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Relevance of circulating antigen detection to follow-up experimental and human cystic hydatid infections

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SUMMARY

We analysed specific antibody (Ab) and circulating antigen (CAg) profiles along experimental mouse infection using as control a group of mice immunized with intact but dead parasites. Results from this experiment showed an early major CAg peak followed by a larger Ab peak which partially overlaps with other minor CAg peaks. These results suggest that CAg may be a marker of early mouse infection. In order to study the relevance of these findings in humans we similarly analysed by ELISA 148 sera provided by retrospective post-surgical follow-up of 19 patients. Available records showed that 14 patients developed new cysts one to ten years after surgery while no new disease was observed in the other five. Some of the former patients showed CAg as early as two months after surgery while no CAg was observed in the other five patients at any time. In addition, a collection of 38 sera obtained before surgery were similarly tested and five of them showed only CAg, while 18 showed only Ab and 12 sera showed Ab&CAg. These results in humans are consistent with the findings in the mouse experimental model and suggest that CAg may be an early marker of hydatid infection, thus being relevant for post-surgical follow-up.

Keywords E. granulosus, *circulating antigen, secondary hydatidosis in mice, human diagnosis*

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INTRODUCTION

The immunodiagnosis of *Echinococcus granulosus* infection in humans is normally achieved through specific antibody (Ab) detection. One of the main problems of the immunodiagnosis is the existence of up to 25 percent of surgically confirmed patients showing no detectable Ab concentrations whatever technique or antigen is used (Rickard & Lightowlers 1986, Maddison *et al*. 1989, Hira, Bahr & Behbehani 1990, Siracusano *et al*. 1991; Liu, Lightowlers & Rickard 1992a, Force *et al*. 1992, Barbieri *et al*. 1993, Lightowlers & Gottstein 1994).

This problem of false negatives may be due to secretion/excretion by the parasite of high levels of circulating antigen (CAg) which generate circulating immunocomplexes (CIC) after reacting with Ab, interfering in this way with Ab detection (Craig & Nelson 1984, Craig, Macpherson & Nelson 1986).

Several authors have demonstrated the existence of CAg and CIC in hydatid patients (Bekhti *et al*. 1977, Richard-Lenoble *et al*. 1978, Pini, Pastore & Valesini 1983, Gottstein 1984, Craig & Nelson 1984, Craig 1986, Schantz 1988, Kanwar & Vinayak 1992, Liu, Rickard & Lightowlers 1993, Bonifacino *et al*. 1993, Barbieri *et al*. 1994). The CAg detection has been proposed to increase the diagnosis sensitivity (Shariff & Parija 1991, Liu, Rickard & Lightowlers 1993, Shariff & Parija 1993, Barbieri *et al*. 1994) but it has not been possible to achieve 100%. As the serum samples are taken from patients at different times during infection, a possible association between the infection stage and the false negative problem may exist.

In order to analyse this problem we studied the Ab and CAg profiles during infection in an experimental model to see if there is some association between Ab titres and CAg levels and the duration of infection. Treatment of hydatidosis is widely based on surgery, sometimes combined with chemotherapy with antihelminthics of the benzimidazole

family (Eckert 1986, Teggi, Lastilla & De Rosa 1993, Craig 1993). Surgery is practically the only efficient treatment in spite of the fact that it shows an important risk of spillage of protoscoleces (PSC) which can generate new cysts thus producing secondary infections. It also has been suggested that it is possible to prevent reinfection by means of chemotherapy (Todorov *et al*. 1992, Teggi *et al*. 1993, Sarciron *et al*. 1993). Therefore, it is necessary to achieve an early serologic detection of relapse (either a new cyst grown after surgery or an unnoticed existing cyst before surgery), including its discrimination from a possible chronic humoral response.

Considering the experimental secondary infection as a mimick of what happens after the surgical spillage, the study of the response early post-infection may allow us to detect some parameters that may be indicative of a relapse. The experimental secondary hydatidosis by intraperitoneal (i.p.) inoculation of PSC in mice is a model that has been widely used (Heath 1970, Araj, Matossian & Frayha 1977, Cox, Marshall-Clarke & Dixon 1989, Janssen *et al*. 1992; Liu, Lightowlers & Rickard 1992b, Hernández & Nieto 1994, Díaz, Ferreira & Nieto 1995, Ferragut & Nieto 1996, Miguez, Baz & Nieto 1996).

Comparison of the profile of Ab response as well as CAg levels during the development of mouse hydatid infection, with the data obtained from mice immunized with dead PSC, would provide relevant information on the levels of those two parameters (Ab and CAg) at different stages during the progress of infection in the intermediate host. This may be used to improve immunodiagnosis of the disease in humans.

In this paper, we analyse Ab and CAg profiles weekly during 68 weeks in mice either infected or immunized with dead PSC as well as the relevance of those findings in human serology.

MATERIALS AND METHODS

Human sera

From 38 surgically confirmed hydatid patients 186 serum samples were obtained over the years. Of these sera 148 corresponded to a retrospective post-surgical follow-up of 19 patients. Another 72 sera were obtained from patients with other diseases produced by: *Ascaris lumbricoides* ($n=5$), *Trichinella spiralis* ($n=5$), *Taenia solium* ($n=10$), *Schistosoma mansoni* ($n=5$), *Giardia lamblia* ($n=5$), *Trypanosoma cruzi* ($n=5$), *Fasciola hepatica* ($n=6$), *Entamoeba histolytica* ($n=6$), *Toxocara canis* ($n=5$), *Toxoplasma gondii* ($n=5$), filaria spp. ($n=10$) and mononucleosis ($n=5$). Thirty sera from healthy donors were also included.

Normal sera

Normal bovine serum (NBS) was obtained from Swedish cattle free of hydatid infection. Normal rabbit serum (NRS) was obtained from a healthy adult rabbit.

Antigens

Bovine hydatid cyst fluid antigen (HCFA) was prepared as described by Ferragut & Nieto (1996). Lipoprotein antigenic fraction (HBLF) was prepared from fertile bovine cyst fluid according to Barbieri *et al*. (1993).

Protoscoleces (PSC)

PSC from bovine cysts were obtained as described by Ferragut & Nieto (1996).

Antibodies

Rabbit anti-mouse immunoglobulins were prepared as described by Ferragut & Nieto (1996). Rabbit anti-HBLF immunoglobulins were prepared according to Barbieri *et al*. (1994). Affinity purified anti-HBLF antibodies were prepared from rabbit anti-HBLF antibodies immunopurified using a column (2 ml) of HBLF coupled to CNBr-activated Sepharose (Pharmacia) according to Barbieri *et al*. (1994).

Enzyme conjugates

Rabbit anti-mouse as well as rabbit anti-HBLF immunoglobulins were conjugated with peroxidase (Type VI-A, Sigma, St Louis MO, USA) according to Tijssen (1985).

Experimental design

The experimental protocols (infection and immunization of Balb/c mice) were described by Ferragut & Nieto (1996). Briefly, each of seventeen four-week-old mice was i.p. injected with a suspension of 2000 viable PSC in 0·5 ml of sterile PBS, containing 100 U/ml of Penicillin G and 100 mg/ml of Streptomycin according to Araj *et al*. (1997). Similarly, three four-week-old mice were i.p. immunized with a suspension of morphologically intact but non viable PSC, prepared by treatment of PSC with 70% ethanol. Six uninoculated mice served as sentinels. Mice were weekly bled during 68 weeks, sera were aliquoted and stored at −20°C. Pools were prepared by mixing equal volumes of sera from individuals of each group corresponding to the same sampling day. All mice were autopsied after the 68th week (see Ferragut & Nieto 1996).

ELISA for Ab detection

ELISA for mouse Ab detection was carried out according to Ferragut & Nieto (1996). ELISA for human Ab detection was carried out by incubating for 48 h 100 μl/well of 20 μg/ml of HBLF in 0·1 M glycine pH 8·2 containing 0·5 M NaCl and 1 g/l NaN_3 in polystyrene microtitre plates (Greiner, Germany). After discarding the coating solution, the plates were blocked, washed and serum samples were incubated as described by Ferragut & Nieto (1996). After washing, 100 μl/well of peroxidase-conjugated rabbit anti-human immunoglobulins (Nordic, The Netherlands) were added. The conjugate was appropriately diluted in phosphate-buffered saline (0·01 M phosphate, 0·15 M NaCl, pH 7·2, PBS) containing 0·05% Tween 20 and 1% (w/v) bovine serum albumin (PBS-T-BSA), as previously determined by checkerboard titration. After overnight incubation at 4°C in a moist chamber, plates were washed and finally 200 μl/well of the substrate solution were added. The substrate solution was prepared by dissolving 1 mg/ml of recrystallized 5-aminosalicylic acid (Ellens & Gielkins 1980) in 0·01 M PBS pH 7·1 with 0·01 M EDTA and 20μl of 30% H_2O_2 per 100 ml of solution. After 10 min incubation under shaking, the reaction was stopped by addition of 50 μl/well of 0·1 M NaOH, and the OD at 450 nm (OD_{450}) was measured in a Titertek Multiskan Plus reader (Flow Laboratories, Helsinki, Finland).

ELISA for CAg detection

ELISA was carried out as described by Barbieri *et al*. (1994). Briefly, plates were coated with affinity purified anti-HBLF antibodies and blocked with BSA. Serum samples, diluted in PBS-T-BSA, were subsequently dispensed (100 μl/well) and incubated for three h. Each sample was tested in four twofold serial dilutions (starting dilution 1:20 for human sera and 1:10 for mouse sera). After washing, 100 μl/well of peroxidase-conjugated rabbit anti-HBLF antibodies were added. The conjugate was appropriately diluted in PBS-T-BSA containing 1% NBS and 1·5% NRS. Plates were incubated overnight at 4°C in a moist chamber, after washing 200 μl/well of substrate solution were added. The substrate solution for detection of CAg in mice was 3-methyl, 2-benzothiazolinone hydrazone hydrochloride and 2-dimethylaminobenzoic acid (Ngo & Lenhoff 1980), while the substrate used with human samples was recrystallized 5-aminosalicylic acid (Ellens & Gielkins 1980) as described above. OD_{600} were measured in the case of mouse samples while OD_{450} in the case of human samples.

As several freeze-thaw cycles may affect CAg stability (Barbieri *et al*. 1994) this test was done using serum aliquots with identical freeze-thaw history.

An ELISA to evaluate the interference possibly due to rheumatoid factor in patients sera was carried out using the same protocol, but coating the plates with 10 μg/ml of non-immunized rabbit immunoglobulins in PBS.

Data analysis

Ab concentrations expressed as arbitrary units (au/ml) and OD_{600} corresponding to dilutions of a pool of serum samples from infected mice used as reference serum were correlated by linear regression.

Human anti-hydatid reference serum (Sanchez-Sus, Martinez & Guisantes 1986) was kindly provided by Dr Guisantes (Vitoria, Spain). The values of Ab concentration and OD_{450} corresponding to dilutions of the reference serum were correlated by linear regression.

ELISA (OD) values from every sample was converted to Ab concentrations equivalent to the respective reference for analytical consistency (Malvano *et al*. 1982).

As standard for CAg, HCFA was added to a pool of normal mouse and human sera, respectively, in the range of 0·5 μg/ml to 5 ng/ml.

The values of HCFA concentrations and OD corresponding to dilutions of the standard were correlated by linear regression. ELISA levels corresponding to every sample were converted to CAg concentrations equivalent to the respective standard.

Thirty sera from healthy donors were used to determine the cut-off value calculated as the mean plus 3 standard deviations for Ab detection and the mean plus 2 standard deviations for CAg detection, respectively.

The sensitivity, specificity and diagnostic efficiency were calculated as described by Galen & Gambino (1975). In brief:

$$\text{Sensitivity} = \frac{\text{positive results}}{\text{Number of true positive} + \text{false negative results}} \times 100$$

$$\text{Specificity} = \frac{\text{negative results}}{\text{Number of true negative} + \text{false positive results}} \times 100$$

$$\text{Diagnostic efficiency} = \frac{\text{Number of true positive} + \text{true negative results}}{\text{Number of true positive} + \text{false positive} + \text{true negative} + \text{false negative results}} \times 100$$

RESULTS

Ab response and CAg detection in mice

The HCFA-specific Ab response was weekly analysed by

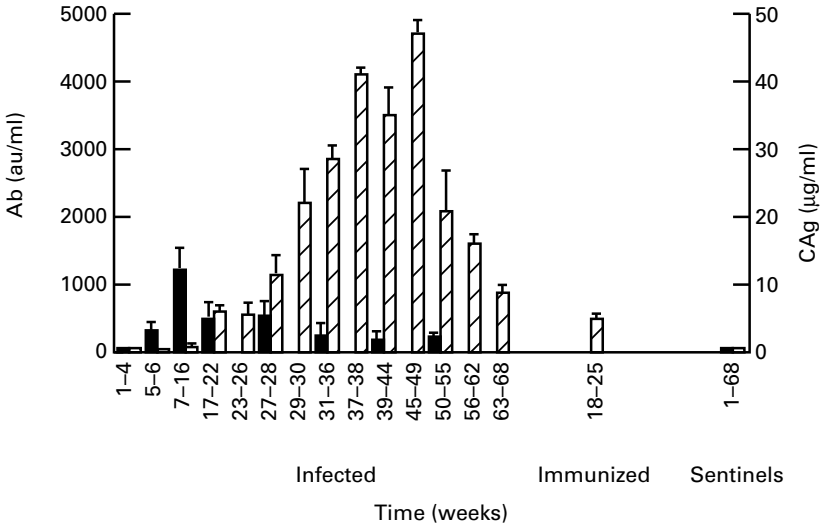

Figure 1 The profiles correspond to the Ab titres (hatched bars) and CAg levels (solid bars) measured during 68 weeks in pooled sera from weekly bleedings of 17 infected mice, three mice inoculated with intact but non viable PSC (immunized) and six sentinel mice.

ELISA during the whole course of the experiment (68 weeks) in pooled sera from the three groups of mice (infected, immunized and sentinels) (Figure 1). The pools from the infected animals showed increasing Ab titres from week 19 up to week 49 and decreasing thereafter up to the end of the experiment. Only one lower titre peak was observed from weeks 18 to 25 in mice injected with dead PSC. Sera from sentinel mice showed background Ab levels during all the experiment.

Capture ELISA was performed for CAg detection also in pooled sera from the three groups of mice (Figure 1). High levels of CAg were observed in infected mice from weeks 5 to 22 p.i. and weeks 27–28. Three minor CAg peaks, exhibiting six times lower CAg concentrations, were observed during the rest of the infection (Figure 1). No CAg was observed during the whole experimental protocol in mice injected with dead PSC.

Ab and CAg detection in hydatid patients

HBLF-specific Ab titres as well as CAg concentrations were analysed by ELISA in sera from 38 pre-surgery samples and from 72 patients with other parasitic and infectious diseases.

Table 1 summarizes the results. Sensitivity for Ab detection was 79% and specificity was 93%, while combining Ab and CAg detection the corresponding results were 92% and 85%, respectively.

Diagnostic efficiency for Ab detection was 88% while combining Ab and CAg detection was 87%.

Sera from five patients infected with filaria spp. were false positive in ELISA for Ab detection. Patients infected with *A. lumbricoides* ($n=3$), *T. solium* ($n=1$), *G. lamblia* ($n=1$) and *F. hepatica* ($n=1$) were false positive for CAg detection.

Table 1 Ab and CAg detection in human sera

	Ab+	CAg+	Ab/CAg+
Hydatid patients $n=38$	30	17	35
Other parasitic diseases $n=72$	5*	6†	11

Specific Ab titres and CAg levels observed in sera sampled before surgery from different surgically confirmed hydatid patients ($n=38$) as well as in 72 sera from other parasitic and infectious diseases. Thirty sera from healthy donors were used to determine the cut-off value. * Filarial infected patients ($n=5$). † Patients infected with : *Ascaris lumbricoides* ($n=3$), *Taenia solium* ($n=1$), *Giardia lamblia* ($n=1$), *Fasciola hepatica* ($n=1$)

Table 2 Performance of immunodiagnostic tests related to cyst location

				Sensitivity (%)	
Cyst location	Ab+	CAg+	Ab/CAg+	Ab	Ab/CAg
Liver ($n=17$)	13	7	15	76	88
Lung ($n=9$)	7	6	9	78	100
Others ($n=12$)	10	4	11	83	93
Total ($n=38$)	30	17	35	79	92

Specific Ab and CAg detection in sera obtained before surgery from different surgically confirmed hydatid patients ($n=38$) with known cyst locations.

The performance of the immunodiagnosis according to cyst location also was analysed (Table 2). In hydatid patients with lung cysts the sensitivity increased from 78% to 100% combining data of Ab and CAg compared to Ab detection alone, while in patients with liver cysts the sensitivity

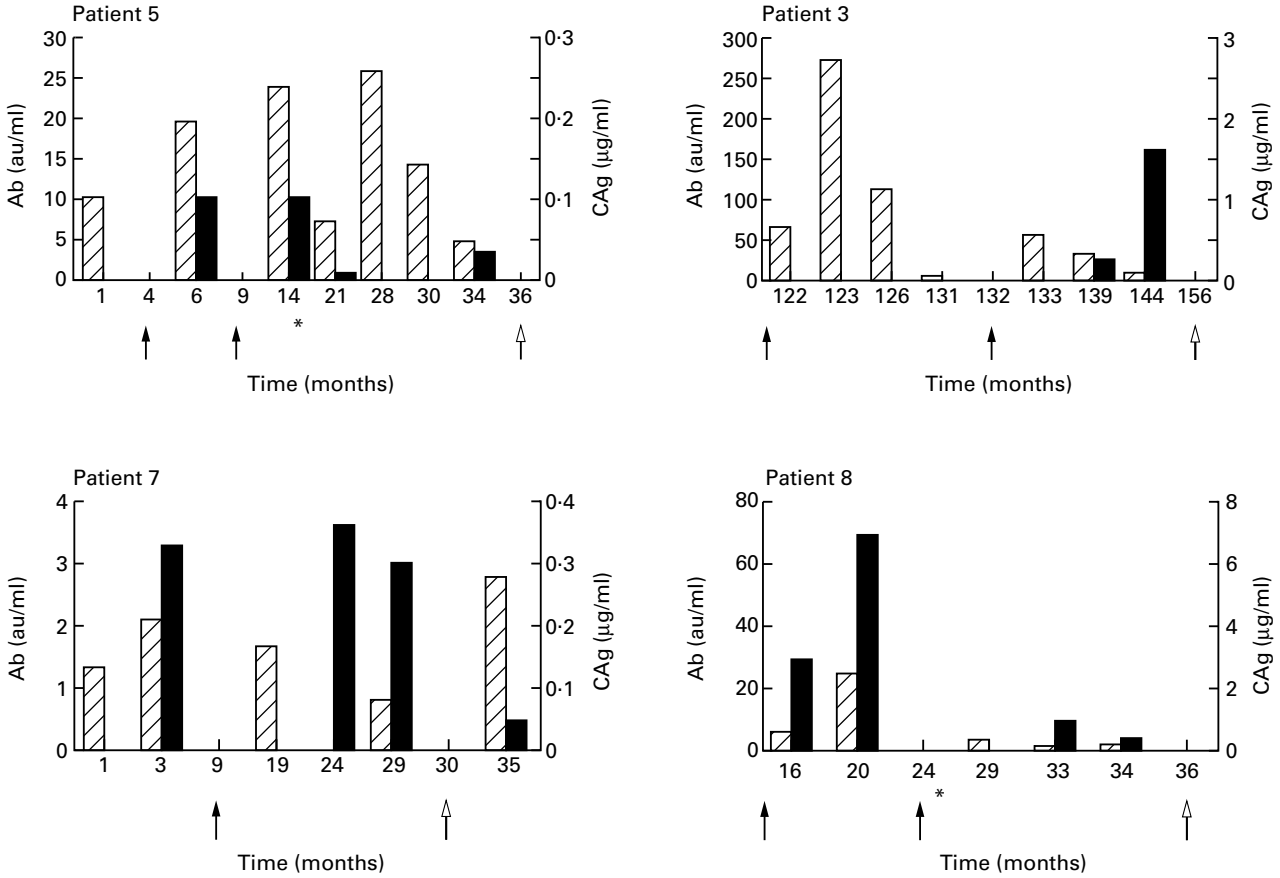

Figure 2 Profiles of Ab (hatched bars) and CAg (solid bars) in sera provided by retrospective post-surgical follow-up of hydatid patients with only liver cysts. ↑ surgery; * treatment; ⇑ new cyst diagnosed. All Ab titres and CAg levels were corrected for the respective background value observed in healthy donors.

increased from 76% to 88%. In patients with other or more than one location the observed increase was from 83% to 93%.

All CAg positive samples were analysed for unspecific reaction possibly due to rheumatoid factor. It was found that sera from 15 patients with other diseases were positive when ELISA was performed to evaluate the interference. This interference was eliminated (sera became CAg negative) in all cases adding 5% NRS to the buffer used to dilute samples and 5% of normal human sera to that used for conjugate dilution.

Follow-up of hydatid patients

One hundred and forty-eight extractions provided by retrospective post-surgical follow-up of 19 patients were analysed by ELISA for Ab and CAg detection. Fourteen patients showed positive CAg results in sera obtained between two and seven months after surgery. All of them developed new cysts one to ten years later. The CAg concentration profiles of some of them are shown in Figures 2 and 3. Five patients showed negative CAg results from 0, 5 to 76 months after surgery and 5–8 years later no disease was observed (data not shown).

The profiles of four patients (numbers 5, 3, 7 and 8) with liver cysts are shown in Figure 2. Patient number 3, showed increased CAg levels concomitant with decreased Ab titres. This patient was operated on twice. In the second operation, the cyst was broken during surgery. Seven months later, positive CAg results were observed and two years later a new liver cyst was observed. Another two patients with liver cysts, numbers 4 and 9 (data not shown), showed increased CAg levels concomitant with decreased Ab levels. On the other hand, patients numbers 13 and 14 with liver cysts (data not shown), showed low Ab titres profiles concomitant with high CAg. Patient number 5 of Figure 2 showed CAg as early as two months after surgery. This figure also shows the profile corresponding to patient number 8, with a liver cyst which showed an Ab peak associated with high levels of CAg.

Figure 3 shows the profiles of other four patients with different cyst locations: patient number 2 with lung cyst,

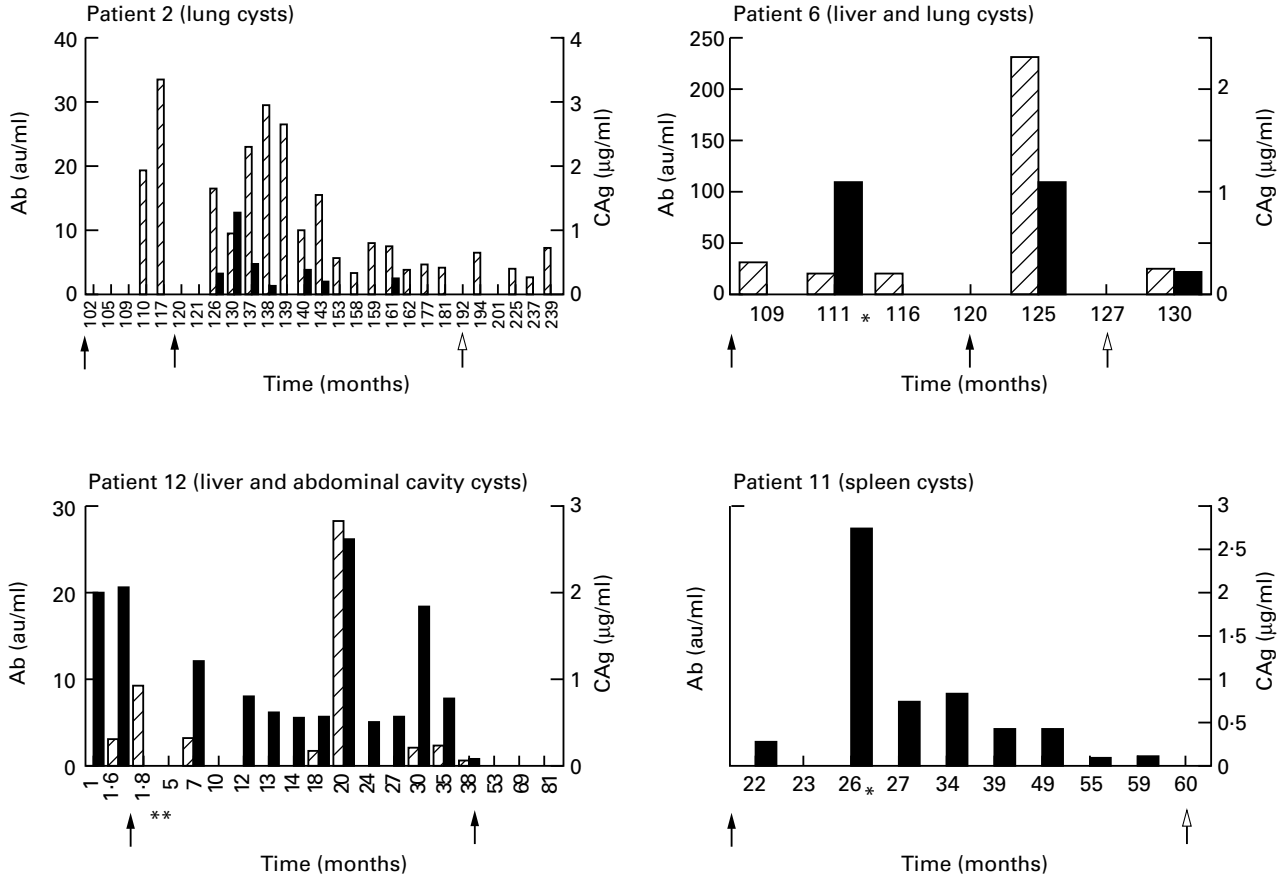

Figure 3 Profiles of Ab (hatched bars) and CAg (solid bars) in sera provided by retrospective post-surgical follow-up of hydatid patients. ↑surgery; * treatment; ⇧ new cyst diagnosed. All Ab tires and CAg levels were corrected for the respective background value observed in healthy donors.

number 6 with liver and lung cysts, number 12 with liver and abdominal cavity cysts and number 11 with spleen cyst. Patient number 11 showed negative Ab results and positive CAg results in several samplings after surgery. Other two patients: number 10 with lung cyst and number 1 with liver cyst (data not shown) showed similar profiles. Figure 3 also shows the profile corresponding to patient number 6 with a peak of Ab titre coincident with high levels of CAg. This patient was operated on for liver and lung cysts, then was operated on again due to a lung relapse.

Patients numbers 2, 12, 5 and 7 showed either periods when both, Ab and CAg were detected, or periods when only Ab was detected as well as other periods when CAg and none or low levels of Ab were detected. Patients numbers 5, 6, 8, 10, 11, 12, 13 and 14 were treated with benzimidazole drugs. After treatment, a decrease of CAg levels was generally observed. Particularly, patient number 11 showed a significant decrease in cyst size (5 to 3 cm) after treatment.

DISCUSSION

An early CAg peak was observed in infected mice between weeks 5 and 22 followed by a large Ab peak from week 19 to week 60, partially overlapping with minor late CAg peaks. Therefore, there were periods of the infection in which both Ab and CAg were detected; others, when high levels of Ab and absence or low levels of CAg were observed, and very late, no CAg was detected while Ab titres declined (Figure 1). These results are consistent with the hypothesis that high levels of Ab can be associated with low levels of CAg probably due to formation of Ag-Ab complexes (Craig & Nelson 1984, Craig *et al*. 1986). On the other hand, in immunized mice no CAg was detected. These results suggest that: a) the existence of CAg in sera of experimentally infected mice could be an early marker of infection, and b) titration of CAg and Ab provides a tool to identify infected mice except during late infection. This last situation is consistent with the hypothesis that the false

negative results observed in some hydatid patients may be associated with late chronic infections.

These results also suggest that the CAg ELISA test could be appropriate to follow hydatid patients after surgery to make an early diagnosis of a relapse as well as to assess the efficacy of chemotherapy.

In order to evaluate the relevance of the serological findings in the experimental mouse infection for human diagnosis, we analysed Ab and CAg profiles in serum samples obtained from hydatid patients before and after surgery. In the latter case, we included serial samples to provide follow-up information. There were 8 Ab false negative patients with the following cyst locations: 4 with liver cyst, 2 with lung cyst and 2 with other or more than one location. The 5 Ab false positive patients had filarial infections (Table 1).

An interference possibly due to rheumatoid factor was described in CAg detection by Nelson & Craig (1984) and Barbieri *et al*. (1994). We detected this unspecific reaction in 15 sera from patients with other diseases. Nonetheless, it was eliminated by addition of NRS to the buffer used in the dilution of the samples and normal human serum to the buffer used to dilute the conjugate.

In CAg detection, 17 hydatid patients showed positive results while 6 false positive results were also observed. Five patients showed positive CAg results and negative Ab results. In addition, 12 patients were positive in both tests, and 18 were Ab positive only. These results are consistent with what was observed in the mouse where the higher CAg concentrations were observed in stages of the infection different from those the higher Ab concentrations.

The sensitivity of the serology for human hydatidosis showed a 13% increase when detection of Ab was combined with that of CAg, in comparison with Ab alone. As far as specificity is concerned, it decreased from 93% for Ab alone to 85% for Ab combined with CAg. The diagnostic efficiency was similar with both approaches. These studies suggest that better sensitivity may be achieved combining the detection of Ab with that of CAg.

Correlation of the previous results with corresponding cyst locations is shown in Table 2. That table shows that when both tests were combined the sensitivity increased in all cases. It is important to note that in the case of patients with lung hydatidosis, the one associated with the major false negative Ab results (Todorov *et al*. 1979), combination of both assays increased sensitivity to 100%, as described by Barbieri *et al*. (1994).

It is interesting to analyse the time profile of CAg concentration after surgery, as it may be an early marker of infection in humans. Several post-surgical samples of 19 patients were analysed to evaluate if CAg can be appropriate to follow hydatid patients after surgery. This was a retrospective study using a sera collection which had been obtained 10 years before. The clinical and surgical history of these patients showed that some of them had been operated more than once.

All the 14 patients showing positive CAg results showed new hydatid cysts between one to ten years later. The profiles of CAg levels and Ab titres of some patients are shown in Figure 2 and 3. Patients with fluctuating titres both of CAg and Ab were observed, as well as patients with positive CAg results in the absence or very low levels of Ab. Relevant differences among the titres of CAg and Ab in the different patients were observed.

There were five out of 19 patients in which CAg was not detected after surgery (data not shown) and none of them showed any symptom of disease up to 5–8 years later.

These results suggest that the post-surgical detection of CAg could be a useful marker for the early diagnosis of hydatid reinfection. The post-surgical follow-up profiles of Ab and CAg in humans were consistent with those in the experimental infected mice. The great majority of follow-up profiles corresponds to patients with liver cysts. Varying Ab and CAg profiles were observed, as shown in the different plots (Figure 2 and 3), suggesting they do not depend on cyst location. Cyst integrity as well as other non-identified factors could explain those differences.

The moment some of the patients were treated with antiparasitic drugs, is also shown in these figures. A general fall of CAg levels was observed after that treatment, as well as increased Ab levels, except for patients numbers 10 (data not shown) and 11. The latter do not show detectable Ab levels in the extractions studied. In the case of patient number 8, Ab levels fall after chemotherapy.

We do not intend to evaluate the drug treatment, as different protocols were used with different patients. Moreover, a more exhaustive study should be made using a standardized protocol to follow treatment. As Ab levels remain high after treatment, also in the five cases with no reinfection, these preliminary data suggest that CAg would be better to evaluate the treatment. It was observed that in patients with bone hydatid cysts treated with albendazole (Bonifacino, Dogliani & Craig 1997), CAg detection may help to monitor the state of the parasite, the course of the disease and its outcome.

In conclusion, the Ab and CAg profiles during hydatid infection suggest that the CAg detection can be a marker of secondary infection. The combination of both Ab and CAg detections may improve the immunodiagnosis of cystic hydatid disease. Finally, the false negative results could be partly due to late chronic infections that, in mice, are associated with low levels of Ab and CAg. This hypothesis is consistent with the results observed in humans with

secondary infection although we do not have similar data from primary human infection.

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