

A new ImmunoCAP assay for detection of *Echinococcus multilocularis*-specific IgE

Nele Wellinghausen, Peter Kern *

Section of Infectious Diseases and Clinical Immunology, University of Ulm, Robert-Koch-Str. 8, D-89081 Ulm, Germany

Received 23 March 2000; received in revised form 5 December 2000; accepted 15 December 2000

Abstract

In alveolar echinococcosis (AE), caused by *Echinococcus multilocularis* (*E.m.*), increased levels of total and parasite-specific IgE are frequently found. These may not only have diagnostic but are also supposed to have prognostic value in the follow-up of AE patients. However, there is no commercial test available for quantification of *E. m.*-specific IgE (sIgE). The only commercial test available is based on *E. granulosus* (*g.*) hydatid antigen, which is not optimal for detection of *E. m.*-specific IgE. Therefore, a new ImmunoCAP with covalently bound crude antigen of *E. m.* was developed in cooperation with Pharmacia Research Forum for the analysis of *E. m.* sIgE. The *E. m.* ImmunoCAP was evaluated in 53 AE patients with different clinical disease progression and 20 healthy controls. Our data showed a higher sensitivity for sIgE determination with *E. m.* ImmunoCAP compared to the *E. g.* ImmunoCAP (73.6% vs 61.5%) and a positive correlation between total IgE and sIgE. Furthermore, there was a significant correlation between sIgE in both tests. In conclusion, the new *E. m.*-specific ImmunoCAP test proved to be a valuable tool for determination of sIgE. It may provide the basis for the development of further *E. multilocularis*-specific IgE immunotests which are essential for evaluation of sIgE during clinical course of AE. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: *Echinococcus multilocularis*; IgE; Diagnosis; Immunoassay

1. Introduction

Alveolar echinococcosis (AE), caused by the larval stage of the fox tapeworm *Echinococcus*

multilocularis, is a life-threatening disease in humans. It mainly affects the liver and has characteristics of a malignant tumor, including infiltrative growth and metastasis formation (Ammann and Eckert, 1996).

As a characteristic feature of helminth infections, high levels of immunoglobulin E (IgE) are frequently found in serum of AE patients (Vuitton et al., 1988; Gottstein, 1992; Dreweck et al., 1997). According to the Th1/Th2 concept, Th2 dominated immune response seems to be associ-

* Corresponding author. Tel.: +49-731-50024420; fax: +49-731-50024422. Present address: Department of Medical Microbiology, University of Ulm, Robert-Koch-Str. 8, D-89081 Ulm, Germany.

E-mail address: peter.kern@medizin.uni-ulm.de (P. Kern).

ated with increased susceptibility to infection, while Th1 cell activation leads to protective immunity against *E. multilocularis* (Gottstein and Hemphill, 1997; Jenne et al., 1997). In contrast to total IgE, specific IgE (sIgE) represents the part of IgE directly involved in immune response against the parasite. Earlier studies have shown that increased levels of sIgE are associated with a progressive state of disease (Vuitton et al., 1988; Dreweck et al., 1997). Measurement of sIgE in AE may also be useful for monitoring of disease progression during follow-up of patients (Gottstein et al., 1984). Despite the importance of sIgE in AE there is no test for detection of *E. multilocularis*-specific IgE commercially available. The only diagnostic test for determination of sIgE (Pharmacia® ImmunoCAP p2) is composed of *E. granulosus* hydatid antigen which shows a certain degree of cross-reactivity with *E. multilocularis* but is not species-specific (Gottstein et al., 1985).

Based on the commercial immunotest, a new diagnostic test for quantitative determination of sIgE and sIgG which employs crude antigen of *E. multilocularis* was developed in this study. The test was evaluated in 53 AE patients and 20 healthy controls and compared to the Pharmacia® ImmunoCAP p2.

2. Materials and methods

2.1. Patients and controls

The study population consisted of 53 patients (29 female and 24 male) with a median age of 57 years (range 25–83 years), all chronically infected with *E. multilocularis* and supervised in our outpatient clinic. Study population included (a) patients cured after radical surgery (group A, $n = 3$), (b) patients with stationary or regressive course of AE (group B, $n = 42$), i.e. a stable course under chemotherapy with benzimidazoles ($n = 30$, median duration of treatment 33.5 months, range 2–186 months), after completion of therapy ($n = 5$) or without treatment ($n = 7$), and (c) patients with a progressive form of disease (group C), characterized by lesions increasing in size, several

complications or metastasis formation ($n = 7$) and patients with relapse after liver transplantation ($n = 1$, classification into group A/B/C followed Dreweck et al., 1997). In group B, 30 patients received chemotherapy during the study; twenty healthy blood donors (10 female and 10 male, median age 57 years, range 35–66 years) with normal liver function tests and without clinical signs of echinococcosis served as controls. Serum samples were stored at -70°C until use.

2.2. Serological tests

Total and antigen-specific IgE (sIgE) were measured with the UniCAP 100 system (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden), according to the manufacturer's instructions. For sIgE determination, the ImmunoCAP p2 (*EgCAPIgE*) that uses hydatid fluid antigen of *E. granulosus* was employed. Compared to the WHO reference IgE standard (Pharmacia & Upjohn, product information UniCAP), concentrations are expressed semiquantitatively in six classes: 0, negative test; 1, 0.35–0.69 kU/l; 2, 0.7–3.49 kU/l; 3, 3.5–17.4 kU/l; 4, 17.5–49.9 kU/l; 5, 50–99.9 kU/l; 6, ≥ 100 kU/l.

2.3. Development of the new ImmunoCAP 'EmCAP' for detection of sIgE (*EmCAPIgE*) and sIgG (*EmCAPIgG*) in serum

Crude *E. m.* antigen was kindly provided by T. Romig (University of Hohenheim, Germany). Briefly (Bilger, 1999), metacestode tissue, passaged in *Meriones unguiculatus* by intraperitoneal transplantation, was cut into pieces of 0.5 cm² in size and pressed through a metal sieve (pore size 250 μm). The tissue was homogenized in a glass homogenizer on ice for 5 min after addition of 1% (v/v) proteinase inhibitor solution 1 (100 mM benzamidine, 100 mM ϵ -aminocaproic acid, 100 mM EDTA) and 2 (100 mM phenylmethylsulfonylfluoride). The tissue suspension was sonicated with pulsed ultrasound of 60 W for 5 min in an ice bath (Branson, Germany). The extract was clarified by centrifugation ($20\,000 \times g$, 4°C) for 20 min and stored in aliquots at -70°C . The extract

was covalently bound to ImmunoCAP solid phase by Pharmacia Research Forum. Results of sIgE detection were reported in kU/l (standard curve 0.35–120 kU/l). For semiquantitative interpretation, values were classified into the above defined ImmunoCAP classes. Concentrations of sIgG were determined quantitatively within a range of 2–200 mg/l. Cut off value of sIgG determination (9.2 mg/l) was determined by ROC analysis. The area under the ROC curve was 0.972 with a standard error of 0.017 and a 95% confidence interval of 0.903–0.996 at the cut off value of 9.2 mg/l sensitivity and specificity were 94.3% and 95%, respectively. Intra- and interassay variance for the *E. m.* ImmunoCAP, determined in three test runs each, were 0.001 and 0.024 for sIgE and 0.063 and 0.09 for sIgG, respectively.

2.4. Statistical analysis

Correlations between test results were determined by the Spearman rank correlation coefficient and differences of $P < 0.05$ were regarded as significant. 95% confidence intervals of sensitivities and specificities were calculated as suggested by Diamond (1989).

3. Results

3.1. Evaluation of the new quantitative EmCAP test

In the EmCAP test, 73.6% of patients (39/53) but none of the controls tested positive for sIgE in serum. The amount of specific antibodies in serum varied considerably (Fig. 1/II) and a median sIgE level of 1.39 kU/l was detected. Concentrations of sIgE correlated significantly with the amount of total IgE in serum ($r = 0.90$, $P < 0.0001$). The group which tested negative in the EmCAP IgE test (14/53) contained patients of different clinical disease state like patients with inactive lesions (group A, 1/3, i.e. 33.3%), patients with stable disease under chemotherapy (group B, 12/42, i.e. 28.6%) but also progressive cases (group C, 1/8, i.e. 12.5%).

In the new ImmunoCAP test not only sIgE but also sIgG can be determined. Here, 94.3% (50/53) of patients tested positive (Fig. 1/III). Several of cured patients (group A, 2/3) and only some of group B patients (3/42, 7.1%) showed negative sIgG levels, sIgG was detectable in all progressive patients. Of those three patients who tested negative in the EmCAP IgG one also had sIgE levels

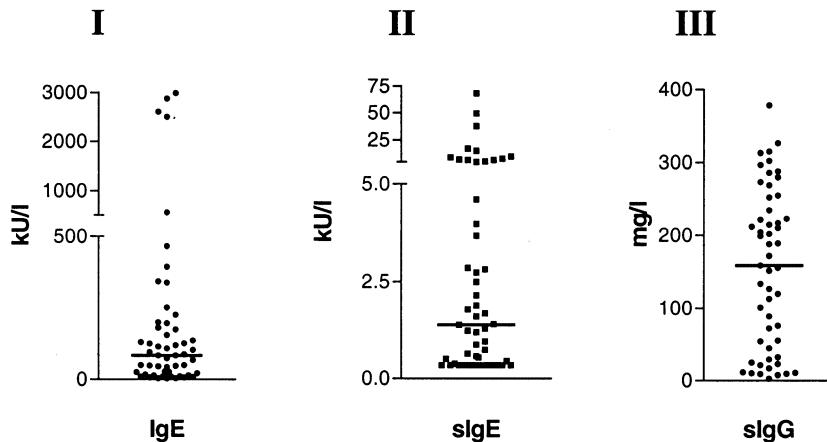


Fig. 1. Distribution of total and specific IgE and specific IgG in serum of AE patients. Concentrations of total IgE (Fig. 1/I), *E. multilocularis*-specific IgE (sIgE, Fig. 1/II) and *E. multilocularis*-specific IgG (sIgG, Fig. 1/III), determined by the newly developed ImmunoCAP assay, in 53 AE patients are shown. The median is marked.

below the detection limit. No statistical correlation between sIgG and sIgE ($\rho = 0.36$) was found.

3.2. Comparison of the new EmCAPIgE with the commercial EgCAPIgE

In the EgCAPIgE, positive results were obtained in 61.5% of patients (32/52) and none of the controls. Comparable to the EmCAPIgE, significant correlation between the EgCAPIgE class and total IgE in serum was found ($r = 0.77$, $P < 0.0001$). In order to determine congruency between the commercial and the new ImmunoCAP test, association between EgCAPIgE and EmCAPIgE was investigated. Thereby, significant positive correlation ($r = 0.84$, $P < 0.0001$) between both tests was found.

4. Discussion

Increased levels of IgE in serum are a characteristic feature of helminth infections (King et al., 1993). In AE, IgE levels are frequently enhanced and a correlation between IgE level and diseases progression has been assumed (Vuitton et al., 1988; Ammann and Eckert, 1996; Dreweck et al., 1997; Wellinghausen et al., 1999). Measurement of IgE may thus be valuable as prognostic marker in the follow-up of patients.

In contrast to total IgE, parasite-specific IgE (sIgE) more closely reflects immunologically relevant immunoglobulin in AE. Thus it might be a candidate for evaluation of disease progression. According to earlier studies, sIgE is detectable in 50–99% of AE patients by RAST or ELISA using *E. multilocularis* antigen isolated from infected rats (Ito et al., 1977), mice (Löscher, 1983), or bovis (Gottstein et al., 1984). Levels of sIgE seem to be related to disease state and to decrease after successful therapy of AE (Löscher, 1983; Gottstein et al., 1984; Dreweck et al., 1997). However, above mentioned studies were done by different non-commercial immunoassays and comparability is therefore limited. Since the only commercially available immunotest for determination of sIgE is composed of *E. granulosus* hydatid

antigen, we applied this assay principle for production of a new immunotest that uses crude *E. multilocularis* antigen. The 'ImmunoCAP' system represents an established method for quantification of specific IgE antibodies and bears several advantages like quantitative interpretation, low detection limit, simple handling, automatization of the analytical process and flexibility in the number of samples. Intra- and inter-assay variances of less than 0.07 and 0.1, respectively, confirmed a satisfactory test validity in our study. In the newly developed EmCAPIgE, a sensitivity of 74% was achieved which was even higher than that of the EgCAPIgE (62%). Patients who tested negative in the EmCAPIgE included patients of all clinical groups but percentage of negative patients was lowest in the group of progressive patients. Furthermore, significant correlation between sIgE in the EgCAPIgE and the EmCAPIgE test have been found. Despite a positive correlation between IgE and sIgE in both tests, calculation of a specific sIgE/IgE ratio did not prove valuable (data not shown). Apart from the above mentioned characteristics the ImmunoCAP system has the advantage that not only IgE and sIgE but also sIgG can be determined in the same assay during one analytical step. Quantitative determination of sIgG might have prognostic value since correlation of sIgG with the clinical course of AE has been suggested earlier (Knobloch et al., 1985). In the present study, sIgG could be detected in 87% and there was a tendency to higher frequency of positive patients in the progressive compared to the cured patients.

Despite the advantages of the new immunoassay, all *E. multilocularis* crude antigens have a certain level of unspecificity (Romig et al., 1999) and, therefore, the assay may be further improved by using purified or recombinant antigens (Gottstein et al., 1991; Helbig et al., 1993; Ito et al., 1999) for coating of the ImmunoCAPs. Thereby, charge variability of the antigen preparation would be minimized and supply of antigen material in sufficient quantities, which is a limiting factor of crude antigen, would be ensured. Furthermore, the specificity of the test has to be evaluated in a larger study with adequate control populations, like patients with CE and other par-

asitic diseases. Unfortunately, there was only available a limited antigen test charge of the new assay and, therefore, adequate controls to determine specificity could not be considered in the present study. Nevertheless, this study confirms that the established ImmunoCAP system provides a valuable tool for the production of new *E. multilocularis*-specific immunotests. Such new tests are essential for evaluation of the clinical significance of sIgE in the follow-up of AE patients and the immunological role of sIgE and sIgG in basic research studies.

Acknowledgements

We gratefully thank G. Burow for helpful co-operation with Pharmacia & Upjohn Diagnostics, Germany, and P. Gebert for excellent technical assistance.

References

- Ammann, R.W., Eckert, J., 1996. Cestodes, Echinococcus. Gastroent. Clin. North Am. 25, 655–689.
- Bilger, B., 1999. Identifizierung und Charakterisierung von Antigenen der frühen Infektionsstadien von *Echinococcus multilocularis*. Grauer, Stuttgart.
- Diamond, G.A., 1989. Limited assurances. Am. J. Cardiol. 63, 99–100.
- Dreweck, C.M., Lüder, C.G.K., Soboslay, P.T., Kern, P., 1997. Subclass-specific serological reactivity and IgG4-specific antigen recognition in human echinococcosis. Trop. Med. Int. Hlth 8, 779–787.
- Gottstein, B., 1992. *Echinococcus multilocularis* infection: immunology and immunodiagnosis. Adv. Parasitol. 31, 321–380.
- Gottstein, B., Hemphill, A., 1997. Immunopathology of *Echinococcosis*. In: Freedman, D.O. (Ed.), Immunopathogenic Aspects of Disease Induced by Helminth Parasites. In: Chem. Immunol. vol. 66. Karger, Basel, pp. 177–208.
- Gottstein, B., Eckert, J., Woodtli, W., 1984. Determination of parasite-specific immunoglobulins using the ELISA in patients with echinococcosis treated with mebendazol. Z. Parasit. 70, 385–389.
- Gottstein, B., Schantz, P.M., Wilson, J.F., 1985. Serological screening for *Echinococcus multilocularis* infections with ELISA. Lancet 1, 1097–1098.
- Gottstein, B., Mesarina, B., Tanner, I., Amman, R.W., Wilson, J.F., Eckert, J., Lanier, A., 1991. Specific cellular and humoral immune responses in patients with different long-term courses of alveolar echinococcosis (infection with *Echinococcus multilocularis*). Am. J. Trop. Med. Hyg. 45, 734–742.
- Helbig, M., Frosch, P., Kern, P., Frosch, M., 1993. Serological differentiation between cystic and alveolar echinococcosis by use of recombinant larval antigens. J. Clin. Microbiol. 31, 3211–3215.
- Ito, K., Horiuchi, Y., Kumagai, M., Ueda, M., Nakamura, R., Kawanishi, N., 1977. Evaluation of RAST as an immunological method for diagnosis of multilocular echinococcosis. Clin. Exp. Immunol. 28, 407–412.
- Ito, A., Ma, L., Schantz, P.M., Gottstein, B., Liu, Y.-H., Chai, J.-J., Abdel-Hafez, S.K., Altintas, N., Joshi, D.D., Lightowlers, M.W., Pawlowski, Z.S., 1999. Differential serodiagnosis for cystic and alveolar echinococcosis using fractions of *Echinococcus granulosus* cyst fluid (Antigen B) and *E. multilocularis* protoscolex (Em18). Am. J. Trop. Med. Hyg. 60, 188–192.
- Jenne, L., Kilwinski, J., Scheffold, W., Kern, P., 1997. IL-5 expressed by CD4⁺ lymphocytes from *Echinococcus multilocularis*-infected patients. Clin. Exp. Immunol. 109, 90–97.
- King, C.L., Low, C.C., Nutman, T.B., 1993. IgE production in human helminth infection. J. Immunol. 150, 1873–1880.
- Knobloch, J., Biedermann, H., Mannweiler, E., 1985. Serum antibodies in patients with alveolar echinococcosis before and after therapy. Trop. Med. Parasitol. 36, 155–156.
- Löschner, T., 1983. Der Radioallergosorbenttest (RAST) in der Diagnostik und Therapiekontrolle der Echinokokkose. Abstract No. 80, Meeting Tropenmed. Gesellschaft, Garmisch-Partenkirchen, 21–23.04.1983.
- Romig, T., Kratzer, W., Kimmig, P., Frosch, M., Gaus, W., Flegel, W.A., Gottstein, B., Lucius, R., Beckh, K., Kern, P., 1999. An epidemiological survey of human alveolar echinococcosis in southwestern Germany. Am. J. Trop. Med. Hyg. 61, 566–573.
- Vuitton, D.A., Bresson-Hadni, S., Lenys, D., Flausse, F., Liance, M., Wattre, P., Miguët, J.P., Capron, A., 1988. IgE-dependent humoral immune response in *Echinococcus multilocularis* infection: circulating and basophil-bound specific IgE against *Echinococcus* antigens in patients with alveolar echinococcosis. Clin. Exp. Immunol. 71, 247–252.
- Wellinghausen, N., Jöchle, W., Reuter, S., Flegel, W.A., Grünert, A., Kern, P., 1999. Zinc status in patients with alveolar echinococcosis is related to disease progression. Parasite Immunol. 21, 237–241.