Diagnostic Value of a Synthetic Peptide Derived from *Echinococcus granulosus* Recombinant Protein

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Abstract

A specific monoclonal antibody (MAb; EG 02 154/12) directed against a protein epitope of *Echinococcus granulosus* antigen 5 was used to screen a cDNA library constructed from *E. granulosus* protoscoleces RNA. One clone designated Eg14 was selected and shown to code for an amino acid sequence partially homologous to that of the clone Eg6 previously identified with the same MAb. Hydrophobic cluster analysis showed that both recombinant antigens may adopt a similar α-helical organization and share a common conformational epitope. A synthetic peptide (89–122) mimicking the conformational site of Eg6 and Eg14 was constructed and demonstrated to be able to inhibit binding of the MAb and human hydatid sera to the Eg6 fusion protein (FP6) or to native hydatid antigens. To assess the diagnostic value of the peptide 89–122, we tested sera from patients infected with different parasites for their antibody reactivity with this peptide in ELISA. A high binding sensitivity and specificity of IgG-A-M antibodies were obtained with *E. granulosus*–infected patient sera. Moreover, the peptide 89–122 was found to be specifically recognized by IgE antibodies from patients with hydatid disease. These results indicate the particular interest of this synthetic peptide as a standardized antigen in diagnosis and treatment surveillance of hydatidosis. (*J. Clin. Invest.* 1992. 89:458–464.) Key words: α-helical organization • hydrophobic cluster analysis • immunodiagnosis of hydatidosis • monoclonal antibody • recombinant protein • synthetic peptide

Introduction

Hydatidosis, caused by the larval stage of the tapeworm *Echinococcus granulosus,* is one of the major public health problems in many areas of the world (1). Because the symptoms vary according to the location of the parasite and its rate of growth, the clinical diagnosis of hydatidosis in humans is often very difficult. The need therefore exists for immunodiagnostic procedures, both for the detection of cases for early treatment and the evaluation of progress in hydatidosis control program.

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Among hydatid cyst fluid antigens (HCF Ag)[1] used in diagnosis of hydatid disease, antigen 5 has been shown to contain epitope(s) with high specificity to *E. granulosus* (2). Hydatidosis is associated with immediate-type hypersensitivity (3, 4) and patients with living *E. granulosus cysts* were found to have a higher concentration of IgE antibodies to HCF Ag. Among these, antigen 5 is the major one combining with reaginic antibodies (5). Also, the evaluation of specific IgE in hydatidosis is considered as useful addition to the classical serodiagnosic tests (5, 6).

Despite important progress made in the improvement of sensitivity of various diagnostic methods, the variability of the source of HCF Ag as well as the fact that HCF can contain host serum components remain the two major problems of effective diagnosis. This stresses the need for a standardized antigen such as a defined *E. granulosus* gene product. We have previously described (7) the specificity of protein epitope(s) of *E. granulosus* antigen 5 defined by a mouse monoclonal antibody (MAb; EG 02 154/12) and recognized by human hydatid sera. Recently, we reported the molecular cloning of an *E. granulosus* protein expressing a specific epitope of antigen 5 (8). The Eg6 fusion protein (FP6) was recognized by EG 02 154/12 MAb and human hydatid sera but few sera from patients infected with other cestodes displayed cross-reactivity with the cloned protein.

Here, we report the molecular cloning and sequencing of a second *E. granulosus* protein identified by EG 02 154/12 MAb. Comparative analysis of the structure of both fusion proteins indicated that they had several common features: a high helical propensity, a similar repartition of hydrophobic residues, strongly evocative of α-helical coiled coil structure, and a common topographical pattern that could explain their common reactivity with the EG 02 154/12 MAb. These data prompted us to select and synthesize a peptide able to mimic the organization and antigenicity of the recombinant antigens. Finally, human antibody response to this peptide was evaluated with the aim to assess its diagnostic value.

Methods

Parasite antigens and sera

HCF was aseptically aspirated from cysts of *E. granulosus* collected from sheep livers and lungs, centrifuged at 40,000 *g,* dialyzed against distilled water, and then lyophilized. Protoscoleces were recovered by sedimentation, washed in 0.15 M NaCl, frozen, and stored in liquid nitrogen. Patient sera were obtained from the diagnosis laboratory of

1. *Abbreviations used in this paper:* CD, circular dichroism; FP6, fusion protein 6; HCF Ag, hydatid cyst fluid antigens; LSA, liver stage antigen; TFA, trifluoroacetic acid.

the Pasteur Institute, Lille, France. Diagnosis was confirmed by parasitological and serological examination. Mouse IgG_1 MAb EG 02 154/12 raised against antigen 5 was previously characterized (7).

λgt11 expression library

Using a modified method of Chirgwin et al. (9), total RNA was isolated from *E. granulosus* protoscoleces as previously described (8). Double-stranded cDNA was synthesized from 300 μg of total RNA (10) and cloned into the EcoRI site of the λgt11 vector (8). The expression library was screened with EG 02 154/12 MAb. After incubation with horseradish peroxidase–conjugated anti–mouse IgG (Diagnostic Pasteur, Marnes-la-Coquette, France), staining was performed with 4-chloro-1-naphtol reagent.

Nucleotide sequencing

cDNA inserts were subcloned in the M13 mp18 vector and both DNA strands were sequenced by the dideoxy chain termination reaction of Sanger et al. (11) using dye-labeled primers (12). Fluorescence of DNA fragments was detected by a model 370A DNA sequencer (Applied Biosystems, Inc., Foster City, CA). The amino acid comparison was made by using Swissprot (Geneva, Switzerland) and National Biomedical Research Foundation (Washington, DC) protein sequence database.

Western blotting

Y1089 lysogenic bacteria were selected on the basis of their temperature sensitivity and bacterial extracts were analyzed in SDS-PAGE under reducing conditions (13). Western blotting was performed according to Towbin et al. (14).

Peptide synthesis

Peptides were synthesized by a stepwise solid-phase method (15) in an automated peptide synthesizer (model 430A, Applied Biosystems, Inc.), according to the butyloxycarbonyl/trifluoroacetic acid (TFA) protocol, on a benzhydrylamine resin (Applied Biosystems, Inc.). Trifunctional amino acids were protected as follows: Arg (*p*-toluenesulfonyl [Tos]), Asp (*O*-benzyl [OBzl]), Glu (OBzl), Lys (2-chlorobenzyloxycarbonyl [2-Clz]), Ser (OBzl). Amino acids were introduced using symmetric anhydride activation in dimethylformamide (DMF) (single coupling), except for Gln, which was introduced using the dicyclohexylcarboiimide/hydroxybenzotriazole activation protocol. At the end of the synthesis, the amino-terminal protecting Boc group was removed, and the peptide was acetylated using acetic anhydride. Final deprotection and cleavage of the peptidyl resins was by high hydrogen fluoride (HF) procedure, for 1 h at 0°C. The cleaved deprotected peptide was precipitated with cold diethylether and then dissolved in 5% acetic acid and lyophilized. Crude peptide was purified by gel filtration (TSK HW40S, Merck, Darmstadt, FRG) followed by reversed-phase HPLC on a 5 μm, 300Å Nucleosil C18 (Macherey Nagel, Düren, FRG) column (0.5 in × 500 mm), using a very resolutive 3-h gradient from buffer A1 (0.05% TFA in water) to 60% buffer B1 (0.05% TFA/75% acetonitrile/25% water), flow rate 2 ml/mn. The hydrochloride form of the peptide was obtained using a step gradient procedure from buffer A2 (pH 3 HCl in water) to buffer B2 (pH 3 HCl 50%/isopropanol 50%), on a 5 μm, 300Å Nucleosil C18 column (0.5 in × 75 mm), flow rate 2 ml/mn. Peptide was checked for homogeneity by analytical reversed-phase HPLC on a 5 μm Vydac C18 column (¼ in × 250 mm), flow rate 0.7 ml/mn. The peptide was eluted as a single sharp peak. Detection was by absorbance at 215 nm.

Analytical methods

Hydrolysis of the peptide and amino acid analysis. Hydrolysis of the peptide was performed with 6N HCl/phenol (10:1) at 110°C for 24 h in evacuated sealed tube. Amino acids were quantified on an amino acid analyzer (model 7 300, Beckman Instruments, Inc., Palo Alto, CA) with ninhydrin detection. The expected amino acid composition was observed.

Molecular mass determination. Plasma desorption mass spectrum was recorded on a ^{252}Cf fission fragment ionization, time-of-flight mass spectrometer (Bio-Ion, Uppsala, Sweden). Approximately 10 μg of peptide were deposited onto a nitrocellulose-backed aluminized foil. The spectrum was accumulated during 10^6 fission events (*m/z* observed, 3898.3; *m/z* expected 3898.4).

Circular dichroism (CD) studies

CD spectra were recorded on a 185 Model II (Roussel Jouan, Longjumeau, France) at room temperature. Peptide concentrations were adjusted from titration of stock solutions by quantitative amino acid analysis after total acid hydrolysis. CD studies were performed on the hydrochloride form of the peptide, 10 mM in NaCl 200 mM in cell path of 0.1 mm, or 1 mM in trifluoroethanol M in cell path of 1 mm. Circular dichroism results were reported in terms of mean residue ellipticity (θ) expressed in deg $dmol^{-1}$. cm^2. Helix contents were calculated from the CD spectra, taking $(\theta)_{222} = -35{,}700$ deg $dmol^{-1}$. cm^2 for 100% helicity.

ELISA and inhibition tests

Plates (Nunc, Copenhagen) were coated with 100 μl of HCF Ag or lysogenic bacterial extract (FP6) at 5 μg/ml of proteins. Human sera were used at 1:100 dilution and bound antibody was detected with horseradish peroxidase–conjugated anti–human IgG-A-M (Diagnostic Pasteur). For inhibition experiments, human sera or EG 02 154/12 MAb were preincubated overnight at 4°C with different concentrations of synthetic peptides. IgE ELISA was performed with human sera diluted at 1:10 in PBS and the peptide 89–122 was coated at 1 μg per well. Mouse IgG_1 anti–human IgE was purchased from Southern Biotechnology Associates, Inc., Birmingham, AL. Values (means of duplicates) were expressed in optical density (OD) at 492 nm±standard deviation (SD).

Results

Selection and sequence analysis of cDNA clones recognized by the MAb EG 02 154/12. The specific MAb to antigen 5 was used to screen the cDNA library constructed from *E. granulosus* protoscoleces RNA. In addition to the Eg6 clone previously characterized (8), a second clone designated Eg14 was selected and further studied. The Eg14 clone contained a DNA insert of 137 bp and exhibited one open reading frame, in phase with the λgt11, encoding for 37 amino acids. Both sequences were represented using the hydrophobic cluster analysis plot described by Lemesle-Varloot et al. (16) (Fig. 1, *B* and *C*). This method, together with more conventional methods for the prediction of secondary structure, suggest an organization in the form of helical stretches. The longitudinal distribution of the hydrophobic cluster along the helical plot is strongly evocative of an α-helical coiled-coil structure, such as the well known rabbit myosin heavy chain of skeletal muscle (Fig. 1 *A*).

Beside these common structural properties, the Eg14 and Eg6 clones showed limited sequence homologies (Fig. 2). However, on the graphic representation, these discontinuous sequence homologies formed two topographical patterns, present on both fragments, that could explain the cross-reactivity observed with the EG 02 154/12 MAb. In addition, the high proportion of charged or polar residues in these homologous structures could account for their presence in predominantly exposed areas on the native antigen.

Selection of a synthetic peptide antigen. Helical organization can be conveniently mimicked by long peptides (17). We therefore selected and synthesized a 34-amino acid peptide, copying the Eg6 clone sequence from amino acids 89 to 122

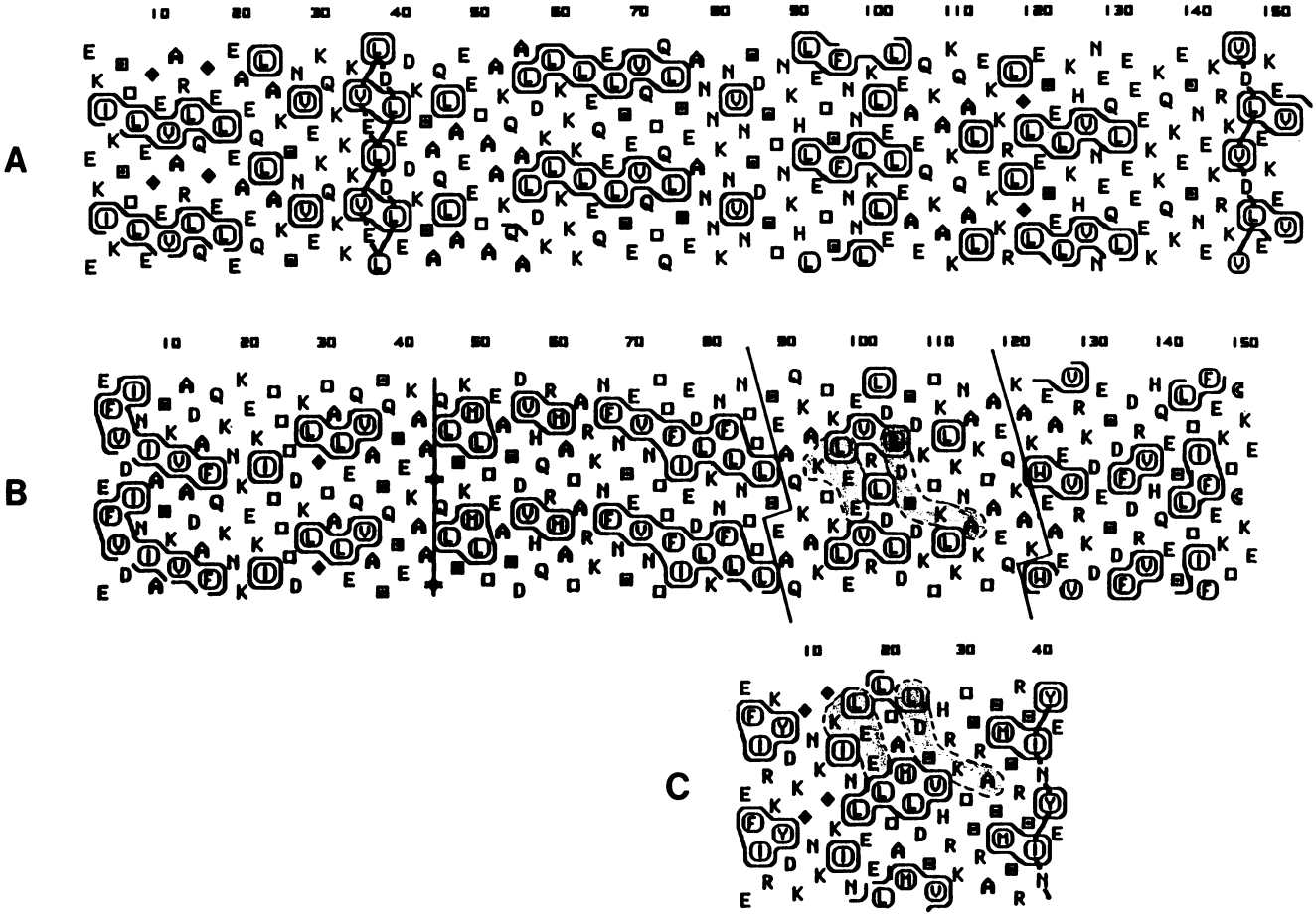

Figure 1. Hydrophobic cluster analysis plot of rabbit myosin heavy chain of skeletal muscle (*A*), Eg6 (*B*), and Eg14 (*C*) *Echinococcus granulosus* recombinant proteins. Adjacent hydrophobic residues are contoured. Some residues are represented by symbols: P (★), G (♦), T (□), S (⊡). The homology of topographical pattern between Eg6 and Eg14 is shaded.

(Fig. 2) and encompassing both homologous patches (Fig. 1 *B*). In order to introduce stabilizing charge interactions between extremities of this peptide designated 89–122 and the helix dipole (18), the amino-terminal end was a negatively charged glutamic residue while the carboxyl-terminal end was a positively charged lysine residue.

Helicity of peptide 89–122. The helical organization of the peptide was assessed by circular dichroism studies, performed at room temperature, in aqueous or aqueous-trifluoroethanol solution. Results presented in Fig. 3 showed that both spectra were characteristic of a significant helical organization, with minima occurring at 202–207 and 222–223 nm, and a positive value near 190 nm. Taking $\theta_{222} = -35{,}700$ deg cm^2/dmol for 100% helicity, we calculated a 14% helix content in aqueous medium, and a 74% helix content in aqueous-trifluoroethanol medium.

Binding inhibition of EG 02 154/12 MAb and human hydatid sera to FP6 or to HCF Ag by a synthetic peptide. The peptide 89–122 was used in ELISA inhibition experiments to assess its capacity to inhibit binding of the MAb and human hydatid sera to FP6 or to HCF Ag. As shown in Fig. 4 *A*, strong inhibitions of binding of the MAb (80%) and human hydatid sera (60%) to FP6 were obtained with the peptide 89–122. This structure was also able to significantly inhibit the binding of these antibodies to HCF Ag (65% and 45% respectively) (Fig. 4 *B*). The binding inhibition was more effective for the MAb than for human hydatid sera. No significant inhibition was observed with an unrelated helix-forming (41 amino acids) synthetic peptide (19) corresponding to the *P. falciparum* liver stage antigen (LSA) (20).

These results suggested that the peptide 89–122 was able to mimic binding site(s) recognized specifically by the MAb and human hydatid sera. Further study of human sera reactivity to this peptide was thus undertaken.

Human antibody response to the synthetic peptide 89–122. The reactivity of sera from patients infected with *E. granulosus*

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1   EFIRKYDKGNKGKINLEELTAMLDSVHRKTSRASMSR          37   Eg 14
        *  *  ***     ***     *     *
80  FLNTTLSEAQKAKTKLEEVRLDLDSDKTKLKNAKTAEQKAK     122  Eg 6
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Figure 2. Amino acid sequence of Eg14 and homology with clone Eg6. The identical amino acids are indicated by asterisks. Nucleotide sequence data of the Eg14 clone have been submitted to the Genbank with the accession number M74034.

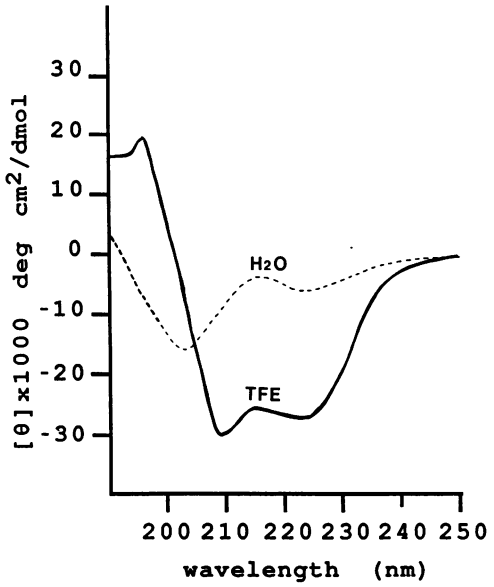

Figure 3. Circular dichroism spectra of the peptide 89–122 in water and in trifluoroethanol (*TFE*).

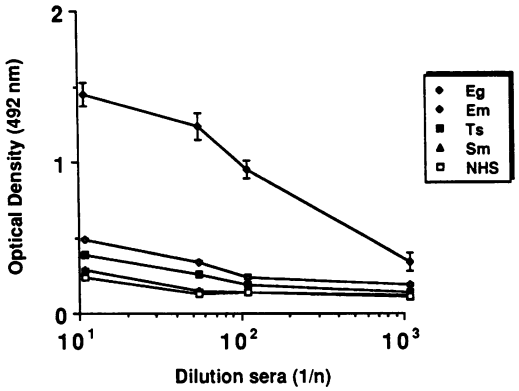

Figure 5. Reactivity of IgG-A-M of various human sera with the synthetic peptide 89–122 used as a solid-phase antigen in ELISA. Results represent a mean±SD of four different human sera: anti-*E. granulosus* (*Eg*), anti-*E. multilocularis* (*Em*), anti-*T. saginata* (*Ts*), anti-*S. mansoni* (*Sm*), and normal human sera (*NHS*).

or other parasites to the peptide 89–122 used as a solid phase antigen was evaluated by ELISA experiments. Using different dilutions of sera, results in Fig. 5 show that a significant antibody binding occurred only with anti-*E. granulosus* sera. Binding levels of anti-*E. multilocularis,* anti-*T. saginata,* and anti-*S. mansoni* sera were lower as were that of normal human sera. Tested against an unrelated synthetic peptide (LSA), all these sera gave a similar low level of background (not shown).

ELISA experiments were also performed to assess the immunodiagnostic value of the peptide 89–122. Two types of human antibody response (IgG-A-M and IgE) were analyzed. Using the upper limit of positive values as three standard deviations above the mean of the control group, a high binding sensitivity (85%) and a good specificity (86%) were observed in IgG-A-M response analysis (Fig. 6). When we compared these results to that obtained with FP6 in Western blotting (Table I), we could observe an improvement of the specificity indicating the particular interest of the peptide 89–122 for diagnosis of hydatid disease.

More interestingly, IgE response to the peptide 89–122 showed a high specificity (100%) for *E. granulosus*-infected patients (Table I). No IgE reactivity could be detected with 52 sera from other parasitic diseases whereas 60% of human hydatid sera (24/40) were positive in this IgE ELISA. Using an unrelated synthetic peptide (LSA) as a control, no significant reactivity was obtained in both ELISAs (not shown).

The sensitivity and specificity of this test were compared to IgE ELISA performed with whole HCF Ag (Fig. 7). The reactivity of IgE to the peptide 89–122 seems to be more specific (100%) for *E. granulosus* while 32% (6/19) of cestode-infected sera cross-reacted with HCF Ag. IgE antibodies from 29 of 40 (72%) *E. granulosus*-infected patient sera were found to react with HCF Ag. The sensitivity of IgE ELISA observed with the peptide 89–122 (60%) remained important in comparison with that obtained with native hydatid antigens.

Discussion

Immunodiagnosis of hydatidosis has been based, during the last two decades, on the detection of circulating antibodies to antigen 5, the major immunogen of *E. granulosus* (2, 21, 22). With the development of new tests with increased sensitivity, there remains the possibility of false-positive reactions assigned mainly to host serum components that could pass through cyst membranes (23–25). Molecular characterization of *E. granulosus* antigens could contribute to the resolution of such problems.

We have previously demonstrated a particular immunogenicity and specificity of a protein epitope on the native antigen 5 (7). Binding of human antibodies from patients with hydatid

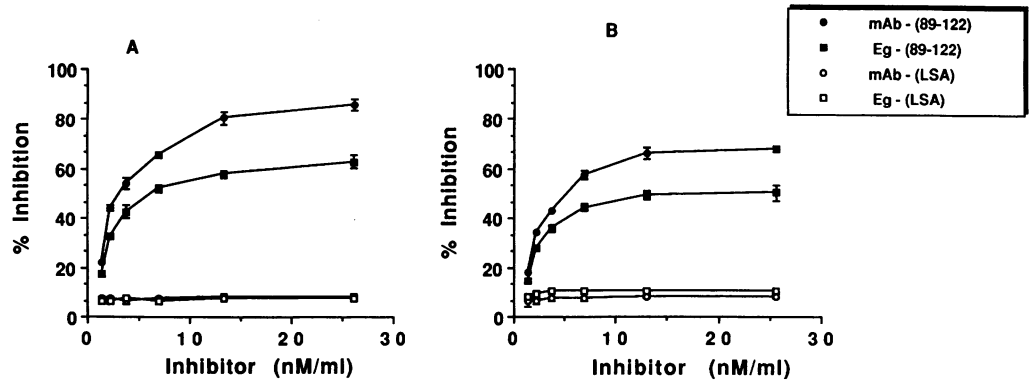

Figure 4. Binding inhibition of EG 02 154/12 MAb and human hydatid sera (Eg) to the fusion protein FP6 (*A*) or to hydatid cyst fluid antigens (*B*) by the synthetic peptide 89–122. A peptide corresponding to the LSA of *P. falciparum* was used as a control.

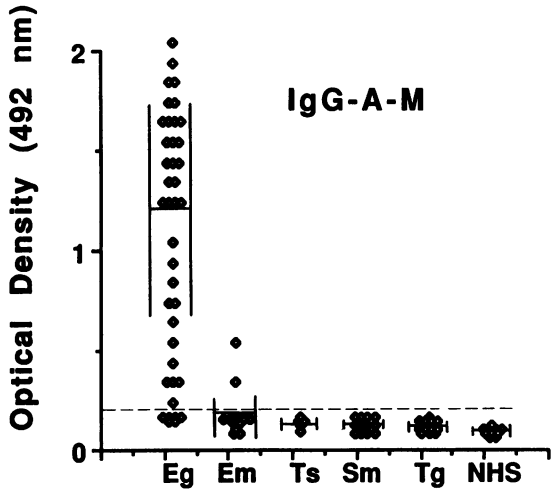

Figure 6. Distribution of IgG-A-M ELISA reactivity of *E. granulosus (Eg)*, *E. multilocularis (Em)*, *T. saginata (Ts)*, *S. mansoni (Sm)*, *T. gondii (Tg)*–infected patient sera and normal human sera (*NHS*) with the peptide 89–122. Dashed line represents the cutoff value calculated as the mean of the control sera (unexposed individuals living in France; $n = 10$) +3 SD.

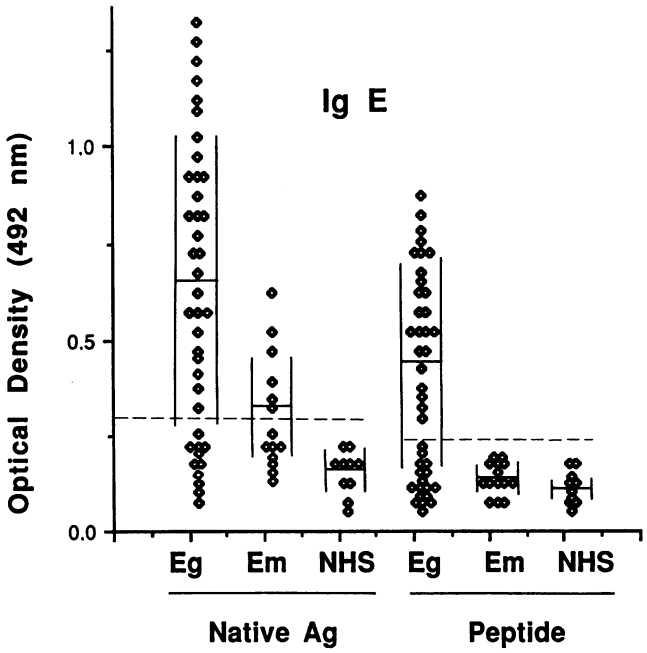

Figure 7. Distribution of IgE ELISA reactivity of *E. granulosus* (*Eg*), *E. multilocularis* (*Em*), *T. saginata* (*Ts*)–infected patient sera and normal human sera (*NHS*) with the peptide 89–122 and HCF Ag (*Native Ag*). Values above the dashed line, corresponding to the cutoff value (mean of the 10 control sera + 3 SD), were considered as positive.

disease to this epitope is specifically inhibited by EG 02 154/12 MAb raised against the antigen 5. With the aim of producing a recombinant antigen for diagnosis of hydatid disease, we have isolated and characterized an *E. granulosus* recombinant protein expressing an immunogenic epitope target of human hydatid sera and EG 02 154/12 MAb (8).

Further screening of the cDNA library with the MAb allowed the isolation of a second clone (Eg14). The latter has no homology with the two known cloned *E. granulosus* antigens that may be involved in protecting the parasite from the host immune system (26, 27). Also, no significant homology can be observed with other published sequences. However, when compared to Eg6, Eg14 clone showed a limited amino acid sequence homology.

Recent work regarding assessment of secondary and tertiary structure of proteins from their amino acid sequence have proved that hydrophobic cluster analysis is a very efficient method of analyzing and comparing protein sequences (28). This method has been applied to obtain structural and functional information for a large number of proteins (16). Using this procedure, Eg6 and Eg14 clones identified by EG 02 154/12 MAb showed a similar repartition of hydrophobic clusters, strongly evocative of α-helical coiled-coil organization. A common discontinuous epitope formed by both sequences could explain their reactivity with the same MAb. According to the recent report of Gras-Masse et al. (17), helical organization of synthetic peptides play an important role in their immunogenicity and antigenicity. Despite the large number of cloned parasite antigens, very little is known regarding immunogenic and antigenic properties of conformational peptides from parasites. Such a study has been recently attempted with *P. falciparum* LSA (19, 20).

As verified by circular dichroism studies performed in aqueous and trifluoroethanol solutions, the peptide 89–122

Table I. Sensitivity and Specificity of Peptide ELISA (IgG-A-M and IgE) and Western Blotting Performed with the Fusion Protein (FP6) Using Different Human Sera

		ELISA of peptide 89–122			
Western blotting of FP6 (IgG-A-M)		IgG-A-M–positive sera		IgE–positive sera	
Human sera	No. (%) positive	No. (%)	OD(492 nm) Mean±SD	No. (%)	OD(492 nm) Mean±SD
Hydatidosis ($n = 40$)	34 (85)	34 (85)	1.18±0.52	24 (60)	0.7±0.24
Alveolar echinococcosis ($n = 14$)	4 (28.5)	2 (14)	0.4±0.14	0 (0)	—
Taeniasis (*T. saginata*) ($n = 5$)	1 (20)	0 (0)	—	0 (0)	—
Schistosomiasis ($n = 18$)	2 (11)	0 (0)	—	0 (0)	—
Toxoplasmosis ($n = 15$)	0 (0)	0 (0)	—	0 (0)	—
Normal human sera ($n = 10$)	0 (0)	0 (0)	—	0 (0)	—

was actually able to adopt an helical organization. Furthermore, the peptide 89–122 was first tested in ELISA for its ability to inhibit the binding of the EG 02 154/12 MAb to the fusion protein Eg6 or to the native HCF Ag. The strong inhibition capacities observed confirmed both the hypothesis of a correct folding of the peptide during interaction with antibodies and the occurrence, within its 34 amino acids, of a major epitope recognized by the MAb.

When used as inhibiting antigen towards the binding of human hydatid sera to the native HCF Ag, the peptide 89–122 was able to inhibit as much as 50% of the reaction. This result could be explained by the existence of several copies of identical, or closely related, cross-reactive epitopes in the native antigen that are mimicked by the synthetic peptide. This explanation is in agreement with the structural hypothesis of a periodic, myosin-related structure. Indeed, the observed ability of the EG 02 154/12 MAb to precipitate antigen 5 in immunoelectrophoresis (7) provide an additional argument in support of this hypothesis.

We have previously reported (8) that the capacity of human hydatid sera to recognize FP6 in Western blotting is correlated with their ability to precipitate antigen 5 in immunoelectrophoresis. Also, human antibodies eluted from FP6 could bind to one subunit of antigen 5 (8). Testing the same sera for their reactivity in IgG-A-M ELISA with a peptide 89–122 derived from FP6, we have found the same correlation. Moreover, the cross-reactions observed with FP6 have decreased with the use of a peptide 89–122 in ELISA. This synthetic peptide appears to mimic probably one of the specific epitopes of antigen 5.

The isotypic profile of human antibody response to HCF Ag has been shown to have an interesting prognostic role in hydatidosis (29–31). According to Dessaint et al. (5), a higher concentration of specific IgE antibodies is detected in patients with living *E. granulosus* cysts. The IgE level seems to decrease with the loss of viability of the cysts after treatment of hepatic hydatid disease with mebendazole (32). In view of this important IgE response, the immediate-type hypersensitivity test, also known as the Casoni test (33), has contributed to the immunodiagnosis of hydatidosis (3, 4, 34), although, the use of whole HCF Ag leads to false-positive reactions with other helminthiasis (35, 36). Among *E. granulosus* antigens identified as allergens (37–39), antigen 5 is a major antigen reacting with reaginic antibodies (5). Using the peptide 89–122 as a solid-phase antigen in ELISA, we could demonstrate that IgE antibodies from 60% of hydatid patient sera bound specifically to this peptide. Such sensitivity remained important in comparison with that obtained with HCF Ag (72%). These data suggest that the peptide 89–122 may represent one major hydatid allergen binding-site recognized by reaginic antibodies from hydatid patient sera.

The use of this peptide as a standardized antigen in immediate-type hypersensitivity test should be of value for the diagnosis of hydatidosis both in humans and animals. Such a test may prove a useful adjunct to detection of specific antibody against this defined peptide in serodiagnosis and treatment surveillance.

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