

Cloning and expression of a cDNA encoding an elongation factor 1 β/δ protein from *Echinococcus granulosus* with immunogenic activity

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SUMMARY

A cDNA clone (Eg EF-1 β/δ) of *Echinococcus granulosus* has been isolated by an expression library screened with immunoglobulin (Ig)E of sera from patients with cystic echinococcosis (CE). The Eg EF-1 β/δ was identified on the basis of sequence homology to the subunits β or δ of the elongation factor-1. The amino acid sequence deduced from this open reading frame is 244 residues long with a predicted molecular mass of 31 kDa. In Southern blot under high stringent condition, Eg EF-1 β/δ hybridized to genomic DNA of *E. granulosus* at two bands of 4 and 2.5 Kb. In immunoblotting analysis, the Eg EF-1 β/δ protein shows immunological reactivity with sera from CE patients: 51.7% of sera contained IgE, 41.7% IgG and 18.3% IgG4 specific to the recombinant protein. We identify the Eg EF-1 β/δ by immunoblotting with specific monoclonal antibody both in protoscoleces and in sheep hydatid fluid. The higher percentage of humoral immune response against Eg EF-1 β/δ observed in CE patients with calcified cysts than in patients with active cysts indicates the possible release of the protein in the hydatid fluid after protoscoleces degeneration suggesting the possible use of this antigen in the immunosurveillance of CE. Overall, these findings seem to assign to Eg EF-1 β/δ a key role in the allergic disorders and in the complex host–parasite relationship in CE.

Keywords *E. granulosus*, elongation factor 1, IgE, immune response

INTRODUCTION

Translation elongation in eukaryotes requires translation elongation factors EF-1 and EF-2, which are functionally and structurally highly conserved among species (Riis *et al.* 1990, Bassel *et al.* 1994). Elongation factor 1, responsible for guanosine triphosphate (GTP)-dependent binding of aminoacyl-tRNAs to the ribosomes, is composed of four subunits α , β , γ and δ . The α chain binds GTP and aminoacyl-tRNAs, the γ chain seems to play a role in anchoring the complex to other cellular components and the β and δ chains stimulate the exchange of GDP bound to the α chain for GTP (Van Damme *et al.* 1991). The β and δ chains are very similar hydrophilic proteins of around 23–31 kDa; their C-terminal part seems important for the nucleotide exchange activity, while the N-terminal section is probably involved in the interaction with the γ chain. The β , γ and δ subunits of EF-1 are predominantly located in the endoplasmic reticulum region, whereas the EF-1 α is present in the cytoplasm and in the nucleus (Sanders *et al.* 1996). To our knowledge, no specific studies on the immunogenicity of elongation factors have been performed.

In this study, we describe the cloning, sequencing and expression of a cDNA encoding a protein from protoscoleces of *Echinococcus granulosus*, displaying sequence similarities with the family of elongation factor 1, in particular with homology with the β and δ subunits (Eg EF-1 β/δ). We screened the expression library with immunoglobulin (Ig)E of sera from patients with cystic echinococcosis (CE) and the recombinant protein isolated beared immunogenic activity. We used immunoblotting to study the serological reactivity of this antigen evaluating the presence of specific IgE, IgG and IgG4 in patients' sera with CE.

MATERIALS AND METHODS

Antigens

Sheep hydatid fluid was collected in Sardinia from fertile

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cysts. Protoscoleces were removed by centrifugation from sheep hydatid fluid, immediately washed several times in phosphate buffer saline and stored in 4M guanidinium thiocyanate containing 0.1M 2-mercaptoethanol and 0.025M Na-acetate (pH 6).

Blood samples

Blood samples were obtained from 60 patients with CE (45 with cysts in the liver, seven with cysts in the lungs, five with cysts in liver and lungs, two with cysts in bones and one with cysts in nervous central system), 15 subjects with atopic disorders (seven with polyspecific allergic reactions, four with monospecificity to *Dermatophagoides farinae* and four with monospecificity to *Parietaria judaica*), 15 patients with other parasitic diseases (four with schistosomiasis, four with trichinellosis, three with filariasis and four with cryptosporidiosis) and nine healthy donors. CE was clinically, serologically and ecographically diagnosed. Hydatid cysts were classified in seven different types according to Caremani *et al.* (1996). In brief, type I are simple cysts, type II are multiple cysts, type III are cysts with detachment of wall, type IV are cyst with mixed pattern, type V are heterogeneous cysts, type VI are hyperechoic cysts and type VII are calcified cysts. All procedures were approved by the local Ethical Committee, and all subjects gave their informed consent to the study.

Antisera

Murine monoclonal antibody to the recombinant Eg EF-1 β / δ protein was obtained with standard procedures. Monoclonal antihistidine antibody was purchased by Qiagen (GmbH, Germany) and used following the manufacturer's instructions. To screen the library a goat antihuman IgE peroxidase labelled serum (Cappel, Cochranville, PA, USA) was used as second antibody. Peroxidase-labelled goat antihuman total IgG (Biorad, Richmond, CA, USA), IgG4 (Oxoid Ltd, Basingstoke, UK) and IgE (Cappel) sera and peroxidase-labelled goat antimouse IgG serum (Biorad) were used as second antibodies in immunoblotting.

Construction of the cDNA library and immunoscreening

Total RNA was isolated from protoscoleces by the guanidinium thiocyanate/phenol/chloroform technique as described elsewhere (Chomczynski & Sacchi 1987). Poly(A)⁺ mRNA was extracted from total RNA by Dynabeads Oligo (dT)²⁵ magnetic beads (Dyna, Hamburg, Germany) according to the manufacturer's instructions and converted in cDNA. The cDNA library was produced

in lamda zap-2 using a λ zap cDNA synthesis kit (Stratagene, USA) according to the manufacturer's instructions. The cDNA clones isolated were screened with a pool of sera from confirmed positive CE patients showing a high positivity in a *E. granulosus* specific IgE enzyme-linked immunosorbent assay (ELISA) ($OD_{280} > 2$) (Afferni *et al.* 1984). The nucleotide sequence of the cloned cDNA insertions was determined by a Sequenase kit (Amersham, Bucks, UK). DNA and translated protein sequences were analysed by the Genetics Computer Group Sequence Analysis Software Package (Devereux *et al.* 1984). The cDNA inserts were used as probes in the standard Southern hybridization analysis. The clone containing the elongation factor-1 cDNA was named Eg EF-1 β / δ .

Subcloning, expression and purification of the recombinant protein

The selected cDNA clone was subcloned into the *Bam*HI/*Kpn*I site of the QIA express vector, pQE31. The 6X fusion protein was expressed in *Escherichia coli* SG130009 cells, purified by affinity of NI-NTA resin for the 6Xhis tag and eluted under denaturing conditions (urea) according to the supplier's (Qiagen) instruction. To remove urea before the immunization of mice the protein was dialysed in PBS for 2 days at 4°C.

Immunoblotting

Immunoblotting was performed as previously described by Siracusano *et al.* (1991) after 12.5% SDS-PAGE in reducing and in nonreducing conditions. For the immunolocalization, protoscoleces and hydatid fluid were used as antigens. When the purified recombinant Eg EF-1 β / δ (3 μ g/lane) was used as antigen, human sera were diluted 1:50 for IgG detection and 1:10 for IgE detection. The specificity of the humoral response was demonstrated by a lack of recognition of another recombinant 6X-histidine-tagged protein expressed and purified in the same manner as the Eg EF-1 β / δ protein.

RESULTS

Molecular characterization of cDNA encoding Eg EF-1 β / δ

The insert of a clone strongly reacting with the pooled human serum was analysed and was observed to contain 801 base pairs (bp) ending with a termination codon at position 733 and a poly(A)⁺ tail and a polyadenylation signal, AATAAA, starting at position 778 (Figure 1). The amino acid sequence predicted from this open reading frame is 244 residues long with a predicted molecular mass of 31 kDa and presents 39% and 38.4% identity and 53.4% and 53.7%

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tttgatgctc actcgtatca ccaatttcca aattatcacg agatggagtc 50
ggcgtagatg cggttccttt ctaaagacat aggaagaca tctgctaccg 100
aacaaaaggg ggattcttac ttggtaaagg agatacagaa agttcgcgcgac 150
aacataaagt cttcacttga aagtaatggg tctgtggaacg agcgaatgga 200
gaagctggaa gcaatcgtaa aggagatgtc tctgcgctgt aacgagttag 250
aagttcaaag ccaacgtctg ggacagattc tgccttctga atcactcaaa 300
ggtgccaac cgattaggcc tgttggtgtc aaggcaccgc cagctgaagg 350
cggtgatgac gatgatattg acctctttgg ttcggaagac gaaggcgaag 400
cggataagtg caagcaaagt atggctgagc agaataaggc agcagcatcg 450
aagaaggaaa aaccggtggc caagtcgatg attgttctgg acgtgaagcc 500
ctgggacgac accacaaaca tggcagagat ggagatgggc gtgctgcaa 550
tcactactga cggctctgctc tggggcacct ctaagctcgt ccctcttgctc 600
catggcatta ataagttgca gatcgctgt gtcgttgaag atgataaggt 650
tggtaccgac ttctcgaag agagcatcat ggagctcgag gactatgtcc 700
aatcgggtgga tgtagcttcc ttaacaaac tgtaaccata tctttctttt 750
gctccacttt gaattagctg aaagtatgaa taaagcgtat caggtttttg 800
taaaaaaa

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FDAHSYHQFPNYHEMESAYMRFLSKDIGKTSATEQKGDSYLVKEIQKVRDNIKS 54
SLESNGSWNERMEKLEAIVKEMSLRVNELEVQIQRLGQILPSESLKGAKPIRPV 108
VVKAPPAEGGDDDDIDLFGSEDEGEADKCKQMMAEQNKAASKKEKPVAKSMIV 162
LDVKPWDDTTNMAEMEKGVRAITADGLLWGTSKLVPLVHGINLQIACVVEDDK 216
VGTD FLEESIMELEDYVQSVDVASFNKL

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Figure 1 Nucleotide sequence of the cDNA clone encoding Eg EF-1 β / δ . The polyadenylation signal and the stop codon are underlined.

similarity with the subunits β and δ of human elongation factor, respectively, with higher identity at the C-terminus (62% with the human δ subunit and 68% with the human β subunit), respectively (Figure 2). The consensus patterns (indicated by asteriks) common to all the proteins belonging to this family were present. The serine at position 128 located N-terminally of an acidic cluster could be involved in regulation by phosphorylation (Janssen *et al.* 1988, Venema *et al.* 1991). We can not determine if the whole open reading frame has been cloned since the sequence identity and the consensus patterns are mainly located in the C-terminal sequence. In Southern blot under high stringent condition, Eg EF-1 β / δ hybridized to genomic DNA of *E. granulosus* digested with *EcoRI* at two bands of 4 and 2.5 Kb, probably the two genes for the β and δ subunits, even though we cannot rule out the possibility that a restriction site in introns might give rise to the obtained pattern (Figure 3).

Molecular and immunological characterization of Eg EF-1 β / δ protein

The purified protein encoded by Eg EF-1 β / δ was analysed by SDS-PAGE under reducing conditions and the molecular size of 33.1 kDa was confirmed by immunoblotting using monoclonal antibody specific to histidine tail and human sera (Figure 4).

Qualitative analysis of IgE, total IgG and IgG4 response determined by immunoblotting showed that 31/60 patients with CE (51.7%) presented IgE, 25 (41.7%) total IgG and 11 (18.3%) IgG4 to the Eg EF-1 β / δ protein. Of the 15 atopic subjects, one serum from a subject with polyspecific allergic reactions presented specific IgE and of the nine healthy donors, two sera had total IgG and one of these had IgE specific to Eg EF-1 β / δ protein as well. Notably, no sera from the patients with other parasitic diseases contained IgG/IgG4 or IgE specific to the recombinant antigen (Table 1).

EgEF1	----FDAHSYHQFPNYHEMESAYMRFLSKDIGKTSATE-----QKGDSYL	41
EF1DHuman	MATNFLAHEKIWFDFKFYDDAERRFYEQMNGPVRGASRQENGATVILRDIAARENIQKS	60
EF1BHuman	--GFGDLKSPAGLQVLNDYLADKSYIEGYVPSQADV-----A----V	36
EF1DXenla	MSAFVITTEQVWLDKYKYDDAEKQYYENLSMGSSASN-----KPHNSPQSA	45
EF1BXenla	-MGFGLKSPAGLKVLKEFLADKSYIEGYVPSQADV-----A----V	37
EF1XCaeel	--MVADVKSAPAGLAAFNTTLAEQAFATGFVLSGEDA-----Q----L	36
.		
EgEF1	VKEIQKVR-----DNIKSSLESNGSWNERMEKLEAIVKEMSLRVNELEVQIQRIGQILP	95
EF1DHuman	LAGSSSGPGASSGTSGDHGELVVRIASLEVENQSLRGVVQELQQAISKLEARLNVLKSSP	120
EF1BHuman	FEAVSS-----PPPADLCHALRWYN-----HIKSYEKEKASLPGVKKALGKYGP	80
EF1DXenla	ASALS-----SGDGSELAARVANLEQENQSLHKVVKDLQSAISKLESRLSTLEKSSK	98
EF1BXenla	FDALSA-----APPADLFHALRWYN-----HIKSYEKQKSSLPGVKKALGNYGP	81
EF1XCaeel	FAALGS-----APNAST-YPN-----VARWYANVASYTDAERKTWASAGG	75
.		
EgEF1	SES-----LKGAKPIRPVVVKAPPAEG-----GDDDDIDLFGSEDEGEADKCKQM	140
EF1DHuman	GHRATAPQTQHVSMPMRQVEPPAKKPATPAE-----DDEDDIDLFGSDNEEDKEAAQL	174
EF1BHuman	-----ADVEDITTGSGATDSK-----DDDDIDLFGSDDEEESEEAKRL	117
EF1DXenla	SQKPAAASQPAIEVAARVQKVQVTPAAKEENG TGEDDDDDDDIDLFGSDNEEDAEAARI	158
EF1BXenla	-----VNIEDTTGSAKETKE-----EDDDIDLFGSDDEEESED AKRV	120
EF1XCaeel	-----S-----APAAAAAD-----GDDFDLFGSDDEEEDA EKAKI	105
	* ** *****. * *	..
.		
EgEF1	MAEQNKAAASKK-EKP--VAKSMIVLDVKPWDDTTNMAEMEKGVRITADGLLWGTSKL	197
EF1DHuman	REERLRQYAEKKAKKPALVAKSSILLDVKPWDDETDMAQLEACVRSIQLDGLVWGASKLV	234
EF1BHuman	REERLAQYESKKAKKPALVAKSSILLDVKPWDDETDMAKLEECVRSIQADGLVWGSSKL	177
EF1DXenla	REERLKQYAEKSKKPGVIAKSSILLDVKPWDDETDMAKLEECVRTVQMDGLVWGSSKL	218
EF1BXenla	RDERLAQYEAKSKKPTLIAKSSILLDVKPWDDETDMGLEECLRSIQMDGLLWGSSKL	180
EF1XCaeel	VEERLAAYA EKKAKKAGPIAKSSVILDVKPWDDETDLGEMEKLVR SIEMDGLVWGGA LI	165
	*. ** * *** * .* *.. ***.** **.	
.		
EgEF1	PLVHGINKLQIACVVEDDKVGTD FLEESIM-ELEDYVQSVDVASFNKL	244
EF1DHuman	PVGYGIRKLQIQCVVEDDKVGTD LLEEIT-KFEEHVQSVDIAAFNKI	281
EF1BHuman	PVGYGIKKLQIQCVVEDDKVGTD MLEEQIT-AFEDYVQSM DVAAFNKI	224
EF1DXenla	PVGYGIKKLQIQCVVEDDKVGTD ILEEIT-KFEDYVQSVDIAAFNKI	265
EF1BXenla	PVGYGIKKLQIQCVVEDDKVGTD VLEEKIT-AFEDFVQSM DVAAFNKI	227
EF1XCaeel	PIGYGIKKLQII TVIEDLKVSVD DLIEKITGDFEDHVSVD IVAFNKI	213
	* ** **** * ** ** * * * * * * *	

Figure 2 Comparison of amino acid sequences of Eg EF-1 β/δ (Eg EF1) and the EF-1 δ and β from human (EF1DHuman and EF1BHuman), *Xenopus laevis* (EF1DXenla and EF1BXenla) and *Caenorhabditis elegans* (EF1XCael) using a multiple (CLUSTAL computer program) alignment that gives the highest number of identical residues (*). Conserved residues (dots).

The pattern of distribution of specific immunoglobulins shows that patients with calcified cysts and patients with cysts of various types, almost one calcified, had the highest percentage of sera positive for IgE (cysts of various types 75%, type VII 73%, type IV 44%, type I 38% and type II 25%), IgG (type VII 63%, cysts of various types 58%, type II 50%, type IV 44% and type I 24%), and IgG4 (type VII 31%, cysts of various types 28%, type I 14% and type IV 11%) specific to Eg EF-1 β / δ (Figure 5).

Immunolocalization of the native protein in *E. granulosus* protoscoleces and hydatid fluid

The monoclonal antibody specific to Eg EF-1 β/δ was used in immunoblotting to investigate the localization of this protein in the protoscolex and in hydatid fluid and to determine the molecular mass of the native antigen. The monoclonal antibody recognizes two subunits of approximately 25 and 40 kDa both in protoscolex and in hydatid fluid in reducing conditions while in hydatid fluid

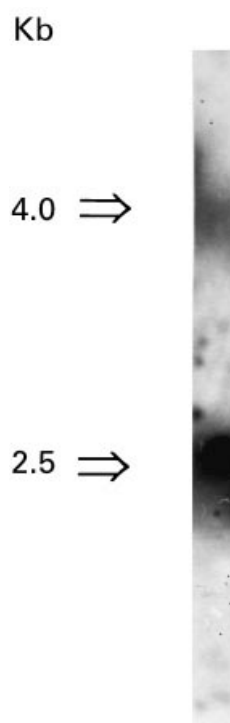


Figure 3 Southern blot analysis of *E. granulosus* genomic DNA. DNA from *E. granulosus* was digested with *Eco*RI. After electrophoresis and blotting, the filter was hybridized to the ^{32} P-labelled Eg EF-1 β/δ cDNA. Molecular weights are indicated on the left.

in nonreducing condition it recognizes a broad band with high molecular weight (>200 kDa) and a subunit at 40 kDa (Figure 6). The presence of two distinct subunits at 25 and 40 kDa might be explained by the binding of the monoclonal antibody with both the β and δ subunits of elongation factor-1.

DISCUSSION

Cystic echinococcosis shares with other helminthiasis three responses typical of immediate hypersensitivity reactions: elevated IgE/IgG4 antibodies production, eosinophilia and mastocytosis (Williams 1979, Afferni *et al.* 1984, Aceti *et al.* 1993). Moreover IgE, which decreases rapidly in serum of patients after surgery or with success of chemotherapy, may be a useful marker indicating the outcome of CE (Aceti *et al.* 1993, Riganò *et al.* 1995a). The immunoscreening of an *E. granulosus* expression library with IgE from CE patients permitted us to isolate a cDNA clone which shares substantial identity with the β and δ subunits of the elongation factor 1. In immunoblotting analysis, the Eg EF-1 β/δ recombinant protein shows immunological reactivity with sera from CE patients: 51.7% of sera contained IgE, 41.7%

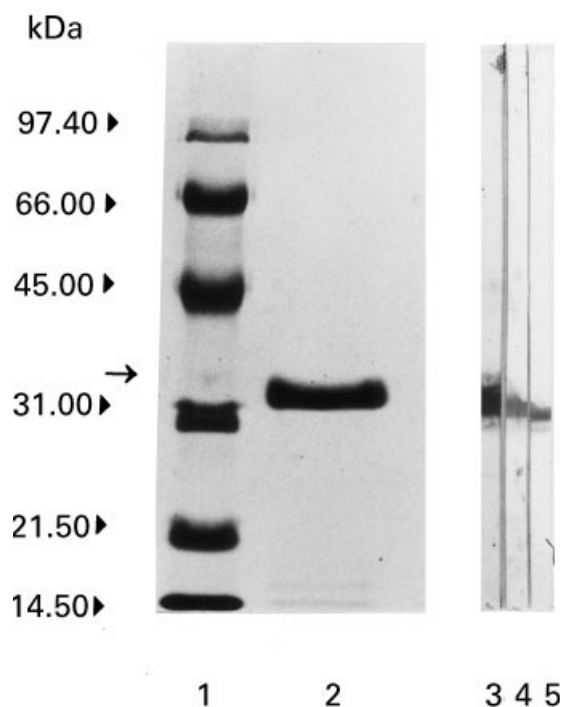


Figure 4 Western blot showing the molecular mass (33.1 kDa) of the recombinant protein encoded by the cDNA of clone Eg EF-1 β/δ following subcloning into pQE-31, induction with isopropyl- β -D-thiogalactoside and purification under denaturing conditions. The purified, recombinant protein (3 μ g) was subjected to SDS-PAGE and stained with Coomassie Blue (lane 2) or transferred to nitrocellulose and revealed with the monoclonal antibody specific to 6X-histidine (lane 3), human serum immunoglobulin (Ig)G (lane 4), human serum IgE (lane 5). Molecular weights (lane 1) are indicated on the left.

IgG and 18.3% IgG4 specific to the recombinant protein. In a previous work, we found that 86.2% of CE patients resulted IgE positive to antigen 5 and antigen B of *E. granulosus* hydatid fluid (Siracusano *et al.* 1993). The high IgE response found in this study seems to assign to Eg EF-1 β/δ a key role in the allergic disorders in cystic echinococcosis. Among the IgE positive patients, six were negative for total IgG against Eg EF-1 β/δ , suggesting the possible occurrence of a direct splicing IgM vs IgE (Vercelli *et al.* 1998). In a recent study, using sheep hydatid fluid as antigen in ELISA, we observed a significant correlation between IgE and IgG4 production (Riganò *et al.* 1995b). The lack of association between the IgE and IgG4 production found in this study might be explained by the nature of the antigen. It would be worth investigating the role of native and recombinant antigen in stimulating distinct patterns of immunoglobulin production as the recombinant protein cannot fold and express the conformational B-cell epitopes the way that the natural EF-1 proteins can. In the immune response to infections, the cytokines have a role in

	No of serum samples	No of positive serum samples in immunoblotting		
		IgG	IgG4	IgE
Patients with cistic echinococcosis	60	25	11	31
Patients with other parasitoses	15	0	0	0
Patients with atopic disorders	15	0	0	1
Healthy donors	9	2	0	1
Sensitivity		41.7%	18.3%	51.7%
Specificity		92.8%	100%	94.8%

Table 1 Pattern of IgG4 and IgE reactivity to Eg EF-1 β/δ of 60 cystic echinococcosis patients and 39 control subjects

regulating antibody isotype production. In recent research, we provided evidence that Th1 and Th2 cytokine patterns are involved in the immune regulation of CE (Riganò *et al.* 1995b, 1996). Further investigations are necessary to clarify the role of this antigen in the modulation of immune response in particular enhancing a Th2 activation through IL-4 production.

Regarding the IgG determination in Eg EF-1 β/δ immunoblotting, we found high specificity (94.8%) and low sensitivity (41.7%), probably due to the high purity of the recombinant antigen. This finding seems to exclude the possible use of this test in the routine immunodiagnosis of CE but it might be employed in areas endemic for cross-reactive parasitic diseases.

The presence of autoantibodies was demonstrated in patients with CE suggesting its possible association with immune evasion strategies of the parasite (Pini *et al.* 1983, Mori *et al.* 1986). The presence of homology between Eg

EF-1 β/δ and human EF-1 is intriguing in view of a possible autoimmune response. The study of molecules shared by different species, particularly the identification of conserved epitopes able to stimulate the immune system and to cause the allergic reaction, could be useful to clarify the complex host–parasite relationship.

The β , δ and γ subunits of EF-1 are conserved, constitutive proteins predominantly located in the endoplasmic reticulum region (Sanders *et al.* 1996). Maneu *et al.* (1996) isolated from a *Candida albicans* cDNA expression library a clone coding the EF-1 β by immunoscreening with a polyclonal antibody against cell wall material suggesting the

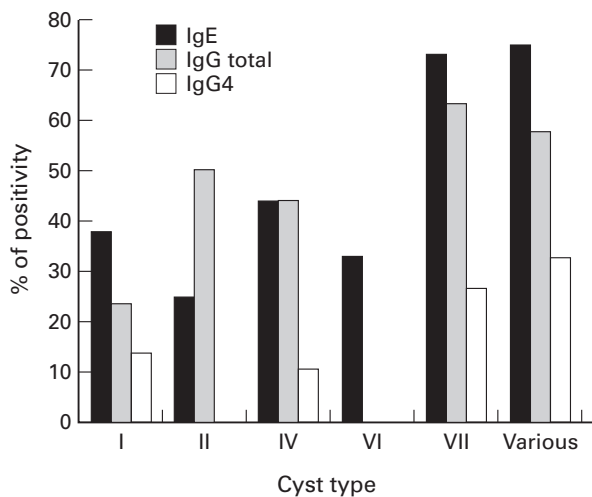


Figure 5 Pattern of distribution of immunoglobulin (Ig)E, total IgG and IgG4 specific to Eg EF-1 β/δ in the 60 cystic echinococcosis patients divided according to the type of cysts following the classification of Caremani *et al.* (1996).

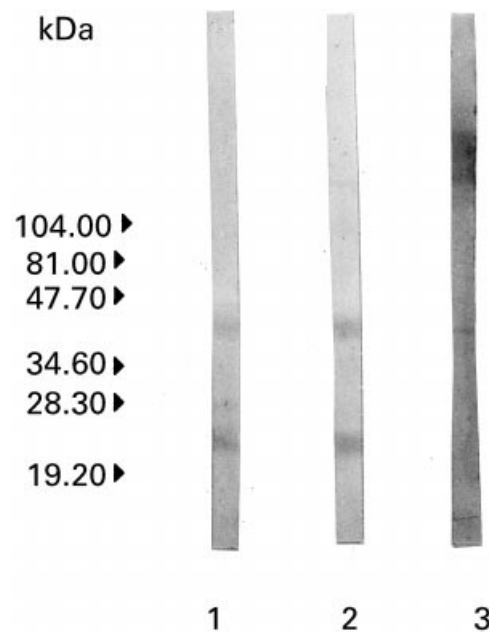


Figure 6 Immunoblotting after SDS-PAGE of protoscoleces (lane 1) and hydatid fluid (lane 2) under reducing conditions and hydatid fluid under nonreducing conditions (lane 3) revealed with the monoclonal antibody specific to Eg EF-1 β/δ . Molecular weights are indicated on the left.

presence of this subunit also in the cell wall. We identified the Eg EF-1 β / δ by immunoblotting with specific monoclonal antibody both in protoscoleces and in sheep hydatid fluid. The higher percentage of humoral immune response against EgEF-1 β / δ observed in CE patients with inactive cysts (type VII) than in CE patients with active cysts (mainly type I–II) indicates the possible release of significant amounts of protein in the hydatid fluid after protoscoleces degeneration. It remains to be clarified whether elongation factor release occurs only in deteriorating cysts during ageing, calcification or drug treatment or whether it is an active process of secretion with significance for the host–parasite relationship. Again, further investigations need to clarify the possible use of this antigen in the immunosurveillance of CE.

To date, all recombinant *E. granulosus* antigens of potential interest for immunodiagnosis have been employed to investigate the IgG response (Facon *et al.* 1991, Shepherd *et al.* 1991, Esteves *et al.* 1993, Frosch *et al.* 1994, Ersfeld & Craig 1995). This is the first report describing an *E. granulosus* recombinant antigen recognized by serum IgE/IgG4 from CE patients. The immune interactions that are necessary to eradicate invasive helminth parasites are extremely complex and are likely to require antibodies of specific isotypes and multiple types of effector cells. Having recombinant antigens involved in the immune response should help to define the precise role that specific antibody isotypes and the cytokines play in human helminth infection.

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