DIFFERENTIAL IMMUNODIAGNOSIS BETWEEN CYSTIC HYDATID DISEASE AND OTHER CROSS-REACTIVE PATHOLOGIES

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Abstract. We assessed an Echinococcus granulosus hydatid fluid antigen-ELISA (EgHF-ELISA) as a serologic prescreening test for E. granulosus infections, supplemented by more specific confirmatory tests, including arc-5 immunoprecipitation and antigen B subunit 8-kD immunoblotting. The diagnostic sensitivity of the EgHF-ELISA was 91%. With regard to the test specificity of the EgHF-ELISA (overall = 82%), we observed relatively frequent cross-reactions in tumor patients (6%) and in patients with other parasitic diseases. Cestode-related cross-reactivity can be resolved by the complementary use of E. multilocularis-specific antigens or Taenia solium cysticercosis–specific immunoblotting. Immunoblotting based upon the detection of antibody reactivity to the 8-kD antigen of EgHF; or if appropriately detectable, to the 29-kD and 34-kD bands exhibited a 91% diagnostic sensitivity and an overall specificity of 97% or 94%, respectively. Thus, immunoblotting provided a 99% discrimination between seropositive pre-operative cystic hydatid disease cases and cross-reactive non-cestode parasitic infections or malignancies.

For primary serologic diagnosis and for support of clinical diagnosis of cystic echinococcosis (cystic hydatid disease [CHD]), the selection of a particular immunodiagnostic test involves consideration of the diagnostic operating characteristics of the technique and the purpose for which it will be used. The diagnostic sensitivity and specificity of the tests vary according to the nature and quality of the antigen and the methodologic sensitivity of the selected technology. A concise definition of the sera used for the assessment of test parameters is essential, with special attention paid to the definition of pre- or post-operative situations respective to CHD serum sampling time point. One of the most specific conventional immunodiagnostic approaches for CHD relies upon the demonstration of serum antibodies precipitating an antigen called antigen 5 by immunoelectrophoresis or similar techniques. Diagnostic sensitivities with respect to hepatic CHD have been reported to vary between 50% and 80%. Antibodies to antigen 5 also occurred in serum of human patients with neurocysticercosis and alveolar echinococcosis (AE). Comparative studies showed that 58% of Swiss patients with AE were arc 5-positive compared with 74% of patients with CHD. Resolution of E. granulosus hydatid cyst fluid by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting, resulted in the identification of the arc-5 subunits, including two subunits with relative molecular masses estimated by different laboratories to be between 37 and 38 kD and 20–22–24 kD, respectively. Diagnostic assessment of these two antigens by immunoblotting performed in different laboratories have resulted in the publication of discrepant sensitivity and specificity parameters. The second major parasite antigen in hydatid cyst fluid is a thermostable lipoprotein called antigen B. The major components of antigen B resolve as three bands of 8–12-kD, 16 kD, and 23–24 kD by Western blotting. The apparent 8–12-kD and 23–24-kD bands were assumed to represent identical antigens, respectively. Both antigens proved to exhibit good diagnostic parameters, although a variable range of diagnostic sensitivities and specificities have been documented by different groups. One of the major obstacles encountered in the evaluation of these serologic procedures was the lack of definition of the diagnostic status of the sera used and the lack of performance under real routine diagnostic conditions. Thus, only a few studies have included malignancies as one of the most common pathologies to be taken into account in the differential diagnosis of space-occupying lesions. With regard to CHD serology, the occurrence of false-positive antibody reactions related to malignancies have already been documented. We designed the present study to comparatively assess the diagnostic performance of three different serologic tests applied either as primary screening tests (ELISA) or as confirmation tests for elucidation of the specificity of a seroreaction positive in the primary screening (arc-5 immunoprecipitation and immunoblotting).

MATERIALS AND METHODS

Antigens. Crude E. granulosus hydatid cyst fluid antigen (EgHF) was obtained and processed for storage at −80°C until used as described previously. Only fertile hydatid cysts were selected for the present study, originating either from naturally infected Swiss cattle, Swiss horses, or Egyptian camels.

Sera. Pre-operative sera used for assessing diagnostic parameters of hepatic CHD were obtained from 35 patients with 1–7 hydatid cysts localized in the liver (32 patients), liver and lungs (2), or liver and heart (1). All blood samples were diagnostic and drawn before any clinical or surgical intervention. Post-operative sera were obtained from 52 patients with 1–5 hydatid cysts localized in the liver (46 patients), liver and lungs (1), liver and heart (1), lungs (2), heart (1), and neck (1). All blood samples were drawn between one month and three years after surgical intervention. For 35 of these post-operative cases, incomplete surgery was reported. This included not completely removed cysts that showed a cyst relapse between one month and three years after initial surgery, additional nonresectable cysts, or initially non-detectable cysts. For the other 17 cases, the cysts were completely resected and no relapses were reported within three years after surgery.

Sera used for assessing test specificities and cross-reactions due to other parasitic infections were obtained from...
patients with clinically, parasitologically, and/or histologically proven infections. The following parasite species (number of investigated patients) were included: hepatic *E. multilocularis* (19), *Fasciola hepatica* (6), *Schistosoma* spp. (6), *Taenia solium* (12), nematodes (20), *Strongyloides stercoralis* (4), *Toxocara canis* (4), *Trichinella spiralis* (4), *Onchocerca volvulus* (4), *Ascariis lumbricoides* (4), and *Entamoeba histolytica* liver abscess (6). These sera had been selected based upon high antibody reactions against antigens of the respective homologous parasite species tested by ELISA.

The sera used for assessing potential nonspecific reactions related to malignancies were obtained from 270 patients (133 males and 137 females; mean ± SD age = 57.5 ± 13.5 years) admitted to the outpatient Clinic of the Institute of Oncology, University Hospital of Berne. Inclusion criteria were 1) a histologically confirmed malignancy, 2) no history or radiologic finding suggestive of hydatid disease, and 3) an age between 18 and 85 years. Malignancies included gastrointestinal (GI) tract neoplasm (47 cases), lymphoma (68 cases), breast cancer (45 cases), lung cancer (28 cases), prostate/testis cancer (15 cases), sarcoma (9 cases), leukemia (5 cases), nasopharyngeal neoplasm (2 cases), histiocytosis (1 case), gynecologic neoplasm (14 cases), melanoma (9 cases), myeloma (9 cases), bladder/kidney neoplasm (5 cases), pancreatic neoplasm (3 cases), central nervous system neoplasm (3 cases), and other neoplasms (7 cases). This group of patients was chosen in the context of another complementary study (Pfister M, unpublished data).

The sera used for the determination of normal ranges and parameters respective to the different antigens were obtained from 200 healthy Swiss blood donors matched by age and sex. Some of the sera included in this study (other diseases and normal ranges) had previously been used for a similar purpose in a study on AE.17

This investigation was approved by the Ethic Committee of the University of Berne. Informed consent was obtained from all patients with echinococcosis and all patients with malignancies. Patients who contributed serum for the assessment of cross-reactivities had already provided consent in the context of a previous study with a similar objective.17

**Enzyme-linked immunosorbent assay.** The ELISA with EgHF antigen was performed exactly as previously described.17 Microplates were coated with 2.5 μg of EgHF protein. Serum dilutions were 1:100 and the conjugate used was goat anti-human IgG (γ-chain specific) (no. A-5403; Sigma, St. Louis, MO) linked to alkaline phosphatase. Reading of color reactions was done at 404 nm using a Dynatech MR7000 reader and Dynatech Biocalc8 Software (Dynatech Produkte AG, Embrach, Switzerland). Threshold values were defined by adding three standard deviations to the mean absorbance value at 404 nm of 200 negative control sera from 200 Swiss blood donors. The calculation procedures have been previously described.17

**Arc-5 electrosyneresis.** Immunoprecipitation of the arc-5 was performed by electrosyneresis18 with the same antigen used for immunoblotting and included a serum immunoprecipitating the arc-5 as a positive control. The arc-5 positivity was determined upon identity with the arc-5 line coprecipitated by the control serum.

**Immunoblotting.** All SDS-PAGE chemicals were obtained from Fluka Chemie, Buchs, Switzerland). Gradient SDS-PAGE was performed as described previously19 and adapted to *E. granulosus* as described by Maddison and others.9 The EgHF antigen was adjusted to a final SDS concentration of 2.5% with a solution of 10% SDS in 9 M urea buffered with 0.01 M Tris-HCl, pH 8.0, and heated for 20 min at 65°C. The samples (10 μg of protein/cm slot) were then electrophoresed in a 5–20% linear gradient gel, using a 3% stacking gel. The SDS-PAGE–resolved components were electrophoretically blotted onto a sheet of nitrocellulose as described elsewhere.20 Nitrocellulose strips were exposed to sera diluted 1:100 with phosphate-buffered saline containing 0.3% Tween 20 and 5% skim milk powder. Antibody bands with bound antibodies were visualized using a goat anti-human immunoglobulin–peroxidase conjugate (γ-chain specific) (no. A-6029; Sigma) and a precipitating chromogenic substrate including H2 O2 and 3,3′-diaminobenzidine. Pretrained standard protein ladder markers were obtained from Gibco-BRL (no. 10748–010, Life Technology, Basel, Switzerland).

**Protein measurement.** Protein concentrations were estimated using the Bio-Rad Bradford protein assay kit (Bio-Rad AG, Glattbrugg, Switzerland) and bovine plasma gammaglobulin as a standard.

**Statistical analysis.** Data are expressed as the mean ± SD. The chi-square test (2 × 2 tables), Fisher’s exact test (two-tail), and analysis of variance, including the Tukey post hoc test, were applied as appropriate. Confidence intervals (CIs) for clustered data were set at 95% and calculated as described elsewhere.21 The SYSTAT statistics software program (version 6.01) was used for data analysis (SYSTAT, Inc., Evanston, IL).

**RESULTS**

The EgHF-ELISA was preliminarily assessed for its threshold parameters discriminating between positive and negative serology. This was done by testing 200 Swiss blood donor sera from healthy individuals who exhibited no hepatic symptoms and no history indicating any potential risk of *E. granulosus* infection. Test parameters matched those described earlier.17 Inter-test and intra-test variations, assessed by determination of the coefficients of variation concerning reference negative and positive sera tested in triplicate on each test plate, showed the same ranges as described earlier17 and thus were considered acceptable.

Diagnostic sensitivities and specificities respective to the different test systems (EgHF-ELISA, arc5 electrosyneresis, and immunoblotting) and to patients with different diagnostic stages of CHD or other putatively cross-reacting parasites are listed in Table 1. The EgHF-ELISA exhibited a diagnostic sensitivity of 91% with regard to the diagnostic sera obtained before any initiation of treatment of CHD. With regard to post-operative sera, the diagnostic sensitivity was 96%. Patients with AE at the diagnostic stage exhibited a very high EgHF-ELISA cross-reactivity with 95% of the respective sera being serologically positive. The relative cross-reactivity with *T. solium* cysticercosis sera was 100%. In this context, it should be mentioned that these sera had been preselected based on seropositivity to a homologous *T. solium* cysticercus antigen. Furthermore, cross-reactions...
were also observed with non-cestode infections such as fascioliasis and especially nematode infections (60% cross-reactivity), with the most cross-reactive sera from patients with onchocerciasis, strongyloidiasis, and ascariasis. The overall test specificity of the EgHF-ELISA was 82%. The arc-5 electrosyneresis showed a diagnostic sensitivity of 63% with the CHD diagnostic sera. Post-operative CHD sera exhibited an arc-5 diagnostic sensitivity of 58%. Patients with AE were arc-5 cross-precipitating in 42% of the cases, whereas all other parasitic sera showed no arc-5 cross-precipitation. Sera from patients with cysticercosis sera were not tested by arc-5 electrosyneresis because of insufficient amounts of some samples. Immunoblot analysis of immunoreactivity to the 8-kD antigen showed a diagnostic sensitivity of 71% for diagnostic CHD cases and 60% for post-operative CHD cases. Sera from hepatic AE patients were cross-reactive with the 8-kD band in 53% of the cases, whereas only one of 12 sera from patients with cysticercosis showed cross-reactivity with the 8-kD band. Non-cestode serum demonstrated no cross-reactivity with the 8-kD band.

A preliminary evaluation of the 8-kD immunoblot had been initiated in our routine diagnostic laboratory. Analysis of the immunoblot data had provided some indications that a 29-kD band and a 34-kD band may exhibit relatively high sensitivity and specificity characteristics. Therefore, all immunoblot strips from the present study were additionally read with the criterion of positivity by being reactive with either one or more of the three bands of 8, 29, and/or 34 kD. A representative selection of respective immunoblot data are shown in Figure 1. When the 29-kD and 34-kD bands were used for interpretation of results (positive versus negative in the immunoblot), a significant increase was demonstrated in the diagnostic sensitivity of the immunoblot test. With respect to the diagnostic pretreatment CHD sera, the increase was from 71% to 91%; the increase was also observed post-operatively (from 60% to 81%). However, cross-reactivity with AE and cysticercosis sera also increased significantly (from 53% to 84% and from 8% to 33%, respectively).

When sera from 270 patients with malignancies were tested, a relatively high cross-reaction rate of 6.3% was determined by the EgHF-ELISA. The mean ± SD age of the patients with a positive EgHF-ELISA reaction was 59.0 ± 3.3 years (10 males and seven females) compared with 57.4 ± 0.9 years (123 males and 130 females) in the patient group with a negative EgHF-ELISA reaction: the differences were not statistically significant. When the distribution of the different types of neoplasms among these 17 EgHF-positive patients was analyzed, the relative frequency was highest for the GI tract (35% within the group of ELISA-positive patients versus 16% in the ELISA-negative group; P < 0.05), followed by lymphoma (12% versus 26%, not significant) and breast cancer (12% versus 10%, not significant). Patients with a positive EgHF-ELISA reaction had (statistically) less extensive disease, including metastasis, compared with patients with a negative EgHF-ELISA reaction (24% versus 55%; P < 0.05). Importantly, in all sera from the 17 patients with a positive EgHF-ELISA reaction, the immunoblot test result remained negative with respect to the diagnostically relevant 8-kD band, as well as with respect to the additional 29-kD and 34-kD bands. Thirteen of the 17 patients in question showed no reactivity and showed negative results in the immunoblots. Sera from two patients showed a single band reactivity with a 13-kD band and two patients showed reactivity with a 27-kD band, respectively; both were not related to the 8-, 29-, and 34-kD diagnostic bands.

When the cross-reactivity data obtained from non-CHD patients with either parasitic diseases or malignancies were combined, an overall specificity of 82.3% (95% CI = 6.3–95%) was demonstrated for the EgHF-ELISA. The test specificities were 96.8% (95% CI = 47.4–100%) for the 8-kD antigen immunoblotting and 93.8% (95% CI = 15.8–100%) for the combined 8-, 29-, and 34-kD immunoblotting.

Among those sera that were tested by immunoblotting, there were two sera that exhibited an unreadable situation in that the serum demonstrated a high background staining of the nitrocellulose strips. This background staining did not allow us to reliably assess the presence or absence of specific immunoreactive bands. One serum was from a post-operative case of E. granulosus case, and the other was from a

<p>| Table 1: Serologic reactivity of cystic hydatid disease (CHD) and alveolar echinococcosis (AE) sera and some selected non-Echinococcus parasitic sera in the E. granulosus hydatid fluid antigen (EgHF)-ELISA and in confirmatory tests (arc-5-electrosyneresis and immunoblotting) |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Disease</th>
<th>No. of patients tested</th>
<th>No. (%) positive in the EgHF-ELISA</th>
<th>No. (%) positive for arc-5³</th>
<th>Immunoblot: No. (%) positive for the 8-kD band</th>
<th>Immunoblot: No. (%) positive for one or more of the 8/29/34-kD band</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHD pre-operative (diagnostic)</td>
<td>35</td>
<td>32 (91)</td>
<td>22 (63)</td>
<td>25 (71)</td>
<td>32 (91)</td>
</tr>
<tr>
<td>CHD post-operative</td>
<td>52</td>
<td>50 (96)</td>
<td>30 (58)</td>
<td>31 (60)</td>
<td>42 (81)</td>
</tr>
<tr>
<td>AE</td>
<td>19</td>
<td>18 (95)</td>
<td>8 (42)</td>
<td>10 (53)</td>
<td>16 (84)</td>
</tr>
<tr>
<td><em>Taenia solium</em> cysticercosis</td>
<td>12</td>
<td>7 (58)</td>
<td>ND</td>
<td>1 (8)</td>
<td>4 (33)</td>
</tr>
<tr>
<td>Schistosomiasis</td>
<td>6</td>
<td>2</td>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fascioliasis</td>
<td>6</td>
<td>4</td>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nematodes</td>
<td>20</td>
<td>12 (60)</td>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amebic liver abscess</td>
<td>6</td>
<td>0</td>
<td>ND</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Malignancies: gastrointestinal tract neoplasm</td>
<td>47</td>
<td>6 (13)‡</td>
<td>0</td>
<td>0</td>
<td>0§</td>
</tr>
<tr>
<td>Malignancies: lymphoma</td>
<td>68</td>
<td>2 (3)¶</td>
<td>0</td>
<td>0</td>
<td>0#</td>
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<tr>
<td>Malignancies: Others</td>
<td>155</td>
<td>9 (6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* ND = not done.  
† Toxocariasis 0/4; strongyloidiosis 4/4; onchocerciasis 4/4; trichinellosis 3/4; ascariasis 3/4.  
‡ P < 0.05.  
§ Not significant.  
# Two sera exhibited a single 27-kD band reactivity each.
case of *E. multilocularis*. Both sera are listed as immunoblot-negative CHD and AE cases, respectively, in Table 1.

**DISCUSSION**

Immunodiagnosis is an important tool in the diagnosis of an *E. granulosus* infection and of CHD. Thus, in addition to imaging techniques, a reliable serodiagnosis improves prognosis for the patients in question in that surgical and medical treatment can specifically be adapted to the CHD problem.\(^{22}\) Conventional CHD serology is based primarily on a screening test such as an EgHF-ELISA and a subsequent confirmation test such as arc-5-electrosyneresis or immunoblotting for the 8-kD antigen.\(^9\) In contrast to AE or cysticercosis serology,\(^{23-25}\) relatively few immunodiagnostic studies have been reported that reliably address routine application and evaluation of the diagnostic test. In central Europe, these include primarily the differential diagnosis of AE, cysticercosis, and other parasitic infections with potential cross-reactivity, other hepatic disorders including malignancies that may mimic cyst-like structures in imaging procedures, and malignancies that may putatively lead to false-positive reactions such as those described by Dar and others.\(^{15}\) The present study was designed to assess the diagnostic performance of an EgHF-ELISA complemented by two confirmatory tests (arc-5 immunoprecipitation and 8-kD immunoblotting test). Our evaluation strategy included discrimination between diagnostic sensitivity and diagnostic sensitivity of the EgHF-ELISA. Thus, we showed that the sensitivity for diagnosing CHD cases before treatment was significantly lower when compared with patients after treatment. Several of the CHD cases investigated showed negative serology before treatment and had already seroconverted a few weeks after surgical intervention, thus emphasizing this diagnostically relevant feature.

Primary screening tests such as the EgHF-ELISA usually exhibit some lack of specificity. This was clearly confirmed by the 95% cross-reactivity observed with AE sera and the high level (58%) of cysticercosis cross-reactivity. There was also a marked cross-reactivity with sera from patients with schistosomiasis, fascioliasis, and several nematode infections. To elucidate potential specificity, cross-reactivity, or false-positivity of the serologic reactions, we assessed arc-5-electrosyneresis and 8-kD immunoblotting with the respective sera. Arc-5 immunoprecipitation was demonstrated in only 63% of the pre-operative CHD sera compared with 71% in post-operative cases. The diagnostic sensitivity of the 8-kD immunoblot was slightly higher for the pre-operative cases (71%), but the rate of 8-kD band positivity decreased with the post-operative samples. It should be noted that the results of both tests matched each other at a high rate. There were only two arc-5-positive pre-operative sera that were negative in the 8-kD immunoblot, whereas five immunoblot-positive sera demonstrated no arc-5-positivity. It should also be mentioned that among the immunoblot-negative echinococcosis sera, there were two sera that showed a peculiar high background staining of the nitrocellulose strips, thus not allowing any reliable assessment of any immunoreactivity with the 8-kD band and other bands. We have observed similar phenomena, although very rarely, in other immunoblot systems, such as in *T. solium* cysticercosis and toxocariasis immunoblots (Gottstein B, unpublished data). However, the frequency of the problem appears to affect less than 1% of the sera, and is thus of minor im-
portance in routine diagnosis. Both test systems (arc-5-electroosyneresis and immunoblotting) also showed a marked cross-reactivity with AE sera, but no cross-reactivity was observed with any non-cestode sera. During our test evaluation, we observed a consistent banding pattern for Echinococcus sera with the 29-kD and 34-kD bands of the EgHF antigen. When these two bands were included as diagnostic markers in addition to the 8-kD band, an increase in diagnostic sensitivity was obtained for pre-operative CHD sera (from 71% to 91%) without losing specificity concerning non-cestode cross-reactivities. It should be noted that the 29-kD and 34-kD bands appeared as relatively weak bands. We have also observed that the intensity of these two bands varied considerably depending on the batch and on the host origin of the hydatid fluid used. Therefore, some limitations may be encountered in using these two bands in routine immunodiagnosis, especially when the diagnostic laboratory does not have access to the appropriate antigenic sources.

In sera from patients with malignancies and not infected with E. granulosus, the frequency of cross-reactions with the EgHF-ELISA was higher compared with sera from healthy subjects (6.3% in 270 tumor patients versus 0.5% in 200 healthy Swiss adult blood donors; P < 0.05). Conversely, the immunoblot test result remained negative with respect to the diagnostically relevant 8-kD band in all 17 patients with a positive initial EgHF-ELISA result. These findings confirmed the relative specificity already reported by Maddison and others.9 Our results demonstrated that this 8-kD antigen could identify patients with a malignancy displaying a cross-reaction with the EgHF antigen from actual CHD patients. Sera from patients with malignancies never cross-reacted with the 29-kD and 34-kD bands. When nonspecific bands of other molecular sizes were included, only four of the 17 tumor patients studied showed any band reactivities. Two patients with GI tract neoplasms recognized the same 13-kD band, and two patients with lymphoma recognized the same 27-kD band. Immunostaining at low intensity appeared within the 14–120-kD region with sera from patients with other parasitic diseases, such as schistosomiasis, fascioliasis and filariasis, but no reaction was seen with the 8-, 29-, or 34-kD bands.

We conclude that the EgHF-ELISA, which is widely used as a serologic prescreening test for E. granulosus infections, is associated with relatively frequent cross-reactions in tumor patients and in patients with other parasitic diseases, although the test exhibited a relatively high and satisfactory diagnostic sensitivity with regard to the pretreatment phase of CHD. Specificity problems may be overcome by testing EgHF-ELISA-positive sera in a second step with a confirmatory test. For this, we recommend using an immunoblotting-based detection of antibody reactivity to the 8-kD antigen of EgHF, and/or, if appropriately detectable, to the 29-kD and 34-kD bands. Arc-5 precipitation techniques exhibited a lower diagnostic performance in a secondary confirmation assay and may be replaced by immunoblotting. Combining the EgHF-ELISA and immunoblotting enables one to discriminate between CHD and cross-reactive non-cestode parasitic infections or malignancies.

To exclude cross-reactions with antibodies in sera of patients with cysticercosis, the corresponding cysticercosis-specific immunoblots20,28 should also be performed, at least for those cases in which no clinical information is available to the diagnostic laboratory. To elucidate putative AE, the use of specific tests such as the Em2env-ELISA17 is recommended.

Additional issues, such as standardization of the EgHF-ELISA and immunoblotting test procedures and standardization of antigen preparations, should be addressed in future investigations. These have been neglected in studies of CHD serology, but are essential for quality assessment of immunodiagnosis in certified laboratories.

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