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Assessment of *Echinococcus granulosus* Somatic Protoscolex Antigens for Serological Follow-Up of Young Patients Surgically Treated for Cystic Echinococcosis▽

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Echinococcus granulosus **protoscolex soluble somatic antigens (PSSAs) were assessed for their prognostic value in the serological follow-up of young patients treated for cystic echinococcosis (CE), compared to conventional hydatid fluid (HF) antigen. Based on different clinical courses and outcome of infection, as well as imaging findings, patients were retrospectively classified into two different groups including either cured CE (CCE; i.e., absence of active cysts or presence of inactive cysts, respectively) and noncured CE (NCCE) patients still presenting active cysts at the end of an up to 5-year follow-up period. An immunoglobulin G (IgG)-PSSA enzyme-linked immunosorbent assay (ELISA) showed a gradual decrease in antibody levels in CCE cases, reaching seronegativity in 20% of the cases at least within 5 years postsurgery. In comparison, the conventional IgG-HF ELISA showed a significantly lower progressive decrease in antibody levels, serology becoming negative in only 15% of CCE patients at the endpoint of the follow-up period. Serological analysis of PSSA by immunoblotting yielded an interesting immunoreactive double band of 27 and 28 kDa that, in 15 (75%) of 20 CCE cases, exhibited a rapid decrease and subsequent disappearance of respective antibody reactivities within 3 years postsurgery. Conversely, anti-27- and -28-kDa antibody reactivity strongly persisted until the endpoint of the follow-up period in all of the five NCCE patients. Further analysis of the 27- and 28-kDa doublet by using affinity-purified antibodies showed that the double band was not detectable in HF. Furthermore, a predominantly IgG4 subclass-restricted humoral immune response against the 27- and 28-kDa antigens was demonstrated in seroreactive CE patients. Overall, an anti-27- and -28-kDa response appeared to correlate with cyst activity. In conclusion, PSSA represents a useful candidate to carry out a serologic follow-up of CE subsequent to treatment and deserves further respective evaluation for other age groups of CE patients.**

Cystic echinococcosis (CE), caused by infection with the larval stage of the cestode *Echinococcus granulosus*, is a zoonosis that is present in many parts of the world and is highly endemic in certain regions. Thus, the pastoral areas of the Mediterranean basin are markedly distressed by the problem. The parasite is mainly transmitted in a synanthropic cycle generally involving dogs and livestock animals (sheep, cattle, goats, and camels) (39). Despite progress in chemotherapy, conventional surgery and laparoscopic or direct percutaneous intervention remain the optimal treatment procedures for CE in humans (11). One of the problems that can be encountered after treating CE patients is the risk of postsurgical relapses or treatment failure due, e.g., to nonradical surgical procedures or perisurgical spillage of parasite material, especially protoscoleces. Relapses in the form of newly developing cysts have been reported and may affect between 2 and 25% of cases after therapy, according to previous studies (14, 29, 39, 40). Therefore, postsurgical follow-up of CE patients for years is indicated, with the aim of detecting newly growing cysts as soon as possible. A posttreatment follow-up method to prognostically determine the efficacy of treatment should therefore include markers that allow the detection of newly growing or relapsing cysts and tracking of previously undetected but still viable cysts. The most conventional tools used to follow up CE patients include imaging techniques such as X-ray, ultrasonography, computed tomography, and magnetic resonance imaging (39). In some cases, however, the prognostic efficiency of imaging tools is significantly hampered, e.g., when small changes cannot be visualized or cannot be interpreted with regard to a concise discrimination between dead and still viable metacestodes (37). Therefore, the availability of an immunodiagnostic test to support this discrimination may be of valuable clinical help. In this respect, the selection of an appropriate immunodiagnostic test involves consideration of the diagnostic operating characteristics of the technique and the purpose for which it will be used. The diagnostic sensitivity and specificity of the tests vary according to the nature and quality of the antigen and the sensitivity of the method selected. A concise definition of the sera used for the assessment of test parameters is essential, with special attention paid to the definition of pre- or postoperative/posttreatment situations with respect to CE and the

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serum sampling time point. Conventional serological methods have so far only been of limited help in prognostically classifying the status of a CE patient during follow-up. Therefore, there is still a need to develop or improve immunodiagnostic tools in order to meet the requirements expressed by clinicians. One approach consists of searching for and identifying new antigens that specifically allow the classification of patients into cured and noncured categories.

The enzyme-linked immunosorbent assay (ELISA) and immunoblotting are among the tests widely used to follow up CE patients conventionally by including crude hydatid cyst fluid, which has so far been the major source of antigen used for this purpose (2, 13, 15, 30, 32, 41).

Alternatively, purified fractions of antigen 5 or B have been similarly tested (1, 9, 10, 27, 34, 36), However, all of these antigens still exhibit some problems, mainly related to temporally delayed reactions to clinical changes (2). Thus, the persistence of specific antibodies against cyst fluid antigens for several years after recovery makes it difficult to predict the outcome of surgery early enough (5, 21, 29). Improved immunodiagnostic performance was achieved by using a synthetic peptide derived from the antigen B sequence (20); however, this peptide was not yet evaluated for the postoperative follow-up of CE patients.

Studies in the field of veterinary medicine have shown that many proteins of *E. granulosus* protoscoleces are immunogenic, and such antigens have been applied in the serodiagnosis of canine intestinal echinococcosis (4, 6, 16, 18, 25) and also in the detection of CE in sheep (24). However, relatively few studies have been undertaken to use soluble protoscolex antigens in the immunodiagnosis of human echinococcosis (3, 12, 27, 31), although whole protoscoleces represent the major antigenic source used to successfully carry out the indirect immunofluorescent-antibody test. Conversely to primary diagnostic investigations, scarce information is available about the use of soluble protoscolex antigen to carry out serologic follow-up studies. The main subject of the present study was the evaluation of using ELISAs and immunoblotting to detect protoscolex soluble somatic antigens (PSSAs) for postsurgical or posttreatment follow-up of young CE patients. The latter approach was also used to search for new antigen markers that may discriminate between cured and noncured patients or even progressive courses of disease. Approximately 10% of CE cases occur in children, but in certain areas, e.g., North Africa, the respective percentage is much higher (2). Furthermore, CE serology in children has been shown to exhibit more problems with respect to diagnostic test sensitivity than serology in adult CE patients (25).

MATERIALS AND METHODS

Sera from CE patients. A total of 125 sera were collected from 25 young CE patients during a clinical follow-up period as defined in Table 1. All of the patients but one had been subjected to surgery, and some additionally underwent chemotherapy following the diagnosis of CE, which corresponded to the time point when the patient entered the present study. It has to be noted that some of the patients exhibited, at entrance, a relapse situation in that they had already been previously diagnosed with CE and then treated (Table 1). The CE cases used in the present study included 15 male and 10 female children or adolescents (age range, 4 to 15 years; average, 9 years). In each case, a first serum sample was obtained at the time of diagnosis of CE (or relapsing CE), prior to clinical intervention. The number of follow-up sera and time intervals were variable between the patients and are documented in Table 2. For most cases, the time points were 1 week after surgery; 1, 6, and 12 months after surgery; and then once a year. All serum specimens were stored at −20°C prior to laboratory testing. Clinical examinations were carried out in the pediatric surgery service of the E. P. S. Hospital of Monastir (Tunisia). Standard laboratory investigations were run in the Laboratory of Diagnostic Parasitology of Monastir University.

Patients' clinical files were retrospectively reviewed for the anatomical location and number of cysts, results of radiologic investigations according to World Health Organization guidelines (39), clinical complications, and putative benzimidazole treatment. Twenty of the 25 CE patients achieved a cure or a fully stabilized stage of infection (no disease) without any clinical or radiological evidence of recurrence or cyst activity during and at the endpoint of the study, with 2 patients (no. 5 and 6) showing total calcification of the initial lesions. Another 5 of the 25 CE patients were still sick at the end of the study, as demonstrated by persisting disease either upon the development of new cysts (patients 22 and 23) or enlargement of existing cysts (patients 21, 24, and 25). One case (patient 24) was considered inoperable and involved disseminated liver cysts. This patient was submitted to continuous albendazole treatment (Zentel; 10 mg/kg/day) for 5 years (Table 1). Thus, the grouping of the patients was retrospectively determined and based upon their clinical status at the endpoint of the study period (5 years).

Antigen preparation. Protoscoleces were collected from fresh fertile sheep liver cysts (G1 genotype) directly at the abattoir of Sousse, Tunisia. Viability was determined by the vital coloration approach with 0.2% eosin staining. Furthermore, viability was confirmed microscopically upon flame cell activity and peristaltic motility of the protoscoleces. Respectively validated batches of protoscoleces were submitted to several washing cycles with a sterile physiological sodium chloride solution, followed by three washings with phosphate-buffered saline (PBS; pH 7.2). Subsequently, the parasites were sedimented at 10,000 × *g* for 10 min and the pellet, supplemented with an equal volume of sterile PBS (pH 7.2), was stored at −80°C until further processing. A PSSA was obtained by performing three freezing-thawing cycles (37°C water bath, liquid nitrogen), followed by two sonications for 30 s at 57 W in a Sonifier B-12 cell disruptor (Bransonic Power Company, Danbury, CT). The treated fluid was sedimented at 10,000 × *g* at 4°C for 10 min, and the supernatant was supplemented with 1% of a 100 mM PMSF (phenylmethylsulfonyl fluoride) solution; subsequent storage was in liquid nitrogen until use. *E. granulosus* hydatid fluid (HF) antigen was obtained and processed as previously described (2), and the same batch of antigen as used in that previous study was used.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. PSSA was separated on a 5 to 20% linear gradient polyacrylamide gel as described by Gottstein et al. in 1986 (22), by using reducing and nonreducing conditions separately. Briefly, this was achieved by diluting PSSA in either (i) 10 mM Tris-HCl with 10% SDS and 9 M urea and heating it for 20 min at 65°C or (ii) a similar buffer but with 5% β-mercaptoethanol and heating it for 5 min at 100°C. Proteins were loaded at a concentration of 100 μg/gel (preparative 14-cm slot). Electrophoresis was run at 500 V for 2 h at 4°C.

Electrophoretically resolved proteins were transferred onto nitrocellulose membranes as described by Gottstein et al. in 1986 (22). Nitrocellulose strips (3 mm wide) were then incubated with human sera diluted at 1:100 in PBS (pH 7.2) containing 0.3% Tween 20 and 5% skim milk powder overnight at 4°C. After three washing steps, the strips were incubated with goat anti-human immunoglobulin G (IgG)-horseradish peroxidase conjugate (diluted 1:400) for 2 h at room temperature (Sigma A6029). Immunoreactive bands were visualized with a chromogenic substrate including 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.03% H_2O_2.

ELISA. Nunc Immulon ELISA microplate strips were coated by incubation at 100 μl per well with either PSSA or HF diluted in ELISA coating buffer (0.1 M $NaHCO_3$-Na_2CO_3 [pH 9.6] containing 0.02% NaN_3) at a concentration of 2.5 μg/ml (PSSA) or 10.0 μg/ml (HF). Incubation was overnight at 4°C. ELISA strips were washed three times with washing buffer (PBS, 3% Tween 20) and blocked with blocking buffer consisting of PBS (pH 7.2) with 0.05% bovine hemoglobin and 0.02% NaN_3. Sera were diluted 1:100 in blocking buffer, and alkaline phosphatase-labeled anti-human IgG conjugate (Sigma A5403) was used at a 1:2,500 dilution. All incubations were done at 37°C for 30 min. Plates were finally washed four times and subsequently incubated with the substrate for 15 min. The reaction was finally stopped with 10 μl of 3 N NaOH. Absorbances (A_{405}) were read with a microplate reader (Dynex Technologies Bioconcept). The results were expressed in arbitrarily defined antibody units (AU) as previously described (23). On each ELISA plate, we incorporated highly, intermediately, and weakly positive control sera. The intermediately and weakly positive controls quantitatively represented approximately 50 and 10% of the reactivity of the highly positive control serum, respectively. Considering these thresholds and based upon em-

TABLE 1. Summary of clinical features of CCE and NCCE patients[a]

Patient group and no.	Age (yr)/sex[b]	Location of cyst(s)[c]	Cyst type(s)	Previous surgery[d]	Albendazole treatment period (mo)[e]	Follow-up period (mo)
CCE						
1	12/M	Lung (1, 1)	CE1	Yes (lung)	None	60
2	13/M	Lung (1, 1)	CE1 (fissured)	No	18	60
3	6/M	Lung (1, 1)	CE1	No	None	60
4	4/M	Liver (1, 1)	CE1	No	None	36
5	10/M	Multiple[f] (4, 3)	2 CE2, 1 CE4	Yes (lung)	36	60
6	7/M	Pleura (3, 2)	2 CE1	Yes (lung)	56	36
7	6/M	Liver (2, 2)	2 CE1	Yes (lung)	None	36
8	11/F	Liver (4, 4)	3 CE1, 1 CE3 (fissured)	No	5	36
9	15/M	Liver (1, 1)	CE1	No	None	36
10	6/F	Liver (1, 1)	CE1	Yes (lung)	6	24
11	5/F	Liver (3, 3)	2 CE1, 1 CE2	No	8	24
12	4/M	Liver (1, 1)	CE1	Yes (lung)	None	48
13	4/M	Liver (1, 1)	CE1	Yes (liver)	1	36
14	13/F	Lung (1, 1)	CE1	No	None	24
15	10/F	Lung (1, 1)	CE1 (fissured)	No	None	48
16	13/F	Lung (1, 1)	CE1	No	None	24
17	5/M	Liver (1, 1)	CE1	Yes (lung)	None	24
18	13/F	Lung (1, 1)	CE1	No	None	24
19	5/M	Lung (2, 2)	2 CE1	No	None	12
20	10/F	liver (1, 1)	CE4	No	2	36
NCCE						
21	11/F	Liver (9, 2)	1 CE3, 1 CE5	Yes (lung)	60	12
22	11/F	Liver (2, 1)	CE1	No	None	12
23	11/M	Liver (2, 1)	CE1	No	12	24
24[g]	10/M	Liver (6, 0)	Disseminated	No	60	60
25	10/M	Multiple[h] (3, 2)	1 CE1, 1 CE3 (fissured)	No	21	24

[a] Cyst type (CE) corresponds to the sonographic classification according to the World Health Organization (39).
[b] Age at diagnosis is shown. M, male; F, female.
[c] The values in parenthesis are the total number of cysts and the number of cysts operated upon.
[d] Prior to the study diagnosis, the patient had already been diagnosed with and surgically treated for CE; therefore, the study diagnosis was based upon a relapse situation in these patients.
[e] The albendazole dose was 10 mg/kg/day.
[f] Peritoneum and liver.
[g] Patient treated only with chemotherapy (no surgery).
[h] Liver and spleen.

pirical experiences, we clustered all AU values into high, intermediate, and weak classes of reactivity.

Affinity purification of antibodies. Affinity purification of specific antibodies against the 27- and 28-kDa double bands was carried out as described by Müller et al. in 1992 (26). Briefly, the bands corresponding to the 27- and 28-kDa molecules were cut out from the nitrocellulose following SDS-PAGE and blotting of PSSA. This was achieved by the following steps. After blocking of the nitrocellulose with PBS containing 0.3% Tween 20 and 5% skim milk powder, cut strips were incubated with a pool of highly positive CE patient sera at a dilution of 1:10 in PBS with 0.3% Tween 20 overnight at 4°C. After washing with PBS-Tween three times for 20 min each, the bound antibodies were eluted with 900 μl of low-pH buffer (50 mM Tris, 50 mM glycine, 0.05% Tween 20, pH 2.6) for 5 min on ice with vortexing. The strips were then removed, and 100 μl of 1 M Tris base was added to the eluted antibody solution for neutralization. The solution was then sedimented (14,000 × *g* for 10 min) in order to remove potentially remaining nitrocellulose particles. Subsequently, bovine serum albumin was added to a final concentration of 0.1%. Affinity-purified antibodies were aliquoted and stored at −20°C. Affinity-purified antibodies were used for subsequent immunoblotting at a dilution of 1:50.

Antisera and conjugates. Biotin-labeled mouse anti-human IgG1, IgG2, IgG3, or IgG4 monoclonal antibody and streptavidin-alkaline phosphatase were used to determine IgG subclasses (Sigma catalog no. B6775, B3398, B3523, and B3648). Nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indolylphosphate (BCIP) was used as the substrate for isotype-specific immunoblotting.

To demonstrate the whole antigenic profile of PSSA as a reference to the affinity-purified antibody profile, a hyperimmune rabbit anti-sheep HF polyclonal antibody was used. It was revealed with an anti-rabbit IgG antibody-alkaline phosphatase conjugate diluted 1:1,000 (Promega catalog no. S3731).

Protein concentrations. Protein concentrations were determined with the Bio-Rad Bradford protein assay with bovine plasma immunoglobulin as the standard.

Statistical analyses. Statistical analyses were done with the EpiInfo 6 program. A *t* test was used to compare the sensitivities of groups of patients during each follow-up period. $P \leq 0.05$ was considered statistically significant. Differences were compared by chi-square tests or Fisher's exact probabilities with 95% confidence intervals. The agreement between the two tests was calculated with the kappa factor (κ). $\kappa > 0.7$ reflected concordance between ELISA and immunoblotting.

RESULTS

ELISA. Posttreatment evaluation of antibody levels in CE patients was carried out from the time of diagnosis (day 0 [D0]) until the end of the follow-up period (Table 1). For the PSSA ELISA, antibody levels were arbitrarily clustered into highly positive (70 to 180 AU), intermediately positive (25 to 65 AU), weakly positive (1 to 20 AU), and negative (<1 AU) groups; for the HF ELISA, the arbitrary clusters were as follows: 85 to 185 AU, highly positive; 25 to 80 AU, intermediately positive; 1 to 20 AU, weakly positive; <1 AU, negative (Table 3).

At the time of diagnosis, D0 (prior to surgery), PSSA ELISA results exhibited the whole range, from high to intermediate to negative, of antibody levels for both CCE and NCCE patients,

TABLE 2. IgG levels obtained by PSSA ELISA, HF ELISA, and 27-28 kDa immunoblotting[a]

Patient group and test	Day 0		Day 7		1 Mo		6 Mo		12 Mo		24 Mo		36 Mo		48 Mo		60 Mo	
	AU	I	AU	I	AU	I	AU	I	AU	I	AU	I	AU	I	AU	I	AU	I
CCE																		
P1 PSSA	40	+					60	+			0	−	0	−			0	−
HF	20	+					110	+			20	+	0	−			0	−
27-28 IB		−						+				−		−				−
P2 PSSA	120	+					30	+			30	+	30	+			20	+
HF	170	+					50	+			10	+	15	+			10	+
27-28 IB		+						+				−		−				−
P3 PSSA	40	+	60	+									45	+	40	+	35	+
HF	140	+	175	+									75	+	60	+	65	+
27-28 IB		+		+							0			−		−		−
P4 PSSA	0	−					0	−	0	−		−	0	−				
HF	125	+					85	+	50	+	40	+	10	+				
27-28 IB		−						−		−		−		−				
P5 PSSA	150	+							85	+	75	+	65	+			45	+
HF	170	+							150	+	140	+	125	+			110	+
27-28 IB		+								+		+		+				+
P6 PSSA	145	+			180	+			85	+	60	+	55	+				
HF	165	+			185	+			110	+	85	+	95	+				
27-28 IB		−				+				+		−		−				
P7 PSSA	145	+	100	+					25	+	9	+	0	−				
HF	135	+	105	+					60	+	45	+	30	+				
27-28 IB		−		+						+		−		−				
P8 PSSA	130	+	130	+			55	+			25	+	20	+				
HF	160	+	150	+			125	+			35	+	25	+				
27-28 IB		+		+				+				−		−				
P9 PSSA	30	+	20	+	55	+	20	+	5	+								
HF	95	+	90	+	140	+	55	+	30	+								
27-28 IB		−		+		+		−		−								
P10 PSSA	30	+	100	+	135	+			40	+	15	+						
HF	0	−	100	+	125	+			20	+	10	+						
27-28 IB		−		+		+				−		−						
P11 PSSA			170	+	160	+	80	+	50	+	10	+						
HF			165	+	165	+	145	+	120	+	50	+						
27-28 IB				+		+		−		−		−						
P12 PSSA	0	−			55	+					0	−	0	−	0	−		
HF	45	+			125	+					60	+	30	+	0	−		
27-28 IB		−				+						+		+		−		
P13 PSSA	105	+					150	+	125	+	65	+	65	+				
HF	85	+					65	+	45	+	30	+	10	+				
27-28 IB		+						+		+		+		−				
P14 PSSA	0	−	0	−	0	−			0	−	0	−						
HF	20	+	25	+	10	+			0	−	0	−						
27-28 IB		−		−		−				−		−						

P15 PSSA	80	+			30	+			20	+	15	+			10	+		
HF	125	+			115	+			80	+	55	+			55	+		
27-28 IB		+				+				−	−	−			−	−		
P16 PSSA	0	−	0	−	30	+			10	+	15	+						
HF	0	−	70	+	85	+			90	+	75	+						
27-28 IB		−		+		+				−		−						
P17 PSSA	100	+					85	+	40	+	25	+			20	+		
HF	130	+					115	+	70	+	45	+			30	+		
27-28 IB		+						+		+		+				−		
P18 PSSA	70	+	80	+	80	+	50	+			35	+						
HF	70	+	75	+	75	+	45	+			20	+						
27-28 IB		−		+		−		−				−						
P19 PSSA	0	−	0	−	45	+	70	+	10	+								
HF	0	−	45	+	95	+	65	+	35	+								
27-28 IB		−		−		−		+		−								
P20 PSSA	60	+			135	+	90	+			5	+	0	−				
HF	115	+			135	+	125	+			45	+	15	+				
27-28 IB		+				+		+				−		−				
NCCE																		
P21 PSSA	100	+	50	+	175	+	180	+	125	+								
HF	125	+	165	+	95	+	185	+	115	+								
27-28 IB		−		+		+		+		+								
P22 PSSA	40	+			140	+	120	+	115	+	100	+						
HF	35	+			95	+	100	+	155	+	160	+						
27-28 IB		−				+		+		+		+						
P23 PSSA	10	+	140	+	175	+			110	+	100	+						
HF	120	+	125	+	155	+			135	+	155	+						
27-28 IB		+		+		+				+		+						
P24[b] PSSA	150	+	160	+			110	+			95	+					90	+
HF	50	+	175	+			130	+			75	+					135	+
27-28 IB		+		+				+				+						+
P25 PSSA	85	+			85	+	80	+	80	+	85	+						
HF	150	+			105	+	90	+	145	+	140	+						
27-28 IB		+				+		+		+		+						

[a] The results shown are for CCE and NCCE patients before therapy (day 0) and during follow-up (7 days to 60 months). ELISA results are shown as the number of AU and the interpretation (I), where <1 AU was considered negative (−) and ≥1 AU was considered positive (+). Immunoblotting (IB) results were considered positive when at least one of the doublet 27- and 28-kDa bands was recognized. Empty field, not determined, as no serum was available at that time point.

[b] Patient who received chemotherapy only.

TABLE 3. Percentages of seropositivity determined by PSSA and HF ELISAs in 20 CCE patients during follow-up period[a]

Test and no. of AU or parameter	No. (%) of samples								
	Day 0 (n = 19)	Day 7 (n = 10)	1 Mo (n = 11)	6 Mo (n = 11)	12 Mo (n = 13)	24 Mo (n = 17)	36 Mo (n = 11)	48 Mo (n = 4)	60 Mo (n = 4)
PSSA ELISA									
70–180	8 (42)	5 (50)	4 (36)	4 (36)	3 (23)	0 (0)	0 (0)	0 (0)	0 (0)
25–65	6 (32)	2 (20)	6 (55)	5 (46)	5 (38)	5 (29)	5 (46)	1 (25)	2 (50)
1–20	0 (0)	0 (0)	0 (0)	1 (9)	3 (23)	9 (53)	1 (9)	2 (50)	1 (25)
<1 (negative)	5 (26)	3 (30)	1 (9)	1 (9)	2 (15)	3 (18)	5 (46)	1 (25)	1 (25)
Total seropositive	14 (73.7)	7 (70)	10 (91)	10 (91)	11 (85)	14 (82)	6 (55)	3 (75)	3 (75)
HF ELISA									
85–185	12 (63)	7 (70)	9 (82)	6 (55)	4 (31)	2 (12)	2 (18)	0 (0)	1 (25)
25–80	2 (11)	2 (20)	1 (9)	5 (45)	7 (54)	9 (53)	3 (27)	3 (75)	1 (25)
1–20	2 (11)	1 (10)	1 (9)	0 (0)	1 (8)	5 (29)	5 (45)	0 (0)	1 (25)
<1 (negative)	3 (16)	0 (0)	1 (9)	0 (0)	1 (8)	1 (56)	1 (9)	1 (25)	1 (25)
Total seropositive	16 (84)	10 (100)	11 (100)	11 (100)	12 (92)	16 (94)	10 (90)	3 (75)	3 (75)

[a] Day 0, serum samples collected before surgery; day 7, serum samples collected 1 week after surgery; 1 month, serum samples collected 1 month after surgery; 6 months, serum samples collected 6 months after surgery; 12 months, serum samples collected 12 months after surgery, and so on. All intertest differences were significant at $P > 0.05$.

and the same held true for the HF ELISA results. The post-treatment evolution of IgG antibody levels in the PSSA ELISA showed that in many cases immunoglobulin levels increased first during the 6 months following surgery, reaching their highest level at 1 month postoperation. Subsequently, levels started to progressively decrease, reaching seronegativity in 4 (20%) of 20 CCE patients (no. 1, 7, 12, and 20) within 1 and 5 years of serological follow-up, compared to only 3 (15%) of 20 CCE patients (no. 1, 12, and 14), as determined with the HF ELISA. These latter sera were also negative in the PSSA ELISA, and the patients were followed up for another few years, although seronegativity had already been reached at 1, 2, and 3 years after treatment (Table 2). Besides, the PSSA ELISA yielded two patients (no. 4 and 14) who were negative at the time of initial diagnosis and who remained seronegative for the whole follow-up period, i.e., 3 and 2 years postoperation, respectively.

Moreover, the majority of patients who remained PSSA ELISA seropositive at the end of the follow-up period showed a faster decrease in IgG levels compared to the kinetics obtained in the HF ELISA, and the remaining antibodies at the end of the study were mostly at low levels (1 to 20 AU). Two CCE patients (no. 5 and 6) whose antibody levels were still high (106 and 95 AU) in the HF ELISA at the end of the follow-up period showed a more pronounced decrease in the IgG level in the PSSA ELISA, reaching relatively lower levels of 47 and 57 AU at the end of the study, respectively. The final clinical examination of these cases revealed the presence of fully calcified cysts; therefore, these patients were correctly considered clinically cured of hydatidosis.

With regard to the overall outcome of the PSSA and HF ELISAs in reference to the study endpoint situation, 75% of the patients with cured cysts (CCE) were still seropositive by both ELISAs at 4 to 5 years postsurgery. Therefore, the endpoint determination of the antibody level alone did not provide a sufficient prognostic value to decide for individual patients if further treatment needed to be added or treatment needed to be continued (Table 3).

In NCCE patients with still active CE, the postoperative serologic response exhibited only a slight increase or decrease in antibody levels in the PPSE ELISA (Table 2). In all five cases, antibody levels in both ELISAs subsequently remained high until the end of the follow-up period. Thus, the endpoint seropositivity was still 100%.

In general, the HF ELISA exhibited higher antibody levels, as well as higher rates of seropositivity, than the PSSA ELISA at diagnosis and during the whole follow-up period (Tables 2 and 3).

Immunoblotting. The PSSA immunoblot profiles of sera from 25 CE patients (20 CCE and 5 NCCE) were, with respect to the banding pattern, qualitatively and quantitatively very heterogeneous within each patient group. The overall pattern, including bands ranging between 9 and 150 kDa, also fluctuated considerably during the follow-up time course for many individual patients. Interestingly, however, we noticed a double band at 27 and 28 kDa that appeared, under both reducing and nonreducing conditions, to be linked to clinical findings. In CCE patients, this double-band activity progressively disappeared; however, the effect did not start before 6 months postsurgery (Fig. 1A and 2A). In some of the cases, the 27-kDa band disappeared before the 28-kDa band (data not shown). Conversely to CCE patients, the respective bands remained strongly recognizable in all of the five NCCE patients with a still active cyst(s) and stayed strongly reactive until the end of the follow-up period (Fig. 1B and 2B).

Taking the presence of at least one band of the 27- and 28-kDa doublet marks as an indicator of still-active cysts, most of the CCE patients were in accordance with this criterion by exhibiting a significant decrease from 62% at 6 months postsurgery (detectability rate among 20 CCE patients) to 18% at 3 years postsurgery (Fig. 1A). Only one patient (no. 5) showed persistent anti-28 kDa antibody activity even at 5 years postsurgery. The clinical examination of this case yielded the presence of a fully calcified cyst, and the patient was thus correctly classified as clinically cured of hydatidosis. Moreover, patients 4 and 14, who both were negative in the PSSA ELISA at the time of diagnosis and during the whole follow-up period, were

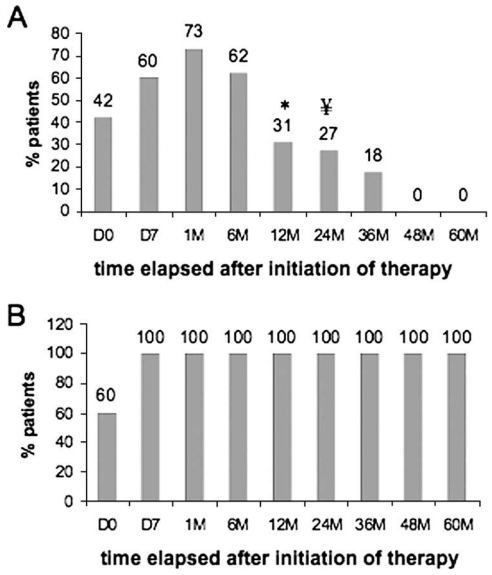

FIG. 1. Temporal evolution of 27- and 28-kDa band recognition by immunoblotting in CCE (A) and NCCE (B) patients after treatment. D0, serum samples collected before surgery; D7, serum samples collected 1 week after surgery; 1 M, serum samples collected 1 month after surgery; 6 M, serum samples collected 6 months after surgery; 12 M, serum samples collected 12 months after surgery, and so on. *, $P = 0.012$; ¥, $P = 0.022$.

both also negative by immunoblotting with regard to 27- and 28-kDa band recognition.

Among the 14 CCE patients who remained positive in the PSSA ELISA, 11 became negative by 27- and 28-kDa immunoblotting, and none of the PSSA ELISA-negative samples was positive by 27- and 28-kDa immunoblotting. Thus, at the end of the follow-up period, an overall immunoblot negativity was found in 15 (75%) of 20 patients.

Conversely, all NCCE patients still reacted with at least one of the doublet bands even at 5 years postsurgery (Fig. 2). A statistically significant difference between the two clinical groups was observed at 12 and 24 months postsurgery ($P = 0.012$ and 0.022, respectively).

No significant correlation was revealed between the PSSA ELISA and immunoblotting results of CCE patients ($\kappa = 0.33$). We could not calculate κ for the NCCE patients because the number of samples was too low.

Immunoaffinity analyses. In order to check if the 27- and 28-kDa molecules can also be found as metabolic products excreted or secreted into the cyst HF, affinity-purified antibodies specific for each of the doublet 27- and 28-kDa bands were used for immunoblotting with cyst HF antigen. A corresponding antibody-binding activity was completely lacking in HF. Moreover, we could demonstrate that affinity-purified antibodies specific to the 28-kDa band cross-reacted with the 27-kDa band and vice versa (Fig. 3A) when applied to PSSA.

Finally, affinity-purified antibodies obtained from a pool of highly seropositive CE patients were also used for the identification of the isotype(s) involved in antigen-binding activity. Immunoblotting revealed that the anti-27- and -28-kDa reactivity of CE patients was restricted to the IgG4 subclass. Moreover, five serum samples collected from the NCCE patients at the end of the respective follow-up periods were individually tested for their IgG4 subclass reactivity. All five sera exhibited strong recognition of the 27- and 28-kDa doublet band (Fig. 3B).

DISCUSSION

In the present study, we used a PSSA to develop a PSSA ELISA and to compare its serological performance with that of a conventional HF ELISA with a view toward following up young CE patients, who represent the age category of patients with the highest degree of difficulty for serological assessment (2). For both ELISAs, antibody levels were very variable at the time of initial diagnosis (D0), prior to treatment. This observation was in line with multiple findings reported elsewhere by others (38). We interpreted high initial antibody reactivity at diagnosis in the PSSA ELISA and also in the 27- and 28-kDa immunoblot assay by the assumption that these patients had exhibited an intensive contact with parasite protoscolex material in the prediagnostic phase of infection. Such high antibody levels were also observed in patients whose cysts were fissured (patients 2, 8, 15, and 25). In such a situation, protoscoleces can easily become accessible to the human immune system after cyst wall fissuration. Follow-up of CCE patients showed that the initial levels progressively decreased in the frame of years postsurgery, conversely to all of the NCCE patients, whose levels remained high until the end of the follow-up period. In these NCCE cases, although some fluctuation of antibody levels was observed during the follow-up period, at the endpoint the concentration was not significantly decreases and actually matched that of CCE patients. Therefore, the PSSA ELISA alone did not allow us to reliably predict the course of CE during follow-up.

Two previous studies by other investigators had used protoscoleces in the primary serodiagnosis of CE. Both showed high diagnostic sensitivities of ELISA of 90.5% (12) and 90% (31). However, neither study tackled the questions of the time that elapsed between surgical intervention and serum collection and of the antibody levels in the follow-up situation.

Immunoblotting with PSSA provided interesting information with regard to the double-immunoreactive 27- and 28-kDa band, which strongly persisted in patients with active cysts and progressively disappeared in CCE patients, an effect that started at 6 months after surgery.

Two CCE patients whose liver and lung cysts were completely removed by surgery both had negative PSSA ELISA and immunoblot assay 27- and 28-kDa findings at the starting time point (day 0) before surgery and at all other time points until the end of the follow-up period. This persistent seronegativity in all tests could hypothetically be related first to a well-protected cyst prior to surgery that did not allow enough antigenic material to be released for the initiation of an appropriate immune response (immune tolerance). Second, as demonstrated by patient 14, who had a complete cyst removal, the persisting seronegativity may indicate that surgery efficiently occurred without damaging the cyst wall, so that leakage of parasite material (including protoscoleces) was not possible. Furthermore, the cyst of patient 14 later proved to be nonfertile (absence of protoscoleces). This could explain the seronegativity against the 27- and 28-kDa antigen, which is

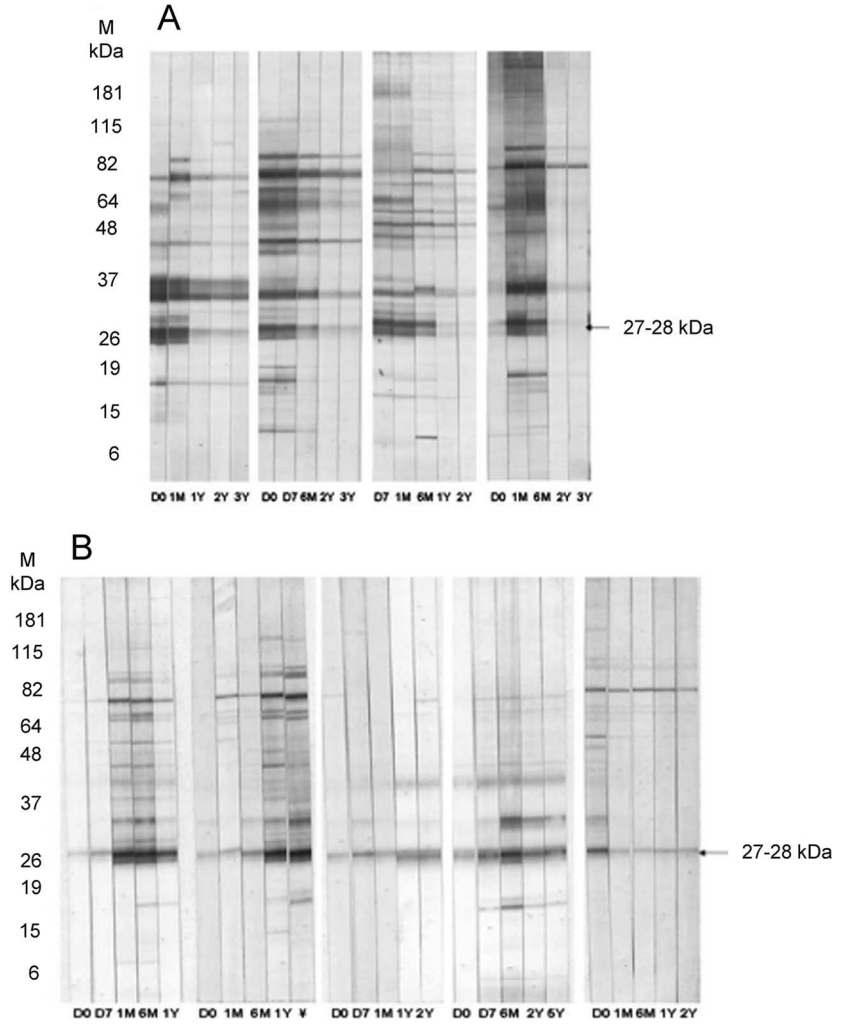

FIG. 2. Follow-up by immunoblotting, exemplified with sera from 4 out of 20 CCE (A) and 5 NCCE (B) patients under reducing conditions (nonreducing conditions not shown). D0, serum samples were collected before surgery; D7, serum samples were collected 1 week after surgery; 1 M, serum samples were collected 1 month after surgery; 6 M, serum samples were collected 6 months after surgery; 1Y, serum samples were collected 1 year postsurgery; 2Y, serum samples were collected 2 years postsurgery, and so on. ¥, sera taken 1 month after a second surgery.

restricted to expression in protoscoleces only. In general, 27- and 28-kDa antigen immunoblotting yielded a lower diagnostic sensitivity at the time of diagnosis and also during follow-up than did the PSSA ELISA. Therefore, for a follow-up, immunoblotting appeared more useful than the PSSA ELISA alone.

The use of affinity-purified anti-27- and -28-kDa antibodies allowed us to demonstrate the lack of these molecules in HF. Consequently, these antigens appeared not to be secreted into HF. This was consistent with the findings of González et al. in 2000 (19), who characterized a 29-kDa antigen from protoscoleces under reducing and nonreducing conditions that may be identical to one of our 27- and 28-kDa antigens. Similarly, a fraction of 27 kDa had been identified by Gasser et al. in 1989 (17) in a study carried out with dogs. This fraction, as an antigen, exhibited a high diagnostic sensitivity (95%) in SDS-PAGE of radioiodinated protoscoleces immunoprecipitated with sera from naturally infected dogs. We postulate that our 27- and 28-kDa antigens are expressed not only in protoscoleces but also in adults and thus may correspond to the above-mentioned adult stage antigen.

Moreover, individually affinity-purified antibodies specific for each band (27 and 28 kDa) reacted simultaneously with both of the bands by immunoblotting. Possibly, the two different molecules have epitopes in common or one of the molecules appears as a breakdown product with a slightly decreased mass. Finally, affinity-purified antibodies obtained from CE patients were uniquely of the IgG4 isotype. This was in line with the findings of Ortona et al. in 2005 (28), who reported on a tegumental protoscolex protein called EgTeg, which appeared upon the screening of an *E. granulosus* cDNA library with IgG4 obtained from CE patients with active disease. In that study, immunoblotting with mouse anti-EgTeg revealed a 27-kDa fraction (among other bands) in the protoscolex extract that also did not appear in HF. More generally, IgG4 subclass antihydatid antibodies have been reported to be associated with cyst development and disease progression, and

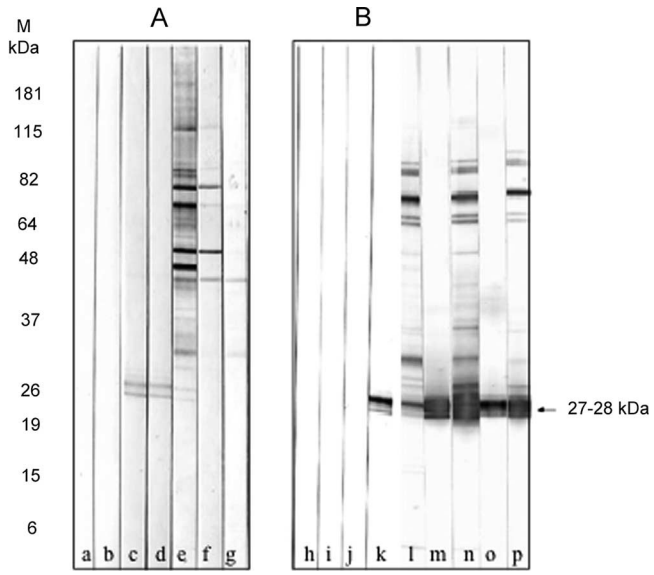

FIG. 3. (A) Immunoblotting of HF (lanes a and b) and PSSA (lanes c to p) probed with affinity-purified antibodies specific to 28-kDa (lanes a and c) and 27-kDa (lanes b and d) bands, as revealed with anti-human IgG-peroxidase conjugate. Lanes e to g: immunoblotting of PSSA probed with polyclonal rabbit anti-sheep HF (lane e), negative control rabbit serum (lane f), and conjugate control (lane g), as revealed with anti-rabbit IgG-alkaline phosphatase conjugate. (B) Immunoblotting of PSSA probed with affinity-purified antibodies specific for either the 27-kDa or the 28-kDa band (lanes h to k) and revealed with anti-human immunoglobulin specific to IgG1 (lane h), IgG2 (lane i), IgG3 (lane j), or IgG4 (lane k). Lanes l to p: sera from five NCCE patients (lane l), serum from patient 21 taken 1 year postsurgery (lane m), serum from patient 22 taken 1 year postsurgery (lane n), serum from patient 23 taken 2 years postsurgery (lane o), serum from patient 24 taken 5 years postsurgery (lane p), serum from patient 25 taken 2 years postsurgery. These strips were revealed with an anti-human IgG4 conjugate.

their usefulness as a marker for parasite development has already been discussed (7, 8, 33, 35). In conclusion, serology with PSSA appears to be a useful approach for postsurgical follow-up, especially when applied in immunoblotting based upon the detection of the 27- and 28-kDa doublet. In light of these findings, we will focus our further studies on more-detailed molecular analyses of these two molecules.

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