Western blot applied to the diagnosis and post-treatment monitoring of human hydatidosis

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Abstract

The serologic diagnosis of hydatidosis (caused by \textit{Echinococcus granulosus}) can be made by different techniques, although the lack of standardization of the antigens affects the sensitivity, specificity and concordance among the different tests. We have applied the Western-Blot (WB) technique, associated with a purified antigen from sheep hydatid fluid, at 60 samples of serum from 14 patients suffering echinococcosis in different bodily locations, monitored for 3 years. The WB test enabled the detection of antibodies in the pre-surgical samples for proteins of 12–14, 16, 20, 24–26, 34, 39 and 42 kDa in molecular weight in 15–96% of the patients. The combination involving 2 of the 3 proteins of 20, 39 and 42 kDa has made it possible to diagnose 100% of the cases. The antibodies specific to proteins 39 and 42 kDa disappeared in less than one year in the patients cured after surgery, while in patients with persistent or recurrent parasitism the bands present before surgery persisted or other new ones appeared. The WB with purified antigens proved to be highly useful in the diagnosis and post-surgical monitoring of hydatidosis patients. The antigen used is proposed as a standard antigen for the diagnosis and follow-up of pre-and post-surgical hydatidosis. © 2001 Elsevier Science Inc. All rights reserved.

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1. Introduction

Serologic tests are useful for diagnosing hydatid disease caused by \textit{Echinococcus granulosus} in humans, since no parasitological diagnosis is possible by non-invasive procedures and the clinical signs of the disease are non-specific. The serologic diagnosis of human hydatidosis is strongly dependent on the antigen used, thus explaining the lack of sensitivity, specificity and concordance among different techniques, standardization of an antigen that enables post-operative follow-up of the disease being necessary. (Mercado et al., 1988; Orduña et al., 1985; Robert-Gangneux & Tourte-Schaefer, 1999; Schantz et al., 1980; Varela-Díaz et al., 1978; Siracusano et al., 1991; Leggatt et al., 1992).

Almost all the serologic tests available have been used for diagnosing hydatidosis (Lightowlers & Gottstein, 1995; Babba et al., 1994; Zarzosa et al., 1999), offering better results, particularly with the use of the B/S-rich fraction or partly purified antigen from hydatid fluid (Siracusano et al., 1991; Rogan et al., 1991; Barbieri et al., 1993; Sbihi et al., 1996, 1997).

One key characteristic of human hydatid disease is the frequency of post-surgical relapse. This makes follow-up of the patient necessary for years after surgery, to detect the appearance of new cysts as soon as possible (Baldelli et al., 1992; Force et al., 1992; Guisantes et al., 1994; Zarzosa et al., 1999). Serology has been one of the methods selected for the post-operative control of hydatidosis. However, the long persistence of anti- \textit{E. granulosus} antibodies after recovery makes difficult the diagnosis of relapse by serology (Todorov & Stojanov, 1979; Zarzosa et al., 1999). In this sense, many serologic techniques have been evaluated (latex agglutination, passive hemagglutination, immuno-electrophoresis and specific IgE, IgM, IgG enzyme-linked immunosorbent assay) in the post-operative monitoring of hydatid disease patients. In this report, we examine the pattern of antigenic bands, useful for the serologic diagnosis of hydatidosis, revealed by immunoblotting analysis and we
report on its the post-operative evolution in patients treated for this disease.

2. Materials and methods

2.1. Parasitic material

The hydatid fluid (HF) was taken from the liver and lung cysts from sheep in the province of Zaragoza (Spain). The supernatant was extracted after centrifugation at 4000 g (30 min) and NaN₃ (1 g/L) were added together with ethylenediaminetetraacetic acid (EDTA, 5 mM) for preventing protease activity. The end product was distributed in aliquots of 20 mL and frozen at −20°C until used.

2.2. Antigen preparation

The hydatid fluid was processed according to the procedure described by Rogan et al. (1991), based on the method of Oriol et al. (1971), in order to obtain a fraction enriched with the subunits of the main 5/B antigens (Sbihi et al., 1996, 1997; Siracusano et al., 1991). After the concentration by dialysis in polyethylene glycol (PEG 20,000), the HF was again dialyzed with an acetate buffer (5 mM) at pH 5.0 for 12 h, and centrifuged at 48,000 g for 30 min. The precipitate was dissolved in phosphate buffer 0.2 M at pH 8.0, boiled 15 min, and centrifuged at 48,000 g for 60 min. The pellet was discarded and the supernatant passed through a G-Sepharose protein column (Pharmacia LKB, Uppsala, Sweden) in order to remove any immunoglobulin G (IgG) contaminant from the host. Finally, dialysis was performed again with phosphate saline buffer (PBS, 0.15M, pH 7.4).

The protein concentration of the antigen preparation was determined by a micro-Lowry assay (Findlay, 1986).

2.3. Sera

A total of 60 serum samples were studied, corresponding to 14 patients (6 males and 8 females) aged 34–77 years (average 59.8, s.d. 15.2). All of the cases presented hydatidosis of different bodily locations, diagnosed by radiologic and serologic procedures (EIA and indirect hemagglutination) in the Hospital Clínico Universitario of Zaragoza (Spain). Of the patients, 13 were treated surgically and all were sampled before treatment. The remaining patient was submitted to medical treatment. All the patients presented cysts in only location: 6 liver, 4 lung, 3 peritoneum and 1 spleen. The patients were monitored for 3 years, providing a mean of 3.2 sera per patient. All the samples were preserved frozen at −70°C until analyzed.

2.4. Western immunoblotting

For Western Blotting (WB), the hydatid antigen (approximately 0.1 μg) was subjected to discontinuous electrophoresis in homogeneous polyacrylamide gels at 12.5% (PhastGel) in the presence of SDS-PAGE, using the PhastSystem equipment (Pharmacia LKB). Protein profiles from the antigen preparation were obtained after staining the gels with silver nitrate (Heukeshoven & Dernick, 1985). The proteins separated were transferred from unstained gels to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, U.S.A.) by means of the PhastTransfer (Pharmacia LKB) following standardized procedures (Towbin et al., 1979). The effectiveness of the transference was verified by staining the membranes with a red Ponceaux solution (0.2% w/v in 3% w/v trichloroacetic acid). Nitrocellulose membranes containing blotted antigen were cut into strips and blocked for 3 h at room temperature in PBS with Tween 20 (0.2% w/v) and defatted dry milk (2% w/v). Afterwards, the strips were washed in PBST (PBS with 0.1% v/v of Tween 20) and incubated for 2 h at room temperature with test sera (dilution 1/50). After washing, the strips were probed with peroxidase-labeled antibodies against human IgG (light chains, Boehringer Mannheim GmgH8) at 1/800 dilution for 2 h, and were developed using diaminobenzidine tetrahydrochloride (Sigma) as substrate (0.05% w/v) and H₂O₂ (dilution 1/5,000).

3. Results

Antibodies against proteins of the following molecular weights were detected: 12–14, 16, 20, 24–26, 34, 39 and 42 kDa. The results of the 13 pre-surgery sera are reflected in Table 1. The bands detected at the greatest frequency were of 16, 20, 39 and 42 kDa. In the 13 patients after surgery revealed two different patterns. In 5 patients without cysts after surgery, no new bands were detected, the existing ones disappeared between 2 and 11 months. In the 8 patients with cysts after surgery (Table 2), the bands of molecular weight greater than 24–26 kDa persisted throughout the study period, while those of lower molecular weight fluctuated. The bands of greater persistence were of 16, 20, 39 and 42 kDa. In the patient medically treated, the bands of 12–14, 20, 39, and 42 kDa persisted.
remained over the entire study period, while those of intermediate molecular weight (24–26 kDa) fluctuated, appearing and disappearing.

4. Discussion

Human hydatidosis is characterized by triggering an intense humoral response with a rise in the titers of specific antibodies (Matossian et al., 1972; Matossian et al., 1976), and thus serologic diagnosis is frequent, using ovine hydatid fluid as antigen. The composition of the hydatid fluid presents qualitative and quantitative differences according to its origin, this affecting the quality of the results (Janssen et al., 1990; Verastegui et al., 1992). Therefore, the search for standardized antigens applicable to the serologic techniques is a major objective in the field of hydatosis. In addition, the post-treatment monitoring of the patient requires a simple, precise and rapid method, to ensure the effectiveness of the treatment and to detect possible relapses. We analyzed the use of WB with a purified antigen following the method of Rogan et al. (1991) in order to evaluate the specificity and to determine its possible application in post-treatment monitoring.

The procedure of purification has provided proteins of the following molecular weights: 12–14, 16, 20, 24–26, 34, 39 and 42 kDa. Most of the antigen extracts containing bands of 12–14 and 20 kDa derived from antigen B (Oriol et al., 1971), while those of 34, 39 and 42 kDa probably came from antigen 5 (D’Amelio et al., 1985; Shepherd & McManus, 1987). According to Sbihi et al. (1996), the proteins of 12–14, 20, 34, 39 and 42 kDa are considered specific and are recognizable by antibodies of the IgG class. Matossian et al. (1972) and Kanwar et al. (1992) refer to the presence of a band of 8 kDa of high specificity in hydatidosis, although Verastegui et al. (1992) show the presence of this band in 12% of the patients with cysticercosis. With the purification procedure used, this protein probably migrates with the 12–14 kDa or shows relatively low sensitivity, as indicated by Verastegui et al. (1992) and Siracusano et al. (1991).

The sensitivity in presurgical samples of the proteins obtained in the present work varied according to the antigen: 92% for p39 and 85% for p42 kDa. These results are similar to those reported by Sbihi et al. (1996) and Siracusano et al. (1991), who used similar purification methods. The sensitivity of the bands p12–14, p20 and p34 in our WB, however, proved less than that described by Sbihi et al. (1996). Perhaps, in our case, such discrepancies might be explained by the presence of antigens of intermediate molecular weight (p16, p24–26 y p30), the diverse bodily locations of hydatid disease in our patients (which can influence sensitivity according to Verastegui et al. (1992), Babba et al., 1994 and Zarzosa et al., 1999), or the origin of the sheep antigen (liver and lung). Nevertheless, the detection of antibodies for 2 of the 3 bands of 39, 42 and 20 kDa have enabled the detection of 100% of the cases.

The most frequent crossed reactions in the WB of hydatidosis are due to Taenia solium (Schantz et al., 1980). Antigen 5 presents a crossed reaction with Echinococcus multilocularis, Echinococcus vogeli, Cysticercus cellulosae and other parasites (Leggatt et al., 1992; Leggatt & McManus, 1994), due to the presence of an common epitope in all of these species, phospholylcholine. Antigen B is less immunogenic, but more specific (Ito et al., 1999; Poretti et al., 1999), and crossed reactions have been described only with E. multilocularis and E. vogeli (Shepherd & McManus, 1987). The antigen-purification method used in the present work reduced the risk of crossed reactions with cysticercosis, leishmaniasis or toxoplasmosis (Sbihi et al., 1996), schistosomiasis, filariasis (Rogan et al., 1991) or other diseases (Siracusano et al., 1991). Nevertheless, the possibility of crossed reactions with other parasites should not pose a problem in areas where the only prevalent species is E. granulosus.

The application of WB for monitoring the surgical treatment is a subject that is not available in the literature. With the antigens used, we have verified that WB technique shows a disappearance of the bands in the case of cures, as well as the persistence and appearance of new bands in the opposite case. This latter situation agrees with the differences in the reactivity of the different isotypes of immunoglobulins with the hydatid antigens, observed by Sbihi et al. (1997) with the WB technique applied to sera of patients suffering hepatic hydatidosis. For these authors, the modifications in the larval activity express different antigens, which would be recognized by different immunoglobulin isotypes of, and evidenced or not according to the time at which the sample is taken. In any case, the changes in the post-treatment WB pattern appear earlier than with the techniques of ELISA or HAI (Doiz et al., 1998; Ravinder et al., 1997; Zarzosa et al., 1999), in which the falls in the antibody levels are slower. The WB with purified antigens appears to be a useful method for diagnosing hydatidosis and for post-surgical monitoring, incapable at the moment of being detected by any other immunologic technique, given that the other techniques react with all the antigens without the ability of observing the appearance or disappearance of the bands observed only with the WB. In this sense, the antibodies against proteins p39 an p42 constitute a good marker for post-surgical monitoring, since in the cases of cured patients these disappear in less than a year, while they remain as long as cysts persist. Further studies are also required to analyze the behavior of these antibodies after medical treatment.
References


