Immunodiagnosis of Gnathostomiasis

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Abstract

Several immunological methods for the diagnosis of gnathostomiasis have appeared in the literature. The oldest method was the skin test, which was established initially by Japanese scientists in the 1960s. It was a convenient and relatively reliable diagnostic means in that period. During the 1980s, ELISA was evaluated by several investigators, and it was found that sensitivity varied from 56% to 100%. The antigen used in the assay was crude larval antigen of *Gnathostoma spinigerum*, crude adult antigen of *G. doloresi* or larval excretory-secretory antigen. The immunoblotting technique was studied during the 1990s. The specific diagnostic band was the protein component with a molecular weight of 24 kDa. The routine current diagnostic method for human gnathostomiasis is the immunoblotting technique. Monoclonal antibodies (mAbs) to the crude soluble extract, 24 kDa protein component of *G. spinigerum* advanced third-stage larvae have been produced. Moreover, hybridoma cell lines, derived from spleen cells of an infected mouse, secreted antibodies that reacted with the cuticle of GsAL3 sections by IFA. These mAbs were produced. However, the diagnostic value of these mAbs is not applicable for the diagnosis of human gnathostomiasis.

Keywords: immunodiagnosis, gnathostomiasis

Human gnathostomiasis is mainly caused by *Gnathostoma spinigerum*. However *G. hispidum* and *G. doloresi* have been reported as causative agents of gnathostomiasis in Japan after the 1980's.

Both cell-mediated immune responses and humoral immune responses against third-stage larvae of *Gnathostoma* sp have been demonstrated in animals and man. Several immunodiagnostic techniques have been used in both animal models and humans in order to obtain a specific diagnosis of human gnathostomiasis. The work was carried out mainly in Japan and more recently in Thailand. The important recorded methods were the skin test, precipitation test, gel diffusion test, indirect fluorescent antibody test, ELISA and immunoblotting technique. Monoclonal antibodies have been produced but have not yet been applicable for the diagnosis of human gnathostomiasis. This paper is a literature review of gnathostomiasis immunodiagnostic techniques.

Skin test

The skin test or intra-cutaneous test was the first immunological method for the diagnosis of human gnathostomiasis in Japan. In the 1960s, it was a convenient and relatively reliable diagnostic technique [1]. The antigen used was prepared from either adults or larvae of *G. spinigerum* or *G. doloresi* [2-3].

The skin test was studied initially by Egashira [4-5] and Yamaguchi [6] using antigen prepared from the adults or larvae of *G. spinigerum*. The sensitivity was reported as 100% in 95 gnathostomiasis cases; however they cross-reacted with *Paragonimus* and schistosome antigen. Ando, in 1957 [7] reported that the cutaneous reaction was elicited more intensely by the protein fraction than by the fat and sugar fraction. This antigen fraction was separated

from the extract of larval or adult *G. spinigerum*. The extract of the esophagus was more antigenic than that of the other parts of the worm. The presence of high antigenicity was also found in the maintaining medium of the worm (*ie*, saline solution).

In mice experimentally infected with advanced third-stage larvae of *G. spinigerum* (GsAL3), Morakote *et al* [8] demonstrated active cutaneous anaphylaxis to larval somatic antigen at the end of the first month of infection. The test was said to be specific for *Gnathostoma* infection, since no reaction was observed with hookworm or *Trichinella*-infected mouse sera.

Precipitation test

In addition to the intradermal test, in Japan, the precipitation test or precipitin ring test was developed as a valuable diagnostic method for gnathostomiasis [9-10]. By Sarles' phenomenon (precipitin formations around the mouth, vulva and anus of AL3 after incubation with rabbit hyperimmune sera), when reacted with human sera, it was demonstrated that the precipitation appeared prominently in the anterior tip of the worm [11-12]. However, non-specificity of the test had been reported [13].

Various fresh and air-dried developmental stages of *G. spinigerum* were tested by circumoval and larval microprecipitation reactions [14]. These developmental stages were unembryonated ova, embryonated ova, first stage larvae and advanced third-stage larvae. The antisera used were obtained from experimentally *G. spinigerum* infected mice, rats, cats and infected humans. The sera were reactive to the body surface of the air-dried preparation of AL3 by larval microprecipitation reactions. The precipitates formed on the larvae were membranous and filamentous precipitates. The filamentous precipitates were distributed randomly on the surface of the AL3 while the membranous precipitates were formed around the esophageal region. This indicated that the epitopes are located exteriorly on the AL3.

Gel diffusion

The Ouchterlony double diffusion test and immunoelectrophoresis have been used as supportive methods for the diagnosis of human gnathostomiasis in Japan [15-17].

In mice, the humoral immune response to early (EL3) and advanced third-stage larvae (AL3) of *G. spinigerum* was studied by Ouchterlony gel diffusion technique [18]. In mice infected with EL3, the antibodies were detected in week 3 and remained up to week 10 after infection. The highest positivity of the sera was demonstrated between weeks 4 to 7. In mice infected with AL3, earlier detection of the antibodies was obtained, *ie*, weeks 2 to 5 after infection. The number of larvae located in muscle correlated with the peak of positive sera. From this experiment, the initial antibody response depended on the stage of worm that was infected, but the duration of the antibody response depended on the number of worms infected.

Radioimmunoassay

In cats infected with AL3, a humoral immune response against somatic antigen of AL3 was demonstrated by radioimmunoassay [19]. The technique was sensitive, in that a 1: 10,000 dilution of sera still gave a positive result.

Complement fixation test

This technique was reported by Yamaguchi [9] as a supportive diagnosis of gnathostomiasis infection.

Indirect hemagglutination antibody test (IHA)

No IHA antibody response was demonstrated in sera of mice either subcutaneously infected with GsAL3 or orally infected with GsEL3 [8]. It was concluded that IHA was insensitive for the diagnosis of gnathostomiasis in mice.

Indirect fluorescent antibody test (IFA)

Morakote *et al* [20] evaluated indirect fluorescent antibody assays for gnathostomiasis. The paraffin sections of GsAL3 were treated with rabbit hyperinfected sera and human gnathostomiasis sera. It was shown that the

target antigen was in the anterior part of the esophagus, the surface of the cuticle and in the cytoplasmic granules of the intestine.

Anantaphruti *et al* [21] studied slide preparations of antigen for the detection of antibodies in the gnathostomiasis sera of rabbits using the indirect fluorescent antibody test. Seven preparations were made from 4 parasitic stages of *G. spinigerum*: eggs, first stage larvae (L1), advanced third-stage larvae (paraffin sections, cryosections, ground pellets) and adult worm cryosection. They found that the smear slide of pellets obtained from sedimenting ground AL3 was the best preparation, followed by AL3 cryosections and L1. In the pellet smear slide, the antigenic sites were observed at either cephalic or body spines as well as body cuticular striations, lips, orifices and cut fragment ends.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA: IgG

IgG ELISA has been trialled for the diagnosis of human gnathostomiasis. The crude soluble extract of GsAL3 was mainly used as antigen to detect serum antibodies. Suntharasamai *et al* [22] studied specific IgG using water extract antigen prepared from AL3 obtained from experimental infected mice and showed that only 56% of the sera of cutaneous migratory swelling patients were positive. The specificity of the test was 84% when compared with sera of healthy controls and other parasitic infections. In 1986, Dharmkrong-at *et al* [23] studied patients' sera from intermittent cutaneous migratory swelling by using 0.85% saline extracted antigen prepared from AL3 obtained from naturally infected eels. The result showed that all sera were positive. However, this study included only a few sera with other parasitic infections.

GsAL3 released excretory and secretory (ES) products in the medium at day 2 up to day 14 of culturing, with the highest peak at day 4. These ES products reacted with antisera of human gnathostomiasis patients when evaluated by ELISA [24].

Maleewong *et al* [25] evaluated ELISA using somatic and ES products as antigen for diagnosis of the infection. It was found that ES antigen gave a similar result to crude somatic antigen. They also indicated that ELISA showed superior results in diagnosis than the indirect hemagglutination test and counter immunoelectrophoresis.

Anantaphruti [26] studied antigens prepared from *G. doloresi* adult worms obtained from infected pigs (Gd antigen) and *G. spinigerum* AL3 obtained from experimentally infected mice (Gs antigen). The worms were prepared and extracted as 0.1% saline extracted antigen. The result showed higher sensitivity but lower specificity was obtained with Gd antigen than Gs antigen. However, in a rabbit immunized with crude extract of *G. spinigerum* larvae, the ELISA values increased gradually after each immunization, with a similar pattern when using Gs antigen and Gd antigen for the assays [27]. A strong antigenic similarity between species was also observed using adult worms of *G. hispidum* and *G. doloresi* as antigen. They showed a close correlation (r = 0.97) between the OD values of gnathostomiasis by ELISA [28].

To evaluate the sensitivity of ELISA for the diagnosis of gnathostomiasis, the use of negative control serum in the assay should be considered. In areas with a high prevalence of various parasitic infections, sera of healthy control individuals may cause a rise in the high OD values in the assay, thus resulting in lower sensitivity for the test [29].

Detection of circulating parasite antigens in murine gnathostomiasis by a two-site enzyme-linked immunosorbent assay was reported by Maleewong *et al* [30]. IgG fractions, prepared from antiserum of a rabbit repeatedly infected with *G. spinigerum*, were used as the capture antibody. Antigen and antibody detection assays of mice infected with 15 GsAL3 were performed during the course of infection. It was found that circulating antigen was detectable at the first week of infection. The amount of detectable antigen increased steadily up to week 4, with the peak at week 3 of infection. No significant amount of circulating antigen was detected after week 4 of infection. However, the serum antibody levels appeared at week 2 and remained

high up to week 8, the end of the study.

In Mexico, 93% of 300 cases of cutaneous larva migrans were positive by ELISA using crude somatic extract of adult *G. doloresi* [31].

In outpatient clinics of hospitals, ELISA has been used for diagnosis and the evaluation of the treatment efficacy of human gnathostomiasis [32-33].

ELISA: IgE

The levels of IgE antibody in patients with parasitologically confirmed gnathostomiasis and in those with migratory swelling were evaluated by ELISA using somatic extract antigen prepared from third stage larvae [34]. It was demonstrated that the total IgE and specific IgE in those sera were markedly higher than those in healthy controls. This indicated that *G. spinigerum* potentiates IgE production. However, there was only a weak correlation between specific IgE titers and total IgE levels, and also between specific IgE and IgG antibody titers.

Western blot analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were analyzed for the diagnosis of the infection.

The crude water extracts of the GsAL3 from naturally infected eels were studied for their protein compositions by SDS-PAGE. It was shown that the extract was highly complex and was comprised of more than 40 protein components with relative molecular weights (MW) ranging from 13 to 150 kDa, in which approximately 20 components were antigenic in humans [35]. The crude extracts of the larvae were evaluated by Western blot analysis for their reactivities against sera from patients with parasitologically confirmed gnathostomiasis, 15 patients with presumptive gnathostomiasis, 64 patients with various parasitic infections and 19 healthy adults. Extensive cross-reactions of the parasite's antigen with sera from patients with other parasitic infections occurred. A MW of 24 kDa antigen was found to react with all parasitologically proven patients, 5 presumptive patients, one of the patient with other parasitic infections (paragonimiasis) and none of the healthy individuals. This 24 kDa component was stated as a potential antigen for the immunodiagnosis of human gnathostomiasis [36].

The 24 kDa protein was further purified; the purification procedures involved gel filtration, chromatofocusing and anion exchange column chromatography [37]. Characterization of the specific antigen was performed by SDS-PAGE, Western blot analysis and isoelectric focusing. It revealed that 24 kDa was a carbohydrate containing protein as revealed by concanavalin A staining, and had a pI of 8.5 as determined by isoelectric focusing. The crude extracted antigen and the partially purified 24 kDa fractions were used as antigens to detect specific antibodies in four groups of individuals: parasitologically diagnosed patients, clinically diagnosed patients, other parasite-infected patients and healthy parasite-free controls. Sensitivity, specificity, and positive and negative predictive values of the assay were compared. The first exclusion peak was obtained from the DE-52 column which contained mostly the 24 kDa band, and when used as antigen in the indirect ELISA it gave the best result, *ie*, 100% specificity and sensitivity and high reproducibility.

Three preparations of crude somatic antigens of *G. spinigerum* were prepared: by extracting advanced third-stage larvae with either distilled water, 1% Triton X-100 or 1% sodium deoxycholate (NaDOC) containing proteinase inhibitors. The protein profiles of the 3 extracts were compared by SDS-PAGE and Coomassie brilliant blue staining, and the reactivities were studied by Western blot analysis. Deterioration of some components was observed in the extraction with Triton X-100 or NaDOC. It was found that distilled water yielded the greatest amount of diagnostic 24 kDa antigen. The strongest reactivity between the patient's antibody and the 24 kDa antigen was observed in Western blot analysis when the fractionated crude water extract was used in the reaction. Little difference in protein and antigenic patterns was found between the extracts of larvae collected from naturally infected eels and from experimentally infected mice. The 24 kDa

diagnostic component was found predominantly in the intestinal organs, such as the esophagus and intestine, and the body fluid of the larvae. The body cuticle and the head-bulb of the larvae contained predominantly high MW proteins, which were weakly antigenic in man [38].

Monoclonal antibodies

Three types of monoclonal antibodies (mAbs) were produced: 1) mAbs to immunized mice with 24 kDa protein fractions of GsAL3 extracted, 2) mAbs to immunized mice with crude soluble extract of GsAL3, and 3) mAbs to an infected mouse with live GsAL3.

The mAbs against the protein components of GsAL3, from the first exclusion peak of the chromatofocusing column chromatography, were produced. The fraction contained 24 kDa glycoprotein, which is the specific antigen of *G. spinigerum* [39]. This mAb was produced from GN6/24 monoclone. It showed specificity to 24 kDa antigen of *G. spinigerum* by Western blot, and gave negative results on ELISA against heterologous antigens. The heterologous antigens tested for specificity were *Angiostrongylus cantonensis, Paragonimus westermani, P. heterotremus, Opisthorchis viverrini, O. felineus, Trichinella spiralis, Schistosoma mekongi, S. mansoni, Echinostoma malayanum, E. ilocanum, E. revolutum, Strongyloides stercoralis, Enterobius vermicularis, Ascaris lumbricoides,* and small intestinal flukes (*Haplorchis taichui* and *Phaneropsolus bonnei*). The mAbs were also proved not to react with the host antigens extracted from eel livers and mouse muscles [40].

Rojekittikhun *et al* [41] described seven monoclonal antibodies: FS-3D11, SS-5H5, SK-6C4, SK-4E1, SK-7G6, SD-8D4 and SA-9B5, specific to *G. spinigerum*. These were established from mice immunized with GsAL3 crude soluble antigens. All of these mAbs belonged to the IgG1 subclass, recognized protein determinants, and only FS-3D11 and SS-5H5 recognized carbohydrate epitopes.

Immunohistochemical localization of antigens was studied by indirect enzyme immunostaining under light microscope using these 7 mAbs. The antigens used were sections of formalin-fixed paraffin-embedded GsAL3 from experimentally infected mice. Each mAb exhibited different reaction patterns and staining intensity in the sections. FS-3D11 bound primarily to the intestinal brush border while SS-5H5 reacted with various tissues of the larvae, including the intestinal epithelium and brush border, lateral cords, muscle, pseudocoel and cuticle. SK-6C4 predominantly stained muscle, SD-8D4 bound to the cuticle and the lateral cords and SA-9B5 reacted primarily with the pseudocoel. The remaining two antibodies, SK-4E1 and SK-7G6, exhibited no labeling. The results suggested that antigens sharing common epitopes were present in various structures of the larvae, with the intestine being the most antigenic site. It also suggested that certain GsAL3 antigens recognized by the mAbs were sensitive to formalin fixation and/or paraffin embedding since for 2 out of the 7 mAbs, staining was negative [41].

Rojekittikhun *et al* [42] studied immunocytochemical localization of GsAL3 antigens by immunogold labeling method, using 7 specific mAbs. The larvae were fixed in paraformaldehyde and embedded in Lowicryl K4M medium and sectioned into 100-120 nm thickness. The gold colloidal particles used for coupling with sheep anti-mouse IgG were 15 nm in size. When the sections of larvae were probed with FS-3D11, the gold particles were concentrated specifically on the intestinal brush border. When SS-5H5 was used, the particles were scattered densely over the brush border and in the cytoplasm of epithelial cells. The rest of the mAbs, which recognized protein determinants, exhibited a lack of labeling. The results suggested that the carbohydrate antigenic determinants were the most stable and most abundant, particularly in the intestines of the larvae.

Purification of crude soluble GsAL3 antigens by mAb affinity chromatography was performed [43]. An immunoaffinity column was prepared by coupling a mAb, SK-6C4 to CNBr-activated Sepharose 4B. The immunological reactivity of this purified antigen was evaluated

by ELISA. The purity and specificity were determined by SDS-PAGE and Western blotting. It was found that this isolated antigen was relatively pure and immunologically specific.

An ELISA using either crude extract or SK-6C4 affinity-purified antigens was studied comparatively for the immunodiagnosis of human gnathostomiasis. Human serum samples tested were: 7 parasitologically confirmed patients, 26 presumptive gnathostomiasis cases, 40 heterologous infections, 22 healthy, parasite-free individuals. Using either GsAL3 crude extracted antigen or SK-6C4 affinity-purified GsAL3 antigen, no significant difference in OD values was observed. Sensitivity, specificity, and positive and negative predictive values, using either antigen were 100%, 98.4%, 87.5% and 100%, respectively [44].

Dantrakool *et al* [45] labeled the surface cuticle protein of GsAL3 with ^{125}I using the IODOGEN method. After labeling, the larvae were extracted in DOC extraction solution. The ^{125}I labeled surface extracted antigen was tested with sera of immunized and infected mice by immunoprecipitation. The result showed that only the sera from infected mice precipitated the 25 kDa protein of antigen, whereas none of those from immunized mice reacted. By IFA, monoclonal antibodies produced from 17 hybridoma of spleen cells from an infected mouse reacted with several tissues of paraffin sections of AL3; esophagus and cuticle, intestinal cells and cuticle or cuticle only. However, when tested by immunoprecipitation, none of these mAbs reacted with surface-iodinated proteins of the larvae, not even the mAbs produced by only cuticle-positive hybridoma cell lines. By Western blot, the mAbs produced by clones derived from one of the IFA cuticle-positive hybridoma cells lines recognized several proteins with MW ranging from 55 to 96 kDa.

Although mAbs to *G. spinigerum* larvae had been produced from 3 different sources, the usefulness of these mAbs for the diagnosis of human gnathostomiasis has not yet been demonstrated. The routine laboratory diagnostic methods for human gnathostomiasis have been used are ELISA and immunoblotting technique, and the latter is preferable. The antigen used is the crude soluble extract of advanced third stage larvae of *G. spinigerum*, run on SDS-PAGE, and transferred to nitrocellulose membrane. The specific diagnostic band of the infection is 24 kDa.

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