Molecular and Immunological Diagnosis of Echinococcosis

BRUNO GOTTSTEIN
Institute of Parasitology, University of Zürich, CH-5057 Zürich, Switzerland

INTRODUCTION

Echinococcosis is an infectious disease caused by the larval (metacestode) stages of various cestode (tapeworm) species of the genus Echinococcus. Two of these parasite species are of medical and public health importance in that they are widely prevalent and may cause severe disease in humans: Echinococcus granulosus is the causative agent of cystic echinococcosis, or cystic hydatid disease; and E. multilocularis in humans causes alveolar echinococcosis, or alveolar hydatid disease. Two other species of the genus Echinococcus, namely, E. vogeli and E. oligarthus, are mainly restricted to sylvatic animals and occur in some areas of Central and South America. As cases of the so-called polycystic echinococcosis (E. vogeli) are very rare in humans and cases of infections with E. oligarthus have not been reported yet, these two parasite species will not be considered in the following text.

A brief characterization of organisms causing echinococcosis is given in Table 1.

Biology of E. granulosus and E. multilocularis

E. granulosus is a small tapeworm (rarely exceeding 7 mm in length) that lives firmly attached to the mucosa of the small intestine in definitive hosts, usually dogs but occasionally other carnivores. Ungulates are intermediate hosts for E. granulosus. E. multilocularis occurs mainly in red and arctic foxes, but dogs and cats can incidentally be involved in the life cycle as definitive hosts (124). Small mammals (microtine and arvicolid rodents, occasionally muskrats, and others) are intermediate hosts for E. multilocularis.

For both Echinococcus species, sexual maturity of the adult-stage tapeworms is reached within 4 to 5 weeks. This is followed by the shedding of gravid proglottids (each containing several hundred eggs) or released eggs in the feces of definitive hosts. Following ingestion of Echinococcus eggs by susceptible intermediate hosts and humans, a larva, the oncosphere, is released from the egg envelope. The oncosphere penetrates through the intestinal epithelium into the lamina propria and is passively transported through blood or lymph vessels to primary target organs such as liver and lungs or, less frequently, to other organs. At these locations, the metacestode stage of the parasite develops. Protoscolices form, and they grow to the adult stage once ingested by a definitive host.

The life cycles of E. granulosus and E. multilocularis are shown in Fig. 1 and 2.

Geographic Distribution

Infections with E. granulosus occur worldwide. A so-called European form (100), primarily involving synanthropic hosts in its cycle, has a nearly cosmopolitan distribution. This form is responsible for major public health or economic problems in many rural areas of the world. A Northern form (100) is prevalent in northern parts of North America and Eurasia. Areas of endemicity are mainly related to tundra and taiga and are delineated by the southern limits of the boreal forest.

E. multilocularis seems to occur only in the northern hemisphere. In North America, the cestode is present in the subarctic regions of Alaska and Canada, including St. Lawrence Island (101) and some other islands (100). The parasite has been discovered in Manitoba, Canada, and North Dakota (78) and, more recently, in Alberta and Saskatchewan, Canada, and Illinois, Nebraska (12), Iowa, South Dakota, Montana, Wyoming, and even South Carolina (73), thus indicating an apparent expansion of the focus within the north central American continent. In Europe, areas with relatively frequent reports of alveolar echinococcosis in humans encompass central and eastern France, Switzerland, Austria, and Germany. These main European areas of endemicity have previously been regarded as iso-
TABLE 1. Brief characterization of organisms causing echinococcosis and the various forms of disease

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E. granulosus</th>
<th>E. multilocularis</th>
<th>E. vogeli</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major definitive hosts</td>
<td>Dog, wild canids</td>
<td>Fox, dog, cat</td>
<td>Bush dog (Speothos)</td>
</tr>
<tr>
<td>Major intermediate hosts</td>
<td>Sheep, cattle, pig, horse, camel</td>
<td>Rodents</td>
<td>Paca</td>
</tr>
<tr>
<td>Adult tapeworm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Length (mm)</td>
<td>2-7</td>
<td>1.2-3.7</td>
<td>3.9-5.6</td>
</tr>
<tr>
<td>No. of proglottids (range)</td>
<td>3 (3-6)</td>
<td>5 (2-6)</td>
<td>3</td>
</tr>
<tr>
<td>Larval form in humans</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shape</td>
<td>Fluid-filled single cysts</td>
<td>Vesiculated tissue, no fluid, central necrotic zones</td>
<td>Multiple small cysts, fluid filled</td>
</tr>
<tr>
<td>Growth</td>
<td>Expansive</td>
<td>Infiltrative</td>
<td>Expansive</td>
</tr>
<tr>
<td>Main organ localization</td>
<td>Liver (60%), lungs (20%), and others</td>
<td>Liver (98%)</td>
<td>Liver and other organs</td>
</tr>
<tr>
<td>Echinococcosis form in humans</td>
<td>Cystic</td>
<td>Alveolar</td>
<td>Polycystic</td>
</tr>
<tr>
<td>Distribution</td>
<td>Worldwide</td>
<td>Northern hemisphere</td>
<td>Central and South America</td>
</tr>
</tbody>
</table>

lated foci, but recent data suggest that they might be connected to each other and to other Eurasian or Asian areas where *E. multilocularis* has been reported. In Asia, *E. multilocularis* occurs in the whole zone of tundra from the White Sea eastwards to the Bering Strait, thus appearing in large parts of the Soviet Union and smaller parts of other countries (109, 140).

**Clinical Manifestations**

*E. granulosus*. In cystic echinococcosis (hydatidosis) of humans, well-delineated spherical primary cysts are formed most frequently in the liver (approximately 65% of the cases) (Fig. 3), but also in the lungs (25%) and other organs such as kidney, spleen, brain, heart, and bone (110). Cysts cause pathological damage or dysfunction mainly by the gradual process of space-occupying repression or displacement of vital host tissue, vessels, or organs. Consequently, clinical manifestations are primarily determined by the site and number of cysts and are quite variable. Accidental rupture of cysts can be followed by a massive release of cyst fluid and dissemination of protoscolices, resulting occasionally in anaphylactic reactions and/or multiple secondary cystic echinococcosis, since protoscolices have the potential of developing into cysts within the intermediate host. Successful surgical removal of hydatid cysts is frequent, so case fatality rates are low (varying between 1 and 4% for cases with first surgical intervention [110]), provided modern medical facilities are available. The public health importance is mainly reflected by the number of infected persons and their diminished capacities, the direct and indirect costs of hospitalization and recovery from surgery, and any residual disability or clinical sequelae (116). Globally, few data on the overall prevalence of human cystic echinococcosis exist. Regions with good documentation of prevalence include the whole Mediterranean area, the Turkana district of Kenya, large foci in South America, and many other zones in all continents. North American experiences have been documented by various groups (24, 29, 76, 112). An updated summary of cases in countries of the European Community and the European Free Trade Association has recently been presented (123). *E. multilocularis*: *E. multilocularis* metacestodes (larvae) in humans are found almost exclusively in the liver, but secondary lesions can form in the lungs, brain, and other organs (110). The hepatic lesion usually consists of a dispersed, spongy, pale tissue consisting of scattered small cysts and vesicles (Fig. 4). The diffuse borders are commonly noted without delineation from the adjacent liver tissue. A central necrotic cavity is often found in the advanced stage of hepatic alveolar echinococcosis. The lesions may be allied with focal zones of calcification. Microscopically, there is evidence of a vigorous proliferation of fibrous and germinative tissue in the periphery of the metacestode but also there are regressive changes centrally. In contrast to infection in rodent hosts, lesions from infected humans rarely exhibit protoscolices, brood capsules, and calcareous corpuscles within vesicles and cysts. At diagnosis of human alveolar echinococcosis, the nonspecific clinical symptoms usually include mild upper-quadrant and epigastric pain, possible hepatomegaly, and obstructive jaundice.

Occasionally, the initial manifestations are caused by metastases localized in the lungs or other organs (7, 109, 110). As in cystic echinococcosis, few data on the overall prevalence of human alveolar echinococcosis exist. Cases in the native Eskimo population at risk in western Alaska, including St. Lawrence Island, have been diagnosed at an average annual rate of 28/100,000 inhabitants (138). In Switzerland, an annual average morbidity rate of 0.18 case per 100,000 inhabitants was reported (37). However, in various cantons of Switzerland, annual morbidity rates are higher (between 0.2 and 0.7 case per 100,000 [122]). Data for some areas of France, Germany, and Austria were similar (139). However, the importance of the disease is not represented by the number of reported cases but rather by the severity of the disease in the individual patient and by a frequently lethal outcome: for cases without radical surgery, mortality was found to be 92% within 10 years after primary diagnosis (114). In recent times, the mortality rate has significantly
decreased to 10 to 14%, most likely because of marked improvements in diagnosis, surgery, and chemotherapy (8).

IMMUNOLOGY

Definitive Hosts

The primary site of host-parasite interaction between the adult-stage echinococcus and its carnivorous host is the mucosa of the gastrointestinal tract. For many years, it was widely held that adult cestodes were non- or poorly immunogenic (67). Thus, little information has been elaborated on the specific immunology of adult Echinococcus infections in definitive host animals. The structures of the adult Echinococcus worm that interact with the intestinal immune system are the scolex, the integument, and all molecules excreted or secreted by the tapeworm. For E. granulosus, there is experimental evidence for induction of an adult-stage-specific humoral immune response (44, 70). The induction of a local immune response, however, does not necessarily imply functionally protective interactions. Acquired protective immunity to experimental E. granulosus infections in dogs has been reported (46), but instead of showing a continuous decline in susceptibility, each dog remained susceptible to a number of infections and then became less susceptible. Movsesijan et al. used 1,000 to 2,500 irradiated E. granulosus protoscolices for oral immunization to demonstrate subsequent induction of protective immunity against challenge infections (93).

Recent evidence suggests not only that new strategies aimed at the vaccination of definitive hosts will have to accurately and specifically elucidate potential immunological modes of protective responses but also that new (especially recombinant DNA) technologies will have to be developed for vaccine antigen production, administration, and presentation to the intestinal immune system. A very promising technological approach in this respect, for instance, is gene expression in biocarriers such as live attenuated Salmonella spp., which may prove ideal for delivering the recombinant parasite antigens to the correct anatomical site in the definitive host (56).

FIG. 1. Life cycle of E. granulosus. 1, Adult tapeworms live in the small intestine of dogs. 2, Proglottid, containing tapeworm eggs. 3, Egg. 4, Ungulates are the main intermediate hosts, with mostly the lungs being affected. Humans (4a) can become infected accidentally as intermediate hosts; target organs for hydatid cysts are mainly the liver and the lungs. 5, Bovine lung harboring hydatid cysts. Cross section (5a) shows the cyst, containing brood capsules with protoscolices. The fluid-filled cyst is surrounded by an outer laminated layer and contains an inner germinal membrane budding into protoscolices containing brood capsules. Multiple detached and sedimented brood capsules form the so-called hydatid sand. Segmentation of hydatid cysts forms so-called daughter cysts (not shown). Courtesy of the Institute of Parasitology, University of Zürich.
Regarding peripheral humoral immune responses in dogs with adult-stage *E. granulosus* infections, various authors were able to demonstrate parasite-specific serum antibodies with diagnostic potential (44, 69, 92, 118, 137). Similar to *E. granulosus*, adult *E. multilocularis* is assumed to induce a humoral immune response in definitive hosts such as foxes. Thus, serum antibodies against an *E. multilocularis*-specific Em2 antigen have been shown to be of practical value for seroepidemiology in fox populations (50).

**Intermediate Hosts and Humans**

Information on the immune response to migrating and subsequently established oncospheres and their development to the *Echinococcus* metacestode in humans is sparse. The diagnosis of echinococcosis is generally based on a fully developed and still proliferating metacestode, which has already induced and potentially influenced an immune response of the host (reviewed in reference 62). Cellular and humoral immune responses in humans, in contrast to those in experimentally infected animals, can vary enormously, as evidenced, e.g., by the different patterns of parasite antigens in different patients and courses of disease (43, 82). These disparities are likely related to human and/or parasite genetic diversity, which is unlike the uniform genetic background of most experimental animals (122).

Investigations of cell-mediated immune response in cases of murine cystic echinococcosis have revealed polyclonal B-cell activation (25), a marked drop of mean T-cell percentage (134) but increase in suppressor cell activity (104), direct splenic T-lymphocyte cytotoxicity to the metacestode (135), and impairment of the host defense potential by the formation of anti-human leukocyte antigen-reactive host antibodies (6). Several researchers have suggested that the host’s capacity for developing a parasite-specific cellular response able to eliminate the parasite may be modulated by parasite-
derived effector substances (9, 36, 82). Local immune modulation by the parasite has been shown to enhance susceptibility to mycobacterial infections close to the site of parasite lesions (39).

With regard to alveolar echinococcosis, most human patients develop parasite-specific serum antibodies, including all isotypes of immunoglobulins; very few patients fail to demonstrate a humoral immune response (52, 60). Antibodies are thought to be involved in immunopathological mechanisms responsible for the occasional chronic granulomatous course of the disease, including immune complex-associated membranous nephropathy (98) and histopathological changes related to the incidence of amyloid and immune complex deposits in the liver, as was found in several Alaskan patients (2).

Most patients show a specific response of peripheral blood mononuclear cells to in vitro stimulation with parasite antigen (17, 54). Information addressing potential immunological events at the site of host-parasite interplay has shown that the periparasitic granuloma in regressive courses of disease is mainly composed of macrophages, myofibroblasts, T cells, and a large number of CD4+ lymphocytes (131). Patients with proliferative metacestodes have increased numbers of CD8+ cells.

Murine models of alveolar echinococcosis have shown that a preexisting larval infection can prevent or suppress the development of a secondary infection (83), but once a primary infection is established in susceptible laboratory rodents, the initial E. multilocularis metacestode appears well protected from the host immune response. It grows and metastasizes despite a marked lymphoproliferative activity in the B- and T-cell areas of lymphoid tissues.

Parasite-specific antibodies alone are unable to control parasite growth, and host tissue infiltration may be due partly to complement-neutralizing factors released by the metacestode that cause complement depletion at the host-parasite interface (61) or partly to the inactivation of C3 as it enters the metacestode tissue (72). Undoubtedly, T lymphocytes play the main role in the immunological control of E. multilocularis infection. Activated macrophages (13) and neutrophils (3, 4) were suggested as key factors in the attack of E. multilocularis metacestode cells. Fragmentary information on cell-mediated immunity in different host cell populations is restricted to more general aspects (16, 79). However, analyses of the actual effector functions of different lymphokines and lymphocyte populations or subsets have not yet provided key findings for understanding the different forms of progression or regression in alveolar echinococcosis.

IMMUNODIAGNOSIS

The clinical signs and symptoms in hepatic cystic or alveolar echinococcosis resemble those of hepatic carcinoma, cirrhosis, or other liver diseases. Noninvasive imaging techniques are primarily applied and can be combined with immunodiagnostic procedures (90). The role of immunodiagnosis is to confirm clinical findings or give diagnostic help by providing detailed information on parasite or host peculiarities (e.g., species differentiation in radiologically unclear cases and determination of the patient’s immune status, etc.). Immunodiagnosis of echinococcosis has been comprehensively reviewed in various articles (80, 103, 110, 111).

FIG. 3. E. granulosus. Cross section through a human liver containing hydatid cysts (hydatid fluid removed). Courtesy of the Institute of Parasitology, University of Zürich.
Antibody Detection in Humans with Echinococcosis for Clinical Diagnosis, Seroepidemiology, and Posttreatment Follow-Up

For primary serological diagnosis and for support of clinical diagnosis of echinococcosis, 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 used most frequently vary according to the (i) nature, purity, and quality of the antigen, (ii) nature of the patient's immunoglobulins (isotypes, etc.) specified in the test, and (iii) sensitivity of the selected technology.

Until a few years ago, most serological tests for immuno-diagnosis of both cystic and alveolar echinococcosis employed E. granulosus antigens because they could be obtained easily and because, in a very early study, E. granulosus hydatid fluid had appeared to be a better diagnostic reagent than antigens prepared from E. multilocularis (96). Cystic echinococcosis also occurs more frequently than alveolar echinococcosis and thus has initiated more investigations in the development of homologous test systems. E. granulosus hydatid fluid antigen, which was generally available, was reported to be diagnostically relatively sensitive (75 to 94%) in the indirect hemagglutination test (11, 64, 113).

One of the most specific immunodiagnostic approaches for cystic echinococcosis (E. granulosus) relies on the demonstration of serum antibodies precipitating an antigen called antigen 5 (19) by immunoelectrophoresis or similar techniques. Experimental studies indicated that antibodies to antigen 5 are among the first detectable after infection (19, 23, 142). Diagnostic sensitivity for hepatic cystic echinococcosis has been reported to vary between 50 and 80% (see references in reference 110). Antibodies to antigen 5 also occur in the sera of human patients with neurocysticercosis (125) and alveolar echinococcosis (127), and comparative studies showed that only 58% of Swiss patients with alveolar echinococcosis had antibodies to antigen 5 compared with 74% of patients with cystic echinococcosis (60).

Subsequently, the antigenic components of E. granulosus responsible for the arc-5 phenomenon were investigated by various approaches that used monoclonal antibodies or immunoblotting or both (20, 35, 82, 119) (Fig. 5). Furthermore, preliminary results had indicated some potential for using purified antigen 5 in highly sensitive techniques such as the enzyme-linked immunosorbent assay (ELISA) (65). Falcon et al. cloned an E. granulosus gene encoding an antigen 5 component and suggested recombinant antigen 5 as the immunodiagnostic reagent (40). However, despite the usefulness of antigen 5 for various immunodiagnostic applications, there is some evidence of a lack of species specificity and of problems of diagnostic sensitivity due to the absence of anti-antigen 5 antibodies in some patients. Consequently, multiple attempts to further characterize E. granulosus antigenic components and to identify corresponding fragments or molecules with optimal diagnostic characteristics were investigated. Shepherd and MacManus (117) initially found a low-molecular-weight subunit of antigen B (97) to be species specific, but this could not be confirmed subsequently (82). Resolution of E. granulosus hydatid cyst fluid by sodium dodecyl sulfate-polyacrylamide gel electrophoresis resulted in the immunoblot finding of a genus-specific diagnostic component with an apparent molecular mass of 8 kDa (86). Further evaluation of this agent will be needed to...
demonstrate its immunodiagnostic suitability. Another approach to improve immunodiagnostic characteristics of *E. granulosus* serological tools was chromatographic fractionation (5) of hydatid cyst fluid. The resulting purified 20-kDa antigen, which resembled antigen A, was first thought to be species specific for *E. granulosus* and was suggested for further evaluation of immunodiagnostic potential.

In summary, the presently recommended strategy for immunodiagnosis of cystic echinococcosis is to rely on a diagnostically sensitive test such as an ELISA, employing hydatid fluid antigen. Positive test results, which depend on the geographical origin of the patient and the implied problems of cross-reactivity due to potential infection with other parasite species, must subsequently be confirmed by tests demonstrating antibody activity against antigen 5 or other specific antigenic components, as discussed above.

Alternatively, *E. multilocularis* metacestode tissue was used as a source of immunodiagnostic *Echinococcus* antigens. Gottstein et al. used affinity chromatography to isolate antigens shared by *E. granulosus* and *E. multilocularis* (called the Em1 fraction) from crude extracts of *E. multilocularis* metacestode tissue (51). This Em1 antigen was used as a reagent for the immunodiagnosis of both cystic and alveolar echinococcosis. It became obvious that, for the diagnostic of alveolar echinococcosis, homologous *E. multilocularis* metacestode antigens were superior to heterologous *E. granulosus* antigens, especially with regard to specificity. Similar findings were described by other groups and have been reviewed by Schantz and Gottstein (110). When crude *E. multilocularis* antigens are used, however, nonspecific and cross-reactions are the cause of difficulties similar to those well known with *E. granulosus* antigens. Subsequent research has thus addressed the question of purifying highly specific antigens from *E. multilocularis*. The first documented attempt used affinity chromatographic procedures to immunosorb cross-reactive antigenic components from a crude *E. multilocularis* metacestode antigen solution (51). The resulting fractions (Em1 and Em2 antigens) were successfully used in an ELISA to correctly differentiate 95% of patients with cystic echinococcosis from patients with alveolar echinococcosis (57). In subsequent studies (31, 49), the antigenic component of the Em2 antigen fraction was purified and characterized by immunochromical means, and a monoclonal antibody was raised against the Em2 antigen.

Attempts to differentiate both forms of echinococcosis serologically have also been undertaken by others (10, 75) but without full documentation of the immunodiagnostic properties of the antigens in question. Furuya et al. used Western blotting (immunoblotting) to analyze banding patterns of serum antibodies from Japanese echinococcosis patients. They reported specific serum antibody activity against 55- and 60-kDa *E. multilocularis* antigens (43). More recently, recombinant DNA technology has been used to synthesize *E. granulosus* and *E. multilocularis* antigens. These will be discussed below.

Another approach to improving the value of immunodiagnostic properties was the individual analysis of antibody classes with respect to parasite antigens. Parasite-specific immunoglobulin E (IgE) has attracted particular attention because of its well-known importance in helminthic diseases such as those caused by *E. granulosus* (1, 18, 34, 87, 99, 136) and *E. multilocularis* (52, 67, 132).

In addition to problems of diagnostic sensitivity and specificity due to cross-reactions, there is accumulating evidence of false-positive antibody reactions not related to infections with heterologous helminth species. Such false-positive antibody reactions have been related to malignancies (30), the presence of anti-P1 antibodies (14), and liver cirrhosis (66).

Early diagnosis of persons with asymptomatic echinococcosis is considered a prerequisite for efficient management and treatment of the disease (71). Consequently, serological screening has been offered to populations and communities in many areas. Techniques used for areas in which *E. granulosus* is endemic have been reviewed by Schantz and Gottstein (110). The tests most widely used were based on detection of arc-5 (reviewed in reference 110). They demonstrated appropriate specificity in that most arc-5-positive persons could subsequently be shown to harbor hydatid cysts (21, 126). However, many studies have clearly demonstrated the limits of using serology alone in epidemiology, especially in areas where seropositivity is low among patients with cystic echinococcosis, such as the Turkana district in Kenya (28). Recent studies therefore employed what can presently be considered the optimal epidemiological tool: ultrasound examination for abdominal cystic echinococcosis combined, if possible, with immunodiagnosis (22, 85, 91, 106).

The use of *E. granulosus* antigens for seroepidemiological investigations in areas in which *E. multilocularis* is endemic permitted the identification of clinical cases (108, 113, 138). More recent findings in similar studies, however, suggested significant improvement not only of diagnostic sensitivity but also of specificity by the application of crude and subsequently purified *E. multilocularis* antigens.

A first direct comparison between homologous purified *E.
multiilocularis Em2 antigen (49) and E. granulosus hydatid fluid antigen was performed by Gottstein et al. in a large-scale serosurvey in Switzerland. This survey showed that E. granulosus antigen exhibits a relatively high degree of false positivity (53). Similar findings were reported from Alaskan studies (77). In general, the epidemiological situation of low prevalence for both cystic and alveolar echinococcosis requires high diagnostic sensitivity and high species specificity, so that positive and negative predictive values result in justifiable clinical investigation of seropositive individuals.

Seroepidemiological studies in Alaska have shown that Em2 ELISA detected not only asymptomatic cases of human alveolar echinococcosis serologically negative by other techniques (58) but also unique cases in which the metacestode had died out at an apparently early stage of infection (102). This finding of spontaneous rejection of the infection may provide valuable information for future research in the immunology of E. multilocularis infection. Another approach used to circumvent specificity problems in seroepidemiology was Western blotting (42), which shows the suitability of immunoblotting to confirm clinical cases of alveolar echinococcosis.

Serological tests used for postoperative monitoring of patients with cystic echinococcosis have been reviewed by Schantz and Gottstein (110). More recent studies have emphasized determination of parasite-specific antibody iso- types (99), but the small contribution of serology to monitoring the course of disease has been discussed and implicitly requires that serology be combined with an instrumental or imaging examination in order to provide an accurate prognosis.

Surgically treated cases of alveolar echinococcosis are usually treated postoperatively with chemotherapy. As only complete surgical removal of the entire parasite lesion offers a prospect of cure, the accurate assessment of the success of the resection is an urgent requirement in the clinical monitoring of patients. Serological tests have generally demonstrated a decrease in the concentration of parasite-specific serum antibody after successful surgery (113). The preliminary use of Em2 ELISA indicated that anti-Em2 antibody concentrations declined dramatically within months after a successful radical operation (77). These findings were confirmed in subsequent larger studies (59).

An exceptional immunological situation is encountered with patients undergoing orthotopic liver transplantation (47, 89). Serum antibodies were artifically reduced or eliminated by abundant blood transfusions coupled with immunosuppressive therapy. Patients with remaining residual foci of extrahepatic parasite tissue had extremely high recurrence rates due to immunosuppression and interruption of chemotherapy with antiparasitic benzimidazoles (15). Such recurrences were generally accompanied by the reappearance of anti-Echinococcus serum antibodies.

Patients with alveolar echinococcosis who received only chemotherapeutical treatment were very difficult to monitor by classical serological means (8, 60, 74, 77, 113). In general, specific antibody concentrations decreased in chemotherapeutically treated patients with regressive forms of disease, whereas specific antibody concentrations in sera of patients with nonresectable lesions and/or palliative surgery and a progressive course of disease remained elevated or increased. To date, the need for clinicians to have a clear predictive interpretation of serology with regard to progressive or regressive disease has not been met by classic serological methods.

Parasite-specific antibody isotypes may correlate better with clinical findings than do results of classic serological tests, especially with regard to IgA and IgE (52). Vuitton et al. reported transient changes in parasite-specific serum IgA and IgM antibodies in patients after chemotherapy with flubendazole (133) and suggested that basophil-bound IgE could be correlated specifically with positive or negative responses to therapy (132).

Antigen Detection in Patients with Echinococcosis

Tests for the determination of circulating immune complexes in patients with cystic echinococcosis have been reviewed by Schantz and Gottstein (110). Soluble E. granulosus antigens circulating in patient sera have been detected in 33 to 85% of serum samples from patients with cystic echinococcosis (27, 48). Generally, it was suggested that the identification of circulating parasite antigens with their potential for immune complex formation in the sera of patients might be useful for monitoring the disease and might reflect more reliably than antibody titers the viability and biological activities of parasites in the host (38). Surprisingly, circulating E. multilocularis antigens have been neglected in this respect.

Lymphoproliferative Responses in Patients with Echinococcosis

Skin tests and basophil degranulation tests have been extensively discussed and reviewed by Schantz and Gottstein (110). Aside from these tests, remarkable developments in basic cellular immunology have attracted the attention of parasite immunologists to parasite-specific host cellular immune responses and have implied cytokine interplay at the site of parasitic lesions as well as at their peripheries (33). In these studies, the in vitro lymphoproliferative response to E. granulosus antigen stimulation was assessed in 40 patients with cystic echinococcosis (120). There was no correlation between serological and lymphoproliferative results. The diagnostic sensitivity of positive test reactions was 75% for both serology and lymphocyte proliferation. Finding seronegative patients with positive proliferation assay results and seropositive patients with negative proliferation assay results suggested that the lymphocyte-specific immunoassays should be used as diagnostic tests.

With regard to E. multilocularis, the relevance of cellular immune responses and reactions is suggested by the important granulomatous infiltration surrounding E. multilocularis lesions in infected human livers (130). The in vitro determination of lymphocyte proliferation to stimulation with E. multilocularis antigens has been proposed as a diagnostic alternative to antibody detection in patients with alveolar echinococcosis (17). The same study addressed the parameters of E. multilocularis-specific cellular immune response during a 2- to 4-year period of mebendazole treatment. A progressive decrease in the capacity to respond to parasite-specific lymphocyte stimulation was observed in most of the patients with regressive disease. On the other hand, an increase of stimulation indices was usually shown to be associated with a progression of the liver lesion. Gottstein et al. showed that the in vitro lymphoproliferative response to E. multilocularis antigen stimulation was very high in cured patients who had radical surgery or in patients with inactive lesions, but the response was significantly lower in patients who had had partial or no surgical resection (54). Distinct differences in the parasite-specific humoral and cellular immune status of patients with self-limited infections and...
other patient groups with different courses of alveolar echinococcosis may provide insight into potentially protective immune mechanisms.

Immunodiagnosis in Definitive Hosts

Definitive carnivore hosts are usually examined for infections with intestinal stages of *Echinococcus* spp. by either examination of purged fecal samples for tapeworms or parasitological examination of small intestines after necropsy. These techniques exhibit some problems of diagnostic sensitivity in cases of infection with low numbers of worms. Antibody detection, therefore, has been experimentally investigated as an alternative for the diagnosis of *E. granulosus* infections in dogs (69, 70). Anti-*E. granulosus* serum antibodies were detected by ELISA by 2 to 3 weeks after experimental infection of dogs with *E. granulosus*. Cross-reactions were evident with the antibody sandwich ELISA from dogs experimentally infected with *Taenia hydatigena* and *T. pisiformis*. Unfortunately, when serological tests for assessing *E. granulosus* infections were evaluated under field conditions in dogs shot in northwestern Turkmenia, where the parasite is hyperendemic, diagnoses of currently infected dogs were not reliable (68).

Gasser et al. demonstrated stage-specific oncospheric humoral immune responses, which strongly suggested that oncospheres from *Echinococcus* eggs actually hatch in the intestines of the specific definitive hosts (44). Identical mechanisms are assumed to occur for *E. multilocularis* (50). In this respect, serum antibodies against the metacestode stage-specific Em2 antigen could be demonstrated in dogs and foxes infected with adult-stage *E. multilocularis*. The corresponding Em2 ELISA was evaluated for assessing fox populations with *E. multilocularis* infection. The species specificity of the test was demonstrated by the absence of cross-reactions with antibodies from carnivores infected with intestinal or tissue-dwelling non-*Echinococcus* cestodes or nematodes. There was experimental evidence that anti-Em2 immunoglobulin synthesis was induced during a postoncospherical development of *E. multilocularis* following an infection of the definitive host with its own viable *E. multilocularis* eggs (31). The value of the test consequently related to (i) the reliable identification of fox populations with or without *E. multilocularis* infections and (ii) the estimation of the prevalence of infection within the fox populations by extrapolation. Thus, the test may be valuable in sequentially assessing the dynamics of prevalence in areas undergoing control campaigns (50).

Alternatives to the coprological or serological diagnosis of adult-stage *Echinococcus* infections in definitive hosts have been proposed, for example, using PCR (polyclonal) ELISA (32). Affinity-purified polyclonal antibodies raised against excretory and secretory antigens of adult-stage tapeworms were used to develop an ELISA that permitted the detection of coproantigens from *E. granulosus* and *E. multilocularis* in dog or fox fecal samples. The absence of cross-reactions with antigens related to most infections with other cestodes or nematodes proved to be genus specific. Conversely, the sensitivity was relatively low in that only hosts with a high intestinal burden of tapeworms (>1,000 *Echinococcus* worms per animal) reacted positively.

Another immunological diagnostic approach has been developed to identify the generic origin of taeniid eggs by using monoclonal oncosphere antibodies (27). Alternatively, molecular diagnostic approaches such as the detection of parasite-specific DNA fragments originating from either parasite eggs or cells of adult tapeworms are rapidly attracting attention, especially with the development of highly sensitive techniques such as the polymerase chain reaction (PCR). Such techniques are discussed below.

Molecular Diagnosis

Recombinant *Echinococcus* Antigens

Techniques in molecular biology exhibit great potential as tools for the synthesis of defined protein antigens. Obtaining a sufficient supply of diagnostically sensitive and specific *Echinococcus* antigens has always been a problem when classic immunochemical methods are used. Cloning and expressing *Echinococcus* genes in suitable vectors may circumvent these problems (81). The logistical approach is generally based on the construction of a cDNA expression library that uses mRNA obtained from the appropriate parasite stage. Libraries are subsequently screened with (polyclonal or monoclonal) antibodies or lymphocytes in order to identify bacterial clones producing recombinant antigens bearing relevant B- or T-cell epitopes. Finally, efficient production of recombinant antigens in an appropriate biological form requires appropriate gene expression systems.

A cDNA library derived from *E. granulosus* protoscoleces mRNA was established by using the *Escherichia coli* expression vector pGT11 and was successfully screened for clones synthesizing antigens suitable for serodiagnosing intestinal adult-stage infections in dogs (45). The subsequent gene expression system selected for the production of diagnostic antigens resulted in the synthesis of glutathione S-transferase fusion proteins. One of the fusion proteins investigated had demonstrated high specificity (100%), although its diagnostic sensitivity was low. Screening an identical library with sera from patients with cystic echinococcosis resulted in the finding of several clones with production of diagnostically highly sensitive fusion proteins (81). The diagnostically misleading reactivity of patient antibodies with glutathione S-transferase from the fusion proteins might be circumvented by using a modified gene expression system (pGEX2), which allows cleavage of the recombinant parasite polypeptide from the glutathione S-transferase (121). The cloning of an *E. granulosus* gene encoding an antigen 5 component has already been mentioned above (40).

The first published *E. multilocularis* cDNA library was constructed by Vogel et al. (128), who also used the *Escherichia coli* expression vector pGT11. An identified *E. multilocularis* species-specific recombinant clone, II/3, demonstrated optimal immunodiagnostic characteristics, as assessed by Western blotting. Poor bacterial expression and lack of an appropriate purification protocol hampered the application of the respective antigen in routine diagnosis. This problem was solved by shortening the initial 1.0-kb cDNA sequence encoding for the antigen II/3 to a 0.6-kb fragment and then subcloning it into plasmid vector pAR3038 (94). The resulting increased bacterial production allowed efficient biochemical purification of the recombinant antigen II/3-10. In the pAR3038 vector, the recombinant antigen II/3-10 was synthesized as a polypeptide fused to a short (11-amino-acid) N-terminal peptide of bacteriophage T7 origin. This short phage peptide was shown to be immunologically irrelevant and thus needed no further modification prior to investigation by ELISA. A preliminary diagnostic evaluation of the recombinant antigen II/3-10 by ELISA resulted in operating characteristics suitable for immunodiagnostic use.
agnosis of alveolar echinococcosis in humans. The production of other recombinant E. multilocularis antigens with immunodiagnostics potential has been reported subsequently (41, 63). Recent comparative experimental results have shown sequence homology between the recombinant antigen II/3 described by Vogel et al. (128) and antigen Em10 described by Frosch et al. (41).

In expression systems such as those given above for the production of E. granulosus or E. multilocularis recombinant antigens, the products usually accumulate within the bacterial cell as a soluble protein or as insoluble precipitates. Consequently, alternative systems such as the insertion of Echinococcus genes into the mglB gene of plasmid pVB2 have been proposed (115). Resulting fusion proteins are linked to the mglB-encoded periplasmic galactose-binding protein, which is excreted into the periplasmic space (95). Recombinant Echinococcus antigens fused to the galactose-binding protein could be conveniently purified in soluble form from a bacterial cell culture supernatant by an osmotic shock procedure. The purified recombinant antigen constituted >50% of total cellular protein and could be applied directly in ELISA. In conclusion, the molecular cloning of Echinococcus genes encoding epitopes with immunodiagnostic potential is promising, not only because the production of antigens in large amounts is facilitated but also because these techniques offer the advantage of producing serological reagents of standardized quality.

DNA Hybridization Techniques and PCR

Molecular biological techniques have evolved rapidly, resulting in technical innovations with potential applications to diagnostic parasitology. The identification of parasite species- or even stage-specific nucleic acid sequences has resulted in the development of DNA probes useful for hybridization to DNA from diagnostic samples. To date, this technology has limited value in that its application focuses mainly on the characterization of Echinococcus isolates or strains, thus providing epidemiological rather than clinical information.

A variety of DNA probes have been developed and used by several groups to characterize, identify, or group different E. granulosus (84, 105, 141) or E. multilocularis (129) strains or isolates. Apart from the restricted availability of specific diagnostic probes, one major problem is the limited sensitivity of hybridization and labeling techniques. Current hybridization techniques do not allow identification of single taenid eggs. The possibility of differentiating single cestode eggs at the species level remains an important goal in parasitic diagnosis (88). These technical limitations can now be essentially eliminated by an extraordinary new tool, the PCR (107). Diagnostic PCR depends on the availability of appropriate target nucleic acid sequences that flank regions of interest, which help in the design of synthetic oligonucleotide primers. By using Taq polymerase (obtained from the thermophilic aquatic bacterial species Thermus aquaticus), which is stable up to DNA denaturing temperatures of 95°C, a millionfold cyclic amplification of target DNA sequences can be obtained. Furthermore, sensitivity can be enhanced by additional techniques such as reamplification with internal primers or Southern dot hybridization labeled with nucleotide probes. On the basis of an E. multilocularis DNA probe, pAL1 (129), the respective nucleic acid sequence was analyzed in order to obtain oligonucleotide primers suitable for use in PCR amplification of specific target sequences from diagnostic Echinococcus genomic DNA (55). Two designed E. multilocularis oligonucleotides, BG1 and BG2, defined a 2.6-kbp fragment in the genome of E. multilocularis. A PCR study including 14 independent E. multilocularis isolates (originating from Switzerland, Alaska, Canada, France, Germany, and Japan) and various other cestodes revealed that the 2.6-kbp PCR product was amplified from the genomic DNA of all E. multilocularis isolates but no other cestode species. Another E. multilocularis primer set, BG1 and BG3, defined a 0.3-kbp fragment that resulted in amplification of a genus-specific PCR product, i.e., from E. multilocularis, E. granulosus, and E. vogeli genomic DNAs only. The diagnostic sensitivity of the E. multilocularis PCR was evaluated experimentally and approached 2.5 pg of template DNA, which corresponds approximately to the DNA content of one single Echinococcus egg (105). The diagnostic application of the E. multilocularis PCR putatively addressed the identification of fine-needle biopsy material obtained from patients with liver lesions of unknown etiology, the rapid and easy identification of E. multilocularis liver lesions from rodents in epidemiological studies, and, perhaps the most promising and important approach, the demonstration and identification of adult-stage parasite tissue, DNA, or eggs in samples derived from feces, small intestines, or anest swabs of definitive carnivore hosts.

REFERENCES


Alveolar echinococcosis in dogs.


