Specific antibodies in serum of patients with hydatidosis recognised by immunoblotting


Departments of Experimental Medicine, General Surgery, Neurology and Gastroenterology, Postgraduate Institute of Medical Education and Research, Chandigarh, and Department of Health Slaughter House, Chandigarh Administration, Chandigarh, India

Summary. Hydatid fluids from sheep, goat, pig and man, after resolution by sodium dodecyl sulphate-polyacrylamide gel electrophoresis under reducing conditions, revealed at least 15 discrete polypeptide bands of 8-116 Kda. By ELISA, sera from all 20 cases of hydatidosis showed anti-hydatid antibody, but so did 11 (73%) of 15 sera samples from cysticercosis patients, eight (67%) of 12 sera from patients with other parasitic infections (amoebic liver abscess or hymenolepiasis) and one (4%) of 25 sera from healthy controls. Antibody to cysticercus antigen was found in 14 (93%) of 15 sera from cysticercosis patients, 17 (85%) of 20 sera from hydatid patients, six (50%) of 12 sera from patients with other parasitic infections and one (4%) of 25 sera from healthy controls. Sera from 17 (85%) of 20 hydatid patients, 11 (73%) of 15 cysticercosis patients and five (42%) of 12 patients with other parasitic infections had antibodies to both hydatid and cysticercus antigens. Sera from 20 surgically confirmed cases of hydatidosis reacted with 12 polypeptides of 8-116 Kda in Western immunoblot with hydatid antigens. Polypeptides of 16, 24, 38, 45 and 58 Kda were recognised by all hydatidosis sera but also by many sera from patients with other infections. However, polypeptides of 8 and 116 Kda were recognised by all hydatidosis sera but not by any sera from patients with cysticercosis, other parasitic infections or viral hepatitis, or from healthy controls. Thus, recognition of 8- and 116-Kda hydatid antigens by a patient's serum appears to be a specific test confirming a clinical diagnosis in an individual case of hydatidosis.

Introduction

Echinococcosis is recognised as one of the world's major zoonoses, affecting both man and domestic animals. Rural populations, especially of underdeveloped countries, are at relatively high risk of acquiring hydatid infection because of close proximity with domestic and wild animals. Disease is mainly due to the physical pressure exerted by the developing hydatid cyst; the dissemination of protoscolices from the primary site may lead to multiple secondary hydatid cysts. The variable sensitivity and poor specificity of several serological tests limit their diagnostic value in suspected cases of hydatidosis. The recognition of antibody to hydatid antigen 5 in the sera of patients with hydatidosis, or of antibody to heat-stable lipoprotein antigen B of Echinococcus granulosus, appeared to provide a specific diagnostic test. However, recent investigations revealed cross-reactivity of antigen 5 or antigen B in other helminth infections. The present study was designed to investigate the identification of hydatid-specific antibody by Western immunoblot assay, for the immunodiagnosis of hydatidosis.

Materials and methods

Antigens

Fertile hydatid cysts were obtained from sheep, goats and pigs slaughtered at a local abattoir, and from human material after surgical removal. Cyst fluid from either source was aspirated aseptically, centrifuged at 2000g for 30 min at 4°C, dialysed extensively against distilled water, lyophilised, and stored at -20°C. Protein content was estimated as described by Lowry et al. Analysis of hydatid cyst fluid. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of lyophilised hydatid fluid was done basically as described by Laemmli. Briefly, 75 μg of fluid was
SPECIFIC ANTIBODIES IN SERUM OF PATIENTS WITH HYDATIDOSIS

Kda

116-

66-

45-

29-

8-

A B C D E

Fig. 1. Antigenic profile on SDS-PAGE of hydatid cyst fluid (HCF): lane A, pig HCF; B, human HCF; C, sheep HCF; D, goat HCF; E, sheep HCF.

subjected to electrophoresis in stacking gels 5% and separating gels 12% under reducing and denaturing conditions in an electrophoretic cell (BioRad Laboratories, USA) at 25 mA for 6 h, and stained with Coomassie Blue 0-25% (fig. 1). The mol. wt standards were carbonic anhydrase (29 Kda), ovalbumin (45 Kda) and bovine serum albumin (66 Kda). The mol. wts of hydatid antigens were estimated by logarithmic plot of migration of the standards.

Serum samples

Samples were obtained from confirmed cases of hydatidosis (20), cysticercosis (15), hymenolepiasis (2), amoebic liver abscess (10) and viral hepatitis (12), and from 25 apparently healthy subjects.

Hydatidosis. These were radiologically and surgically confirmed cases, and microscopic examination of cyst fluid revealed protoscolices of Echinococcus; the sera had anti-hydatid antibodies as assessed by plate enzyme-linked immunosorbent assay (ELISA).

Cysticercosis. In 13 patients a diagnosis of neurocysticercosis was based on clinical symptoms (headache, nausea, vomiting, impaired vision, confusion, dizziness) and computerised tomography compatible with cysticercosis; the sera contained anti-cysticercus antibodies as assessed by plate ELISA. Also, two cases of epidermal cysticercosis were confirmed by histopathology.

Hymenolepiasis. Stool examination revealed eggs of Hymenolepis nana.

Amoebic liver abscess (ALA). These cases had an enlarged and tender liver, palpable abscess, and systemic toxaemia; the aspirated anchovy-sauce pus was either sterile on bacteriological examination or had Entamoeba histolytica trophozoites. The anti-amoebic antibody titres were ≥64 by indirect haemagglutination. The ALA patients made a good clinical recovery with anti-amoebic therapy (metronidazole or emetine).

Viral hepatitis. These patients had detectable levels of hepatitis B surface-antigen by plate ELISA.

Healthy controls. These were aged 17–32 years, resident in India since birth, and showed no abnormality on medical examination. Their sera had no antibody to E. granulosus or Taenia solium; repeated stool examination revealed no ova or cysts of parasites.

Western immunoblotting

The SDS gel proteins were transferred to nitrocellulose paper (NCP) in a transblot cell (BioRad Laboratories, USA) at 200 mA for 3 h12 Non-specific binding sites on NCP strips were blocked with bovine serum albumin (BSA) 3% in Tris saline, pH 7.4, for 3 h at 4°C. Strips were treated for 3 h at 4°C with serum samples diluted 1 in 25 with BSA 0.1%; they were washed three times with Tris saline containing nonidet P-40 0.01% (a non-ionic detergent, to remove excess reactant and to reduce non-specific reactions), and were then incubated with a 1 in 1000 dilution of a conjugate of anti-human immunoglobulin (IgG, IgA and IgM) and horseradish peroxidase (Jackson Immune Research Lab., Baltimore, USA) for 1 h at 4°C. The reaction was developed with 4-chloro-1-naphthol (Sigma) as substrate.

Detection of antibodies by ELISA

Antibodies to hydatid antigen were assayed by micro ELISA, with hydatid cyst fluid as antigen. Anticysticercus antibodies to crude cysticercus antigen were also assayed by micro ELISA.

Data analysis

The sensitivity, specificity, predictive values and diagnostic efficiency of micro-ELISA and immunoblot assays were determined as described by Galen and Gambino.15

Results

SDS-PAGE analysis of hydatid cyst fluid

Hydatid fluid from sheep, goat, pig or man, after resolution by SDS-PAGE under reducing conditions, revealed at least 15 discrete polypeptide bands of 8–116 Kda (fig. 1), with a major complex of c. 66 Kda. A 20-Kda polypeptide was found in the fluid from one of the sheep (fig. 1, lane C).
Table I. Detection of antibodies to hydatid and cysticercus antigens by ELISA

<table>
<thead>
<tr>
<th>Disease (number of cases)</th>
<th>Sera of patients tested by ELISA against hydatid (E. granulosus) antigen</th>
<th>Sera of patients tested by ELISA against cysticercus (T. solium) antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range of OD (Mean, SD) of sera</td>
<td>Range of OD (Mean, SD) of sera</td>
</tr>
<tr>
<td></td>
<td>positive*</td>
<td>positive†</td>
</tr>
<tr>
<td>Hydatidosis (20)</td>
<td>0.61-1.99 (1.26, 0.37)</td>
<td>0.18-1.68 (0.91, 0.42)</td>
</tr>
<tr>
<td>Cysticercosis (15)</td>
<td>0.04-1.50 (0.85, 0.47)</td>
<td>0.24-1.98 (0.41, 0.53)</td>
</tr>
<tr>
<td>Other parasitic infection (12)</td>
<td>0.33-1.08 (0.64, 0.24)</td>
<td>0.14-0.72 (0.46, 0.17)</td>
</tr>
<tr>
<td>Viral hepatitis (12)</td>
<td>0.08-0.42 (0.22, 0.12)</td>
<td>0.09-0.42 (0.23, 0.10)</td>
</tr>
<tr>
<td>Healthy controls (25)</td>
<td>0.02-0.65 (0.25, 0.14)</td>
<td>0.04-0.37 (0.20, 0.12)</td>
</tr>
</tbody>
</table>

* OD ≥ mean + 2 SD for control sera against hydatid antigen.
† OD ≥ mean + 2 SD for control sera against cysticercus antigen.
‡ Amoebic liver abscess (10), hymenolepiasis (2).

Detection of anti-hydatid and anti-cysticercus antibodies by plate ELISA

The plate ELISA, with sheep hydatid fluid as antigen, gave a mean OD value of 0.25 (SD 0.14) with sera from healthy controls; so, a serum with an OD of not less than 0.53 (mean + 2 SD) was considered to be positive for anti-hydatid antibody. All 20 of the sera from cases of hydatidosis contained anti-hydatid antibody. However, 11 (73%) of 15 sera from cysticercosis patients, eight (67%) of 12 sera from ALA and hymenolepiasis patients, and one (4%) of 25 sera from healthy controls also had anti-hydatid antibody (table I).

With cysticercus antigen, the mean OD of sera from healthy controls was 0.20 (SD 0.12); so, a serum with an OD of not less than 0.44 (mean + 2 SD) was considered to be positive for anti-cysticercus antibody. Fourteen (93%) of 15 sera from cysticercosis patients had anti-cysticercus antibody. However, 17 (85%) of 20 sera from hydatidosis patients, six (50%) of 12 sera from patients with other parasitic infections, and one (4%) of 25 sera from healthy controls also had antibody to cysticercus antigen (table I).

The sera from 17 (85%) of 20 patients with hydatidosis contained antibodies to both hydatid and cysticercus antigens; 11 (73%) of 15 sera from cysticercosis patients, and five (42%) of 12 sera from patients with other parasitic infections also had antibodies to both antigens, but none of the sera from viral hepatitis patients or healthy controls had antibodies to both antigens (table II).

Western immunoblot assay with hydatid antigen

The sera from cases of hydatidosis showed 12 polypeptides of 8-116 Kda by Western immunoblot with crude hydatid fluid antigen (fig. 2, lanes A and B). The polypeptides of 16, 24, 38, 45 and 58 Kda were recognised by all sera from hydatid patients but also by some sera from patients with other infections (table III). The 8- and 116-Kda polypeptides were recognised by the sera from all hydatid patients but by none of the controls or patients with other infections.

The 66-Kda polypeptide was strongly recognised by sera from patients with hydatid and other parasitic infections (fig. 2, lanes A–E), but only faintly by sera from viral hepatitis patients (lane F) and healthy controls (lane G).

Diagnostic value of ELISA and of immunoblot

The detection of anti-hydatid antibody by plate ELISA was found to have a sensitivity of 100% but a

Table II. Proportion of patients and controls whose sera had ELISA antibodies to hydatid antigen (HAg) or cysticercus antigen (CAg) or both antigens

<table>
<thead>
<tr>
<th>Disease (number of cases)</th>
<th>Number of sera with antibody to</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>both antigens</td>
</tr>
<tr>
<td></td>
<td>HAg only</td>
</tr>
<tr>
<td></td>
<td>CAg only</td>
</tr>
<tr>
<td></td>
<td>neither antigen</td>
</tr>
<tr>
<td>Hydatidosis (20)</td>
<td>17</td>
</tr>
<tr>
<td>Cysticercosis (15)</td>
<td>11</td>
</tr>
<tr>
<td>Other parasitic infections (12)</td>
<td>5</td>
</tr>
<tr>
<td>Viral hepatitis (12)</td>
<td>0</td>
</tr>
<tr>
<td>Healthy controls (25)</td>
<td>0</td>
</tr>
</tbody>
</table>
Specific antibodies in serum of patients with hydatidosis

Sensitivity Specificity Predictive positive Predictive negative Diagnostic efficiency

Specificity of only 76%; the diagnostic efficiency was 83% (table IV). The plate ELISA with cysticercus antigen had a sensitivity of 94% and a specificity of 63% for anti-cysticercus antibody; the efficiency of this assay was 84%.

Based on the recognition of 8- and 116-Kda antigens by the sera of hydatid patients and their non-recognition by sera from other patients and controls, Western immunoblot assay had both a sensitivity and a specificity of 100% in the diagnosis of hydatidosis (table IV).

Discussion

Anti-hydatid antibodies in the sera of hydatidosis patients have been examined by several serological procedures, with purified thermolabile antigen 5 or thermostable antigen B from the hydatid fluid, or with the crude hydatid fluid as antigen. ELISA has been reported to be relatively sensitive and specific, but variable sensitivity has been ascribed to the source and nature of the hydatid antigen. Except for a minor antigenic difference in one of the hydatid fluids that we obtained from a sheep, we found little difference in antigenic components between the hydatid fluids from sheep, goats, pigs and human cases (fig. 1). We preferred to use

Table III. Patients and controls whose sera reacted with hydatid cyst fluid and its components by ELISA and by immunoblot

<table>
<thead>
<tr>
<th>Disease (number of cases)</th>
<th>Number (%) of sera reacting in immunoblot with hydatid-fluid polypeptide of (Kda)</th>
<th>Number (%) of sera reacting with hydatid antigen in ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 16 24 38 45-52 58 66 68 116</td>
<td>8 16 24 38 45-52 58 66 68 116</td>
</tr>
<tr>
<td>Hydatidosis (20)</td>
<td>20 (100) 20 (100) 20 (100) 20 (100) 20 (100) 20 (100) 20 (100) 20 (100) 20 (100) 20 (100) 20 (100)</td>
<td>20 (100) 20 (100) 20 (100) 20 (100) 20 (100) 20 (100) 20 (100) 20 (100) 20 (100) 20 (100) 20 (100)</td>
</tr>
<tr>
<td>Cysticercosis (15)</td>
<td>11 (73) 8 (67) 10 (67) 7 (47) 8 (53) 6 (40) 3 (20) 15 (100) 4 (27) 0</td>
<td>11 (73) 8 (67) 10 (67) 7 (47) 8 (53) 6 (40) 3 (20) 15 (100) 4 (27) 0</td>
</tr>
<tr>
<td>Other parasitic infection (12)</td>
<td>0 0 0 0 0 0 0 0 15 (100) 0 0</td>
<td>0 0 0 0 0 0 0 0 15 (100) 0 0</td>
</tr>
<tr>
<td>Viral hepatitis (12)</td>
<td>0 0 0 0 0 0 0 0 12 (100) 0 0</td>
<td>0 0 0 0 0 0 0 0 12 (100) 0 0</td>
</tr>
<tr>
<td>Healthy controls (25)</td>
<td>1 (4) 0 0 0 0 0 0 0 25 (100) 0 0</td>
<td>1 (4) 0 0 0 0 0 0 0 25 (100) 0 0</td>
</tr>
</tbody>
</table>

Table IV. Diagnostic evaluation of ELISA and immunoblot assay

<table>
<thead>
<tr>
<th>Criteria</th>
<th>by ELISA</th>
<th>by immunoblot with hydatid-fluid polypeptide of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hydatid antigen*</td>
<td>cysticercus antigen†</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>100</td>
<td>94</td>
</tr>
<tr>
<td>Specificity</td>
<td>76</td>
<td>63</td>
</tr>
<tr>
<td>Predictive positive</td>
<td>50</td>
<td>39</td>
</tr>
<tr>
<td>Predictive negative</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>Diagnostic efficiency</td>
<td>83</td>
<td>84</td>
</tr>
</tbody>
</table>

* For diagnosis of hydatidosis.
† For diagnosis of cysticercosis.
fluid from sheep because of easy availability and the less likely cross-reaction of host antigens with immunoglobulins in human sera. Elevated anti-hydatid antibody was detected by ELISA in the sera of all 20 cases of hydatidosis; but we found a high degree of non-specificity ranging from 73% in cysticercosis to 67% with other parasitic infections (table I). Interestingly, 17 (85%) of the 20 hydatidosis patients had antibodies to both hydatid and cysticercus antigens (table II), and 11 (73%) of 15 cysticercosis patients also had antibodies to both antigens.

Our data clearly indicated that, though ELISA was sensitive, many non-specific results were obtained, as has been observed by others who also concluded that immunological procedures for the detection of antibodies to hydatid antigen have serious limitations because of a high incidence of false positive results. Anti-hydatid antibodies persist for years after the surgical removal of a hydatid cyst. Non-specificity may be caused mainly by a sharing of hydatid antigen with those of other parasites; but other possible reasons are interaction with some blood-group antigens, or with non-specific host proteins in hydatid fluid. Thus, we feel that mere demonstration of anti-hydatid antibodies does not confirm the clinical diagnosis of hydatidosis.

An alternative would be an assay system to identify hydatid-specific antigen in the circulation. Such an immunoassay would require specific antibody; so, a first step would be identification of hydatid-specific antigen(s). Our data show that the polypeptides of 8 and 116 Kda were recognised in Western immunoblot assay by the sera of all 20 hydatid patients, but not by any of the sera from patients with other helminth infections, ALA, or viral hepatitis, or from healthy controls (table III). However, Shepherd and McManus have reported 12- and 16-Kda polypeptides as E. granulosus-specific. This difference in mol. wt between their results and ours may be related to the processing of hydatid cyst fluid before electrophoresis: we have loaded fluid directly on the gel; but they processed the fluid by iodination, immunoprecipitation with pooled patients’ sera and repeated washing with detergent. Such treatment is known to alter polarity, size, configuration and optimum concentration of antigenic molecules. Recently, Maddison et al. reported a specific antigen of 8 Kda, like ours. We conclude that the immunoblot recognition of hydatid-specific 116- or 8-Kda polypeptides by the sera of clinical cases appears to confirm the diagnosis of hydatidosis.

References


