Circulating gut-associated antigens of Schistosoma mansoni : biological, immunological, and molecular aspects

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Chapter 5

Application of the FITC–anti–FITC gold system to ultrastructural localization of antigens

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Chapter 5

Application of the FITC–anti–FITC gold system to ultrastructural localization of antigens

Abstract

We report the application of a fluorescein isothiocyanate (FITC)–anti–FITC method to localize antigens at the ultrastructural level. In the systems studied, the anti–FITC–based detection method displays high specificity and sensitivity. These observations, combined with ease of production and with availability of FITC–protein conjugates, suggest that the FITC–anti–FITC method is a good alternative to presently used methods and widely applicable to immunochemical and immunocytochemical procedures. The same preparation and protocol can be used for light and electron microscopic studies, thereby reducing possible artifacts introduced if different procedures are used. In the present study, two systems were used to test the method. One system used an FITC–labeled monoclonal antibody (McAb) to schistosome circulating cathodic antigen. In this system, the label was detected in the gut of adult *Schistosoma mansoni* by an anti–FITC McAb conjugated to 10–nm gold particles. The second system used human IgM antibodies pooled from patients infected with *Schistosoma mansoni*. In this system detection was accomplished using an anti–human IgM–FITC conjugate followed by the anti–FITC–Au antibody conjugate.

Introduction

One of the drawbacks to immunocytochemical studies using FITC conjugated antibodies is the inability to use the same material for ultrastructural studies. Results from fluorescence studies and electron microscope studies are usually derived from different protocols for specimen preparation and detection, thus introducing the possibility of artifacts if those results are compared. Recently, a fluorescein isothiocyanate (FITC)–anti–FITC system has been developed as an alternate amplification and specific detection method to systems, such as the

avidin-biotin complex, for immunodiagnosis and immunochemical procedures [5,10]. For example, anti-FITC monoclonal antibodies (McAbs) have been employed as a sensitive method for detecting proteins on dot and Western blots [10], for ELISA [5,6], for solid-phase radioimmunoassay [8], or for separating bound from free tracers in competitive immunoassay [7]. Polyclonal anti-FITC antibodies have been used for the simultaneous demonstration of two antigens on the same cell [4], in migration studies of FITC-labeled lymphocytes [15], and in incorporation studies of fluorescently labeled actin into stress fibers [1].

The object of the present investigation was to determine whether the same principle, *i.e.*, the FITC-anti-FITC, could be adapted for electron microscopic immunocytochemical studies by using an anti-FITC McAb labeled with gold (anti-FITC-Au) as a probe. For this study, the probe was utilized to detect an FITC conjugated McAb against *Schistosoma mansoni* gut-associated circulating cathodic antigen (CCA). This particular McAb was selected because previous light and electron microscopic studies [2] would allow for comparisons. Subsequently, the flexibility of the system was tested by using an antibody that was not directly conjugated to FITC. For both light microscopic comparisons and electron microscopic localization, the same specimen of LR White embedded worms was used.

Materials and Methods

Fixation and embedding procedure for worms

Schistosoma mansoni adults were removed from the mesenteric venules of mice infected 8 weeks previously with a Puerto Rican strain of cercaria. The worms were rinsed several times in Earl's lactalbumin hydrolysate at room temperature and fixed in a mixture of freshly prepared 2% paraformaldehyde and 0.2% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.2) for 1 hr at room temperature. During the fixation period, the adult worms were cut into several smaller sections about 2 mm long.

After fixation, the tissue was rinsed with the cacodylate buffer and partially dehydrated in an ethanol series to 70% ethanol. Infiltration with LR White resin (Polysciences, Warrington, PA) was accomplished through a series of three dilutions with 70% ethanol:resin (1:2, 1:3, 1:4), followed by two changes of fresh, undiluted resin for one hr at room temperature and one change at 4°C overnight. After an additional change of resin for one hr at room temperature, the tissue was embedded in Beem capsules, sealed, and polymerized at 55°C for 24 hr [14].

Sections were cut from the same block of LR White embedded worms with a diamond knife on an LKB ultratome and collected either on gelatin coated glass slides (semi-thin

sections) or on 75 × 300 mesh bare grids (silver interference sections). The grids were precleaned with ethanol and distilled water prior to use.

Production and labeling of monoclonal antibodies

Production and characterization of the anti-CCA McAb used in this study has previously been described [2]. FITC conjugates (F/P ratio was 2.5) were prepared according to the technique of The and Feltkamp (1970) [12,13].

The anti-FITC McAb was produced essentially as described by Samuel *et al.* (1988) [10]. In short, hybridoma cells were obtained by fusion of SP2/0 mouse myeloma cells with spleen cells of BALB/c mice that had been immunized intraperitoneally with a conjugate of FITC and keyhole limpet hemocyanin (KLH-FITC). Anti-FITC specificity of the McAb secreted by cell line 256-4A1-A was ascertained by testing in ELISA against several proteins, native and FITC conjugated, including KLH, bovine serum albumin (BSA), and McAb of IgG1 and IgM isotype.

Using McAb produced by hybridoma cell line 256-4A1-A (IgG1 isotype) immunoglobulin-gold (10 nm) conjugates were prepared as follows. A colloidal gold solution is prepared by reduction of a gold chloride solution (1 ml 1% $HAuCl_4$ + 79 ml distilled water) with tannic acid (Aleppo Tannin, Malinckrodt, Edison, NJ) and trisodiumcitrate (for 10 nm gold: 100 μl 1% tannic acid, and 4 ml 1% trisodiumcitrate $\cdot 2H_2O$ in a total volume of 20 ml) at 60°C. The protein-gold conjugate was formed by adding the IgG1 McAb at pH 9.0 in such an amount that the colloidal gold solution was just stabilized [11]. Finally the protein-gold was centrifuged for 60 min at 25 000 × g into a 10% glycerol cushion to remove unconjugated IgGs, metallic gold, and to concentrate the sample. The conjugates were stored at 4°C in 10% glycerol in PBS with 0.1% BSA.

Immunocytochemistry

For light microscopy, direct immunofluorescence was performed on semi-thin LR White sections, as follows: sections mounted on gelatin-coated glass slides were pre-incubated for 5 minutes in 1% BSA in PBS (B/P) at room temperature, then incubated with the appropriate McAb-FITC conjugate (*e.g.*, anti-CCA, 1/100) in PBS/Evans blue (1/10 000) overnight at 4°C. After successive washes in B/P and distilled water, sections were covered with anti-fading solution (1 mg/ml 1,4-phenylenediamine in 90% (v/v) glycerol in PBS, stored in the dark at -20°C), mounted, and viewed in a Leitz fluorescence microscope with excitation and barrier filters of 492 and 520 nm, respectively.

For electron microscopy, all incubations with antibodies were of 60 min duration at room temperature. Ultra-thin sections, mounted on copper grids, were pre-incubated for 5 min in P/B by immersion, then incubated in the appropriate McAb-FITC conjugate (1/200 in B/P). After thorough washings in four changes of B/P with a total time of 20

min, sections were incubated in the anti-FITC-gold conjugate (1/250 in B/P), washed in B/P, then rinsed in distilled water.

In instances when the primary antibody was not conjugated with FITC, the procedure was modified as follows. After pre-incubation with B/P and incubation with the primary antibody (*e.g.*, pooled sera from *Schistosoma mansoni*-infected individuals, 1/1000 in B/P), FITC-labeled swine anti-human IgM (Nordic Immunological Laboratories, Tilburg, The Netherlands) (1/100 in B/P) was used as a secondary antibody. Finally, sections were incubated with the anti-FITC McAb-gold conjugate (1/250 in B/P). Each antibody incubation step of the procedure was followed by thorough washings in B/P.

Sections were stained with aqueous solutions of uranyl acetate and lead citrate and viewed with a Philips 201 electron microscope.

Controls consisted of incubations in unconjugated primary antibodies and/or omission of the secondary antibody step.

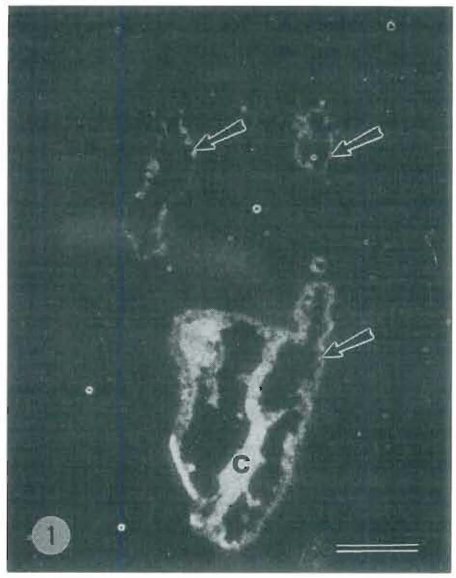
Results

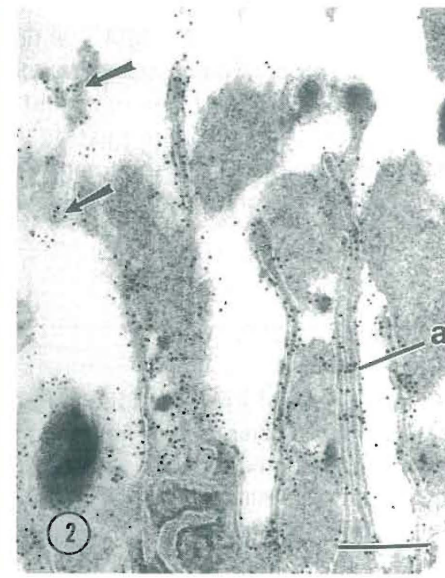
A titration series was used to optimize antibody and conjugate dilutions. Concentrations used gave strongest labeling combined with lowest to negative backgrounds.

In the direct immunofluorescence on semi-thin sections of adult worms using anti-CCA McAb 54-4C2-A conjugated to FITC, fluorescent reaction was seen in the digestive tract, most noticeably in the gastrodermis and contents of the gut (Fig. 1). On sections cut from the same specimen of embedded worms, gold particles were observed on the surface amplifications of the gastrodermis (Fig. 2) after the FITC-anti-FITC-gold procedure. No particles were observed either in the parenchyma or tegument with the concentrations used. Controls with unconjugated McAb 54-4C2-A were negative (not shown).

Figure 3. Section through the gut of adult *Schistosoma mansoni* incubated with pooled sera (1:1000 dilution in B/P) from infected humans. IgM antibodies are detected with swine anti-human IgM-FITC followed by anti-FITC-gold. Gold particles were observed associated with surface amplifications (a) and amorphous material in the gut lumen (arrows). Original magnification × 30 000. Bar = 0.25 μm.

Figure 4. Section through the tegument of adult *Schistosoma mansoni* incubated as described in Fig. 3. Gold particles were observed associated with the plasma membranes of the extensive invaginations of the tegument (arrows). Original magnification × 20 000. Bar = 0.25 μm.





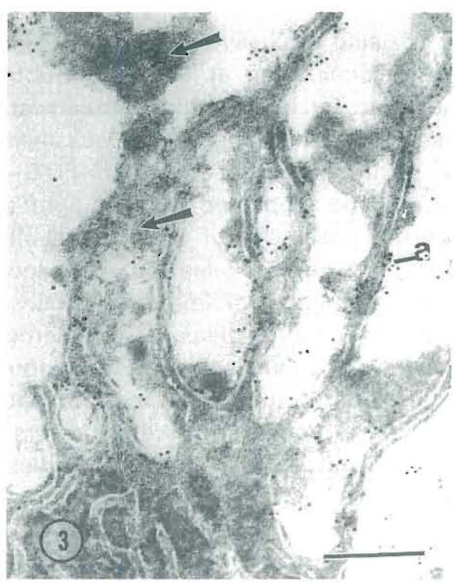


Figure 1. Semi–thin section of LR White–embedded adult *Schistosoma mansoni* labeled with anti–CCA McAb–FITC. Fluorescence is mainly seen on the gastrodermis (arrows) and in the gut contents (c). Original magnification × 300. Bar = 10 μm.

Figure 2. Localization of CCA in adult *Schistosoma mansoni* using anti–CCA McAb–FITC followed by anti–FITC–gold. Gold particles are closely associated with the surface amplification (a) of the gastrodermis and with amorphous material in the gut lumen (arrows). Original magnification × 20 000. Bar = 0.25 μm.

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Antigen specificity of IgM antibodies in serum from *Schistosoma mansoni*–infected humans using the FITC–anti–FITC–gold system is shown in Figs. 3 and 4. Gold particles were observed mainly in the gut lumen and on the surface amplifications of the gastrodermis, as well as on the plasma membrane of the invaginations of the tegument. Background gold particles were reduced by decreasing the concentration of the primary antibody to 1/1000.

Discussion

FITC conjugates of immunoglobulins and other proteins have contributed greatly to localization studies at the light microscopic level. Labeling proteins with FITC is a well–established method [12,13] with distinct advantages over labeling with biotin [5] or gold since conjugation is less laborious and easy to monitor owing to the strong color and fluorescence of the FITC. These features also simplify the purification and characterization of the conjugates.

A specific drawback was the impossibility of using basically the same procedure as in light microscopic studies for studying localization at the ultrastructural level. Application of an anti–FITC–Au conjugate for investigating ultrastructure of cells and tissues in the electron microscope greatly expands the possibilities for localization of antigens. Using the same specimen, the anti–FITC–Au conjugate allows the same FITC conjugate to be employed in both light and electron microscopic studies, and reduces the possibility of artifacts resulting from the necessity of switching protocols from light to electron microscopic studies. In addition, a wide variety of FITC conjugates of immunoglobulins of many different specificities or of a variety of other proteins is commercially available. Furthermore, this method offers new possibilities to perform double labeling experiments with two McAbs at the ultrastructural level, in combination with, for instance, the biotin–avidin system. This procedure therefore offers an alternative to traditional methods and extends flexibility of localization studies at the electron microscopic level.

We have shown an immunolabeling procedure for ultrastructural studies using an anti–FITC McAb conjugated to 10 nm gold to detect the binding specificity of an FITC–labeled anti–CCA McAb to adult worms. The anti–CCA McAb was utilized in this investigation because its specificity can be compared with the previously published report of its localization using a goat anti–mouse immunoglobulin–gold conjugate [2]. Results are reproducible and are in accordance with earlier localization studies using other techniques, including fixation and embedding procedures [2]. The fluorescence intensity in Fig. 1 is slightly reduced when compared with, for example, paraffin sections (6 μm) of Rossman–fixed worms

[3,9]. This reduction is due to surface labeling of the section of the embedded worm, whereas antibodies completely perfuse the Rossman–fixed section.

Electron micrographs are shown of labeled parasite tissue cut from the same specimen of LR White embedded worms both for light microscopic and electron microscopic studies. The embedding method employing LR White resulted in good preservation of antigenicity in the system studied. In control experiments, Lowicryl sections of embedded worms have also been labeled using the same procedure. Results were similar to LR White–embedded sections (data not shown). As generally applies for all methods, applicability of this novel method is limited to antigens that survive fixation and embedding procedures which are needed to preserve ultrastructural details.

The technique can also be used with antibodies not directly conjugated to FITC by simply employing one additional step. In the present investigation, pooled *S. mansoni*–infected human sera were used to exemplify the diversity of the protocol. The epitopes recognized are mainly present in the gut of the schistosome, and to a lesser extent on the tegument and in the parenchyma. This is in accordance with light microscopy, where hardly any fluorescence is observed outside the gut area. The rather widespread distribution of label using pooled *S. mansoni*–infected human sera is not surprising since, in the humoral immune response, antibodies are produced against the entire parasite, although IgM antibodies mainly against antigens present in the gut [3,9].

In summary, the immunolabeling procedure employing anti–FITC–Au conjugates has been shown to be specific and sensitive. In combination with ease of production and availability of FITC–protein conjugates, this FITC–anti–FITC procedure presents a widely applicable immunochemical and immunocytochemical procedure that offers a good alternative to traditionally used methods.

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