minutes. The conjugate was rinsed off and the slides were washed as above. After drying, the slides were overlayed with buffered glycerol 1:2 pH 7.2, covered, and examined under a fluorescence microscope (NIKON Optiphot Epifluorescence microscope equipped with Nikon mercury lamp HBO-100 W/2).

RESULTS

The sera of non-infected and infected rabbits were reacted with crude soluble antigens prepared from GsAL3 by Ouchterlony test. The protein content of antigens as determined by the method of Lowry *et al* (1951) was 6.78 mg/ml and a 1:8 dilution of this was used in the test. A weak precipitin band appeared in sera obtained 4 weeks after the initial infection. The strongest precipitin reaction was observed at week 12 postinfection. Rabbit serum obtained in the 12th week after initial infection was kept for this study.

Seven antigenic slide preparations were tested with rabbit anti-serum by IFAT. Antibodies were detected in 3 preparations of antigens ie L1, AL3 cryosections and AL3 pellets. The specific reaction of L1 antigens was seen as a bright green fluorescence at the cuticular sheath (Fig 1). AL3 cryosections preparations gave a bright green fluorescence at the cuticle, muscle of body wall, esophagus and esophageal lumen (Fig 2). AL3 pellets antigens fluoresced at the spines of both cephalic portion and body, along the transverse cuticular striations, lips, orifices (anus and excretory pore) and cut ends of fragmented larvae (Fig 3).

Preparations of eggs, AL3 paraffin sections and adult cryosections from both male and female worms showed no specific fluorescence when reacted with antiserum from the infected rabbit. All slide antigen preparations were negative with control serum from the uninfected rabbit.

DISCUSSION

In this experiment, egg and adult worm antigens were less reactive than L1 and AL3 when tested by IFAT. Furuno (1959) reported that larval stage antigens have higher sensitivity than adult antigens in precipitin test, while Punyagupta and Pacheco (1961) reported similar results by indirect hemag-

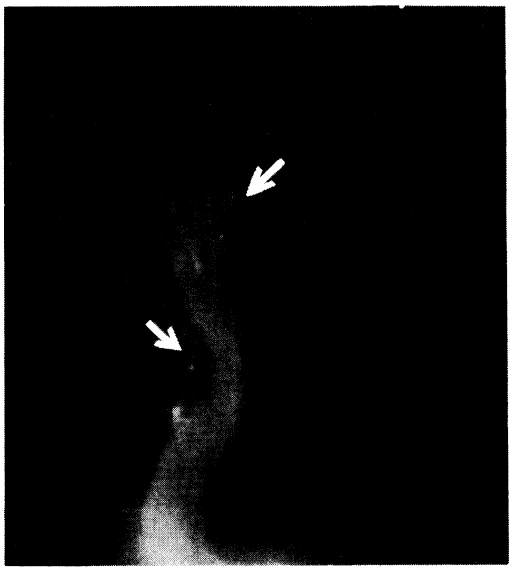

Fig 1—Specific fluorescence of first stage larvae of *G. spinigerum* reacted with rabbit anti-GsAL3 serum, showing bright green fluorescence at the cuticular shealth (arrows).

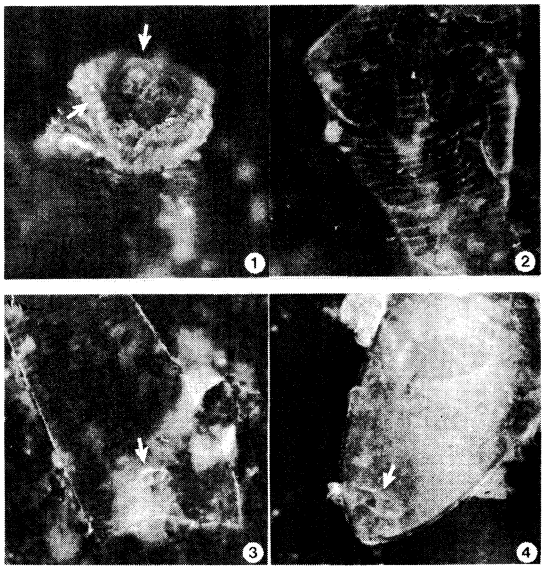

Fig 3—Fluorescent staining of rabbit anti-GsAL3 detected with AL3 pellet antigens was seen at lip (arrow) and cephalic spines (1); cuticular striations (2); orifices (arrows) (3, 4).

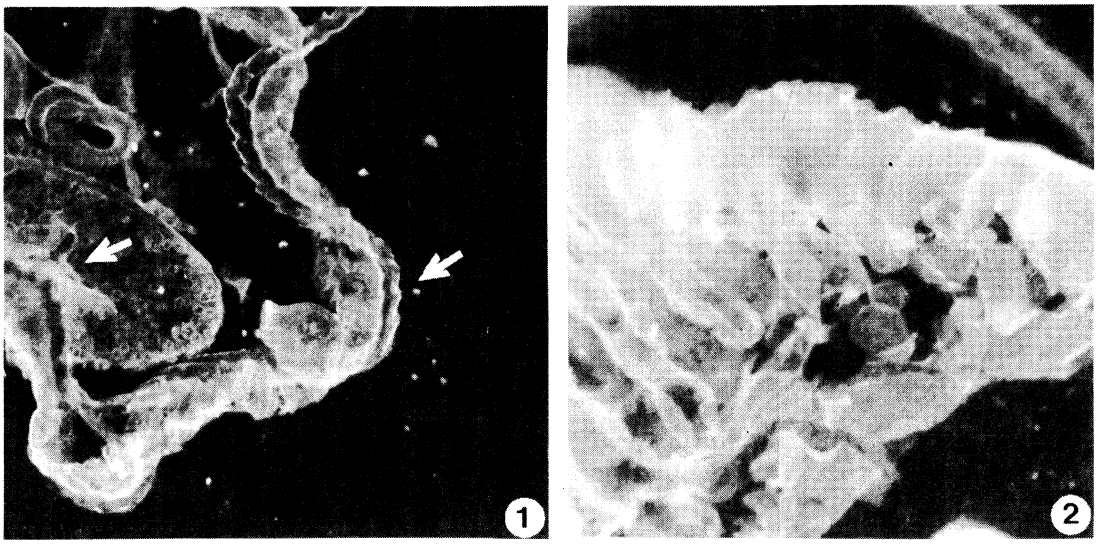

Fig 2—Cryosections of advanced third stage larvae of *G. spinigerum* (GsAL3) reacted with rabbit antiGsAL3 serum; bright green fluorescence was seen at cuticle (arrow), esophagus and esophageal lumen (arrow) (1); muscle of body wall (2).

glutination test. It appears that adult worm antigens are not useful in most serological tests. Au *et al* (1982) showed that adult *Brugia pahangi* antigens were more suitable than those derived from other stages in the diagnosis of human filariasis by IFAT. Kamiya and Tanaka (1969) reported better results obtained with adult *Angiostrongylus cantonensis* than with other stages when tested

with infected rat sera by hemagglutination test. However, in gnathostomiasis, antigens derived from advanced third stage larvae (AL3) gave optimum results.

The method of preparation of antigens also influences the antigenic properties of the parasites. Although AL3 was shown to be a good antigen, cryosections or pellets from ground worms were better than paraffin sections. The reactions seen in paraffin sections from our study consisted of a dull fluorescence, but Morakote *et al* (1989) showed positive fluorescence at the cuticle, esophagus and intestine of paraffin sections of GsAL3 isolated from eel's liver. Beisler *et al* (1984) examined the difference between two preparations of adult *Schistosoma japonicum* against rabbit anti-sera and concluded that frozen acetone-fixed sections showed strong fluorescence at the tegument whereas the reaction in paraffin embedded formalin fixed sections was weak. The gut and stroma of these two preparations remained clearly positive.

Singh *et al* (1980) reported a bright fluorescence at the cut ends of sonicated microfilariae as well as the cuticle. This agreed with our results in using AL3 pellet antigens, ie the fluorescence was seen at the end-pieces of fragmented larvae and at the cuticular striations. Even though the cuticular striations fluoresced dimly in some larval fragments the cut ends fluoresced brightly. Specific fluorescence was also observed at both anus and excretory pore. This finding was also demonstrated in microfilariae of *Dirofilaria immitis* against canine and human anti-sera (Welch and Dobson, 1974).

Although L1, AL3 cryosections, and AL3 pellets were good diagnostic antigens, L1 and AL3 cryosections were easily detached from the slides during manipulation, therefore, requiring more careful handling compared to pellets. Hedge and Ridley (1977) also found difficulty in keeping whole microfilariae on the slides. Our study showed that AL3 pellets were superior to AL3 cryosections and L1 for use in IFAT.

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