Specific Circulating Immune Complexes in Amoebic Liver Abscess

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Amoebiasis occurs worldwide. A recent estimate indicated that 480 million people carry Entamoeba histolytica in their intestinal tract; 10% of the total, i.e., 48 million people, annually have intestinal mucosal or liver invasion (26). Antiamoebic antibodies are regularly produced in symptomatic and invasive amoebiasis but with variable frequencies in asymptomatic amoebiasis. However, the presence of antiamoebic antibodies does not have any correlation with the clinical status or intensity of infection (2, 7, 10, 19, 20, 22–25). Moreover, antiamoebic antibodies are known to persist for years even after successful eradication of the infection (1, 4, 20). Thus, the demonstration of antiamoebic antibodies cannot differentiate between present and past infection, especially where amoebiasis is endemic (1, 4, 8). Circulating immune complexes (CIC) have been documented in many parasitic infections, such as malaria (5), trypanosomiasis (3), schistosomiasis (11), onchocerciasis (12), and toxoplasmosis (17). Increased soluble immune complexes have also been documented in amoebic liver abscess patients and chronic cyst passers (13–15). The specificity of these complexes, in terms of diagnostic applicability by demonstrating the presence of amoebic antigen in CIC, may discriminate present from past amoebic infection. Thus, the present investigation was designed to demonstrate specific CIC in the serum of amoebic patients and to evaluate the diagnostic applicability of the findings.

MATERIALS AND METHODS

Patients. Serum samples were collected from confirmed or suspected cases of amoebic liver abscess, cases of nonamoebic hepatic disorders, and apparently healthy subjects. Criteria for the diagnosis of the cases were as follows. (i) Confirmed amoebic liver abscess (15 cases): clinically enlarged tender liver with palpable abscess and associated systemic toxemia; aspiration of anchovy sauce pus which was sterile on bacteriological examination, or the presence of E. histolytica trophozoites in the aspirated pus; high titers of antiamoebic antibodies as studied by an indirect hemagglutination technique; and good clinical recovery on specific antiamoebic therapy, such as metronidazole or emetine. (ii) Suspected amoebic liver abscess (seven cases): clinically enlarged tender liver with palpable abscess and associated systemic toxemia; aspiration of pus could not be attempted in two cases, and in the other five, bacteriological examination of the pus was not carried out and none had amoebae in the pus; and good clinical recovery on metronidazole or emetine therapy. (iii) Nonamoebic liver diseases (nine cases): these included two cases of hydatid cyst disease confirmed by laparotomy, one case of nonspecific hepatomegaly with no response to antiamoebic therapy and with a final diagnosis of hepatoma, and six cases of viral hepatitis confirmed by demonstration of hepatitis B surface antigen; none had antiamoebic antibodies. (iv) Apparently healthy subjects (10 subjects): these were adults aged 20 to 30 years and residents of India since birth; they had no overt symptoms, and their physical examination indicated that they were normal; their stool samples did not reveal any E. histolytica cysts or trophozoites, and in addition none had any antiamoebic antibodies in their serum.

Preparation of amoebic antigen and antiamoebic antisera. The antigen used was a sonicated extract of axenically grown E. histolytica NIH 200 prepared essentially as described earlier (16). Antisera against the whole amoebic extract were raised in rabbits by three weekly subcutaneous injections of 4 mg of amoebic protein emulsified in Freund complete adjuvant followed by an intravenous injection of 1 mg of protein 1 week later. The animals were bled to death 10 days later, and sera were collected. The immunoglobulin G (IgG) fraction of antiamoebic antisera was prepared by DEAE-cellulose chromatography (6) and used as the solid phase for coating antibody in the micro enzyme-linked immunosorbent assay (ELISA).

Precipitation of CIC by PEG. CIC were prepared by precipitation with 2.5% polyethylene glycol (PEG) (6). Briefly, 0.2 ml of serum was mixed with PEG in Veronal-buffered saline, pH 7.6, (VBS 7.6), and incubated overnight at 4°C. The samples were centrifuged at 2,000 x g for 20 min at 4°C. The precipitates were washed once with 2.5% PEG in VBS 7.6 containing 0.01 M EDTA. Finally, the precipitates were dissolved in 0.2 ml of VBS 7.6 by incubation at 37°C for 1 h.

Determination of amoebic antigen in CIC. The optimal dilutions of the coating antibody and CIC were first deter-
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The wells of the polystyrene microtiter plates were coated with 100 μl of optimally diluted rabbit antiamoebic IgG in carbonate buffer (0.05 M, pH 9.6). After incubation at 37°C for 3 h, the material was drained and the plates were washed with 0.01 M phosphate-buffered saline, pH 7.2, containing 0.05% Tween-20 (PBS-T). The plates were then incubated with 0.5% bovine serum albumin in PBS-T (200 μl/well) for 30 min at 37°C to reduce nonspecific reaction. The plates were washed three times with PBS-T and incubated with 100 μl of serial dilutions of CIC per well for 3 h at 37°C. After a wash with PBS-T, the final incubation was done with 100 μl of optimally diluted peroxidase-conjugated rabbit antibody specific to human immunoglobulin (Dakopatts a/s, Denmark) for 3 h at 37°C. The plates were washed, and enzyme activity was assayed by incubating the wells with 100 μl of substrate and indicator system (6) at 37°C for 10 to 20 min. The reaction was terminated by adding 50 μl of 12.5% sulfuric acid and read visually.

**Determination of antiamoebic antibodies.** Antibodies were determined by the indirect hemagglutination test with axenic *E. histolytica* antigen (21).

**RESULTS**

The use of antiamoebic rabbit IgG as a solid-phase antibody and peroxidase-conjugated anti-human immunoglobulin as the developing-phase antibody clearly distinguished the amoebic liver abscess patients from the controls (Table 1). PEG precipitates, each derived from 0.2 ml of serum, were suspended in the same volume of buffer, and serial dilutions of 1:80 and above were used for the test samples and the controls. The cutoff titer of the precipitate was determined by a standard titration method with precipitates from confirmed amoebic liver abscess patients and healthy subjects. Any titer greater than 1:160 was considered positive for amoebic antigen in the precipitates. Fourteen of the 15 (93.3%) confirmed amoebic liver abscess cases had amoebic antigen in CIC. In addition, four (57.3%) of the seven suspected cases had a titer of 1:320. None of the nine nonamoebic hepatic disease cases or 10 apparently healthy controls was positive for specific amoebic CIC. There was no correlation between the antiamoebic hemagglutination antibody titers and the amoebic antigen titers in CIC (Table 1).

**DISCUSSION**

The main consequences of invasion by *E. histolytica* are amoebic colitis, dysentery, and liver abscess. Demonstration of parasites in the dysenteric or well-formed stool clinches the diagnosis. However, it is difficult to demonstrate parasites in liver abscesses. In the best laboratories, no more than 15% of confirmed cases of amoebic liver abscess had demonstrable *E. histolytica* in the pus (9, 21). Thus, in the majority of such cases, diagnosis remains circumstantial. The level of antiamoebic antibodies is relatively higher in such cases, but it cannot differentiate past or silent from present infection. Thus, the demonstration of antigen in amoebic pus, in tissues, or in the circulation (free or complexed form) would be an ideal choice for diagnosing an individual case of amoebiasis.

Recently, increased serum immune complexes have been used as a tool for the diagnosis of patients with amoebic liver abscess and chronic cyst passers. CIC have been reported to be higher in patients with active amoebiasis, and surprisingly, in cyst passers the prevalence and mean number of CIC were even higher (13). In another study, the presence of CIC was documented in 58% of amoebic liver abscess cases (14). In the present investigation amoebic antigen in CIC could be demonstrated in 93% of the confirmed cases of amoebic liver abscess. The specificity of the amoebic antigen for diagnosis is supported by the fact that amoebic antigen was undetectable by the micro ELISA technique in serum from apparently healthy subjects and from nonamoebic hepatic disorder patients. Our observations tend to support the findings of Pillai and Mohimen (15), who reported the presence of amoebic antigen in CIC with a solid-phase radioimmunossay in the sera of 100% of their patients with amoebic liver abscess and colonic amoebic infection. We observed the presence of specific amoebic antigen beyond the cutoff titer of 1:160 in CIC in 57% of the clinically suspected but nonconfirmed cases of amoebic liver abscess. All of the patients who had amoebic antigen in CIC re-

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. tested</th>
<th>Specific titer (no. of samples)</th>
<th>No. (%) with antiamoebic antibody titer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Specific titer (no. of samples)</th>
<th>No. (%) positive for amoebic antigen in CIC</th>
<th>CIC antigen titer (no. of samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoebic liver abscess</td>
<td>15</td>
<td>≥8,192 (2)</td>
<td>4,096 (3)</td>
<td>1,024 (3)</td>
<td>512 (2)</td>
<td>256 (1)</td>
</tr>
<tr>
<td>Confirmed</td>
<td></td>
<td>4,096 (3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suspected</td>
<td>7</td>
<td>2,048 (1)</td>
<td>256 (3)</td>
<td>64 (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonamoebic hepatic disease</td>
<td>9</td>
<td>32 (2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>10</td>
<td>&lt;32 (10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tbody>
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<sup>a</sup> Titer is the reciprocal of the highest serum dilution.

<sup>b</sup> Antigen titer of 1:320.
sponded to antiamoebic treatment. This observation would indicate that these four (57%) suspected amoebic liver abscess patients in fact had amoebic disease in the liver. The demonstration of amoebic antigen in such a situation would definitely help the clinician in differential diagnosis of an individual case.

It is yet to be seen how long these antigen-specific CIC persist after eradication of the disease. We believe that it is unlikely for the antigen-specific CIC to persist for a long period of time in the host. Nevertheless, our study clearly indicated that demonstration of antigens in CIC would be a good diagnostic method to differentiate acute and active disease from previous infection.

LITERATURE CITED


