

Assessment of monoclonal antibodies to *Echinococcus granulosus* Antigen 5 and Antigen B for detection of human hydatid circulating antigens

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SUMMARY

Four monoclonal antibodies (MAb) to *Echinococcus granulosus* Antigen 5 (Ag5) and Antigen B (AgB) were assessed in an enzyme-linked immunosorbent assay (ELISA) for detection of circulating antigens (CAg) in sera of human patients with *E. granulosus* infection. Around 5.5–8% of 200 sera from 42 surgically proven hydatid patients contained detectable CAg by individual MAb. The combined detection rate for CAg, using four MAb, was 19% (38/200). Although hydatid CAg was detected by MAb in at least one serum sample from 21 of 42 patients, some patients remained negative in the assay regardless of the time when serum samples were taken (pre- or post-operatively), or of the continuing presence of hydatid cysts, their location or fertility. In addition, it was observed that the binding capacity of MAb for sheep hydatid cyst fluid antigen (SHCF) was somewhat reduced in the presence of normal human serum. The CAg detection assay would only be useful for assessment of hydatid infection status in patients with detectable CAg in serum samples.

Key words: *Echinococcus granulosus*, Antigen 5, Antigen B, monoclonal antibody, circulating antigen, hydatidosis.

INTRODUCTION

Diagnosis of *Echinococcus granulosus* infection in humans is normally achieved through detection of specific antibodies using an assay of high sensitivity (e.g. ELISA) for initial screening followed by a test of relatively high specificity (e.g. immunoelectrophoresis (IEP)) for confirmation (Rickard, 1984; Rickard *et al.* 1984). However, many routinely used immunodiagnostic tests do not differentiate *E. granulosus* (unilocular) hydatid disease from *E. multilocularis* (alveolar) hydatid infection in man. Further, detection of antibodies in sera from patients with hydatidosis by ELISA or indirect haemagglutination (IHA) results in false positives due to other parasitic infections such as *Taenia solium* cysticercosis, filariasis and schistosomiasis (Farg, Bout & Capron, 1975; Speiser, 1980; Rickard *et al.* 1984; Craig, 1986). Even specific tests such as IEP, based on the identification of Arc 5, can give cross-reaction with sera from patients with *T. solium* cysticercosis (Schantz, Shanks & Wilson, 1980). False negative results can also occur in hydatid antibody detection assays (Chemtai, Bowry & Ahmad, 1981). This phenomenon of false negatives prompted speculation on the role of circulating antigens (CAg) and circulating immune complexes

(CIC) in 'mopping up' specific antibodies and rendering them undetectable by conventional antibody assays (Richard-Lenoble *et al.* 1978).

CIC and CAg have been demonstrated in sera from hydatid patients in several studies (Bekhti *et al.* 1977; Richard-Lenoble *et al.* 1978; Pini, Pastore & Valesini, 1983; Craig & Nelson, 1984; Gottstein, 1984; Craig, 1986). Craig & Nelson (1984) showed that sera from hydatid patients which were serologically negative in antibody tests often contained measurable CIC and CAg. While sera from human patients with filariasis, taeniasis and fascioliasis cross-reacted strongly with SHCF antigen in ELISA using anti-human IgG, IgM, IgA, IgE class specific conjugates, the CIC in these sera were negative in CAg-ELISA for hydatid diagnosis (Craig, 1986). Gottstein (1984) also demonstrated that sera from patients cured by radical surgery had decreased amounts of CAg compared to samples taken before the operation.

Being of single specificity, MAb provide unique opportunities for improving CAg detection in parasitic infections (Weil *et al.* 1985; Feldmeier *et al.* 1986; Robertson *et al.* 1988). In this report, we assess MAb to *E. granulosus* Antigen 5 (Ag5) and Antigen B (AgB) in an enzyme-linked immunosorbent assay (ELISA) for their potential in detection of CAg in sera of human patients with unilocular hydatidosis.

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MATERIALS AND METHODS

Sheep hydatid cyst fluid

The preparation of sheep *E. granulosus* hydatid cyst fluid (SHCF) has already been described (Liu, Lightowlers & Rickard, 1992a).

Sera

Two hundred sera from 42 surgically proven *E. granulosus* patients of Australian origin were selected from serum stock built up over 10 years for routine diagnosis of hydatid disease. Eighty normal human sera (NHS) collected from individuals with no exposure to hydatid parasites were used as negative controls.

Monoclonal antibodies

Monoclonal antibodies (MAb) to *E. granulosus* Ag5 and AgB were prepared and characterized as described (Lightowlers *et al.* 1989; Liu *et al.* 1992a; Liu, Rickard & Lightowlers, 1992b). Two MAb to Ag5 (24.14 and 61A12, of IgG_{2a} and IgG_{2b} subclasses, respectively) and two MAb to AgB (39B3 and 31.15, of IgG_{2a} and IgG_{2b} subclasses, respectively) were assessed in ELISA for hydatid CAg detection. MAb and normal mouse IgG (NMIgG) were purified from MAb and SP2 myeloma ascites through a protein A-Sepharose column (Pharmacia) for use in the assay.

CAg-ELISA

Polystyrene 96-well plates (Dynatech) were coated in triplicate with 50 µl/well of sera at 1/10 dilution in 50 mM bicarbonate-carbonate buffer (BCB), pH 9.6, for 2 h at 37 °C. After blocking with 100 µl of 1% BSA or 20% FCS in PBS, pH 7.4, for 30 min, 50 µl of MAb (24.14 and 61A12 at 1 µg/ml, 31.15 and 39B3 at 2 µg/ml) were added to two wells and 50 µl of NMIgG (2 µg/ml) to the third well. Following incubation with urease-labelled sheep anti-mouse IgG conjugate (CSL, Australia) at 1/500 dilution (50 µl/well), 50 µl of urease substrate (Chandler *et al.* 1982) were added to each well. The plates were read at an absorbance of either 450 or 590 nm. The arithmetic mean of each hydatid serum tested in duplicate was calculated and where the difference between two readings was > 0.1, the serum sample was retested.

The cut-off points of the CAg-ELISA were established through the reaction of MAb and NMIgG with 80 individual hydatid-free NHS. The arithmetic mean ($\bar{x}$) of absorbance values for each hydatid-free NHS in duplicate, following incubation with MAb and NMIgG in ELISA, was calculated as 0.220, 0.238, 0.269, 0.281 and 0.227 with standard

deviation (S.D.) of 0.021, 0.020, 0.031, 0.033 and 0.021 for MAb 24.14, 61A12, 31.15, 39B3 and NMIgG, respectively. The values of these sera with all MAb and NMIgG were normally distributed as analysed by chi-square (χ^2) test. Therefore, when the values of $\bar{x} + 2\text{s.d.}$ were used as positive cut-off points for hydatid patient sera, an absorbance value above 0.262, 0.278, 0.331 and 0.347 in the CAg-ELISA would indicate a most likely positive reaction for CAg in a serum sample with MAb 24.14, 61A12, 31.15 and 39B3, respectively.

The absorbance values from different test plates were standardized by using a batch of hydatid-free NHS, prepared by pooling 10 sera from individuals having absorbance values with the 4 MAb around the means of the 80 hydatid-free NHS with these MAb, as negative control since it was observed that the absorbance values of NHS with MAb displayed a linear increase with time.

The standardization process involved two steps. Firstly, a correction factor was derived from the mean absorbance value of 80 hydatid-free NHS with MAb, divided by the mean absorbance value of the pooled NHS with this MAb. For example, if in a test plate the mean absorbance value of the pooled NHS with MAb 24.14 was 0.244, the mean absorbance value of 80 hydatid-free NHS with this MAb was 0.220, the correction factor would be: $0.220/0.244 = 0.901$. This factor was used to multiply the values of individual hydatid sera with MAb 24.14 in the same plate resulting in an adjusted value. Correction factors for all plates with other MAb and NMIgG were calculated and used in the same way.

Secondly, by comparing the adjusted value of each hydatid serum with NMIgG to the mean absorbance value of NMIgG with 80 hydatid-free NHS, a second factor was obtained which was used to add to, or subtract from, the adjusted values for hydatid sera with MAb. For example, the adjusted absorbance value of a hydatid serum with NMIgG was 0.207, the mean absorbance value of NMIgG with 80 hydatid-free NHS was 0.227, the factor $0.227 - 0.220 = 0.007$ was added to the adjusted value of the hydatid serum with MAb. If the serum had an adjusted value of 0.195 with MAb 24.14, the final value of this serum with 24.14 would be $0.195 + 0.007 = 0.202$. All absorbance values for each patient serum with other MAb were adjusted in the same manner.

Determination of sensitivity of CAg-ELISA

The sensitivity of the MAb based CAg-ELISA was determined using serial dilutions (from 50–0 µg/ml) of artificially added SHCF in 1/10 NHS in BCB.

Examination of heat and acid treatments of NHS

The effect of heat treatment of human serum on

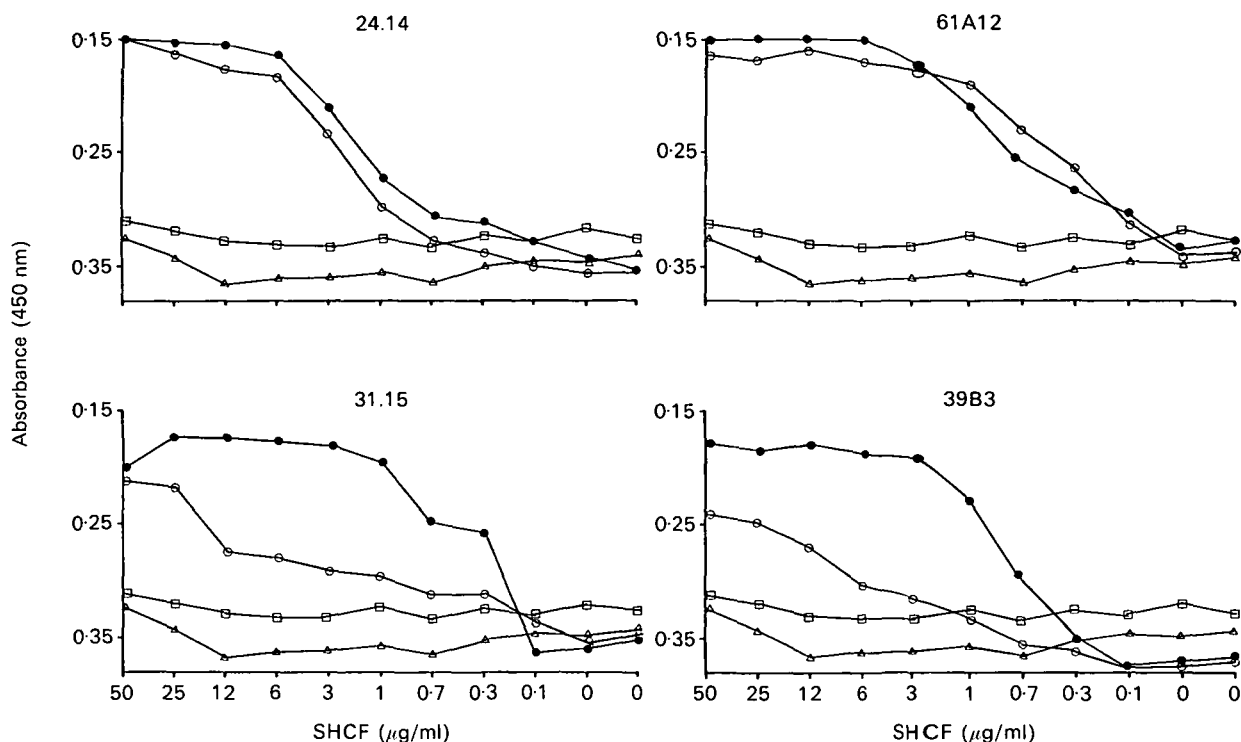


Fig. 1. Determination of sensitivity of CAg-ELISA. Plates were coated with SHCF (50–0 $\mu\text{g/ml}$) in 1/10 NHS in BCB or in BCB only, with the last two wells (shown as 0, 0) being no antigen controls (1/10 NHS in BCB, BCB only). After blocking with 1% BSA (for MAb 24.14, 61A12, and NMIgG) or 20% FCS (for MAb 31.15 and 39B3), the selection of which was based on their giving lowest noise: signal ratios in CAg-ELISA, MAb or NMIgG (at 1–2 $\mu\text{g/ml}$) were added. With urease-labelled sheep anti-mouse IgG conjugate and urease substrate, the plates were read at an absorbance of 450 nm. The heading 24.14 indicates SHCF in 1/10 NHS (○) or SHCF in BCB only (●) were examined by MAb 24.14, and SHCF in 1/10 NHS (□) or SHCF in BCB only (△) were examined by NMIgG. The same applies to headings 61A12, 31.15 and 39B3.

detection of hydatid CAg was examined because recent hydatid patient sera sent to this laboratory were routinely heated to 56 °C for 30 min as a precaution against hepatitis or LAV/HTLV-III virus contamination. In addition, acid treatment of hydatid patient sera was reported by Craig & Nelson (1984) to reduce the background of human sera and enhance the detection of CAg by rabbit antisera in antigen detection assay. Thus acid treatment of NHS was also examined here.

Heat treatment. A vol. of 120 μl NHS was added to 156 μl of SHCF (385 $\mu\text{g/ml}$) and heated at 56 °C for 30 min; after adding 925 μl of BCB, 50 μl volumes of serial dilutions (with SHCF ranging from 50 to 0 $\mu\text{g/ml}$) in 1/10 NHS (heated) in BCB were added to each well in polystyrene plates.

Acid treatment. A vol. of 120 μl NHS was added to 156 μl of SHCF and 600 μl of 3 N glycine, pH 3, and incubated for 1 h at room temperature; after adding 325 μl of BCB, the acid-treated serum-antigen mixture was diluted in 1/10 NHS (acid treated) in BCB as above.

Heat and acid treatments. A vol. of 120 μl NHS was added to 156 μl of SHCF and heated at 56 °C for

30 min, then added to 600 μl of 3 N glycine, pH 3, and incubated for 1 h at room temperature. After adding 325 μl of BCB, the serum-antigen mixture was diluted in 1/10 NHS (heat and acid treated) in BCB as above.

No treatment. A vol. of 120 μl NHS was added to 156 μl of SHCF and 925 μl of BCB, and then diluted in 1/10 NHS in BCB as above.

SHCF alone was also treated as above to use as control.

RESULTS

Determination of sensitivity of CAg-ELISA

MAb 24.14 and 61A12 detected 0.75 $\mu\text{g/ml}$ and 0.19 $\mu\text{g/ml}$ SHCF in the absence of NHS, but they could only measure 3.12 $\mu\text{g/ml}$ and 0.39 $\mu\text{g/ml}$ SHCF in the presence of NHS (Fig. 1). Similarly, MAb 31.15 and 39B3 were capable of detecting 0.39 $\mu\text{g/ml}$ and 0.75 $\mu\text{g/ml}$ SHCF without NHS, but only 1.56 $\mu\text{g/ml}$ and 6.25 $\mu\text{g/ml}$ SHCF with NHS (Fig. 1). NMIgG also showed higher non-specific binding with SHCF in the presence of NHS than in the absence of NHS (Fig. 1).

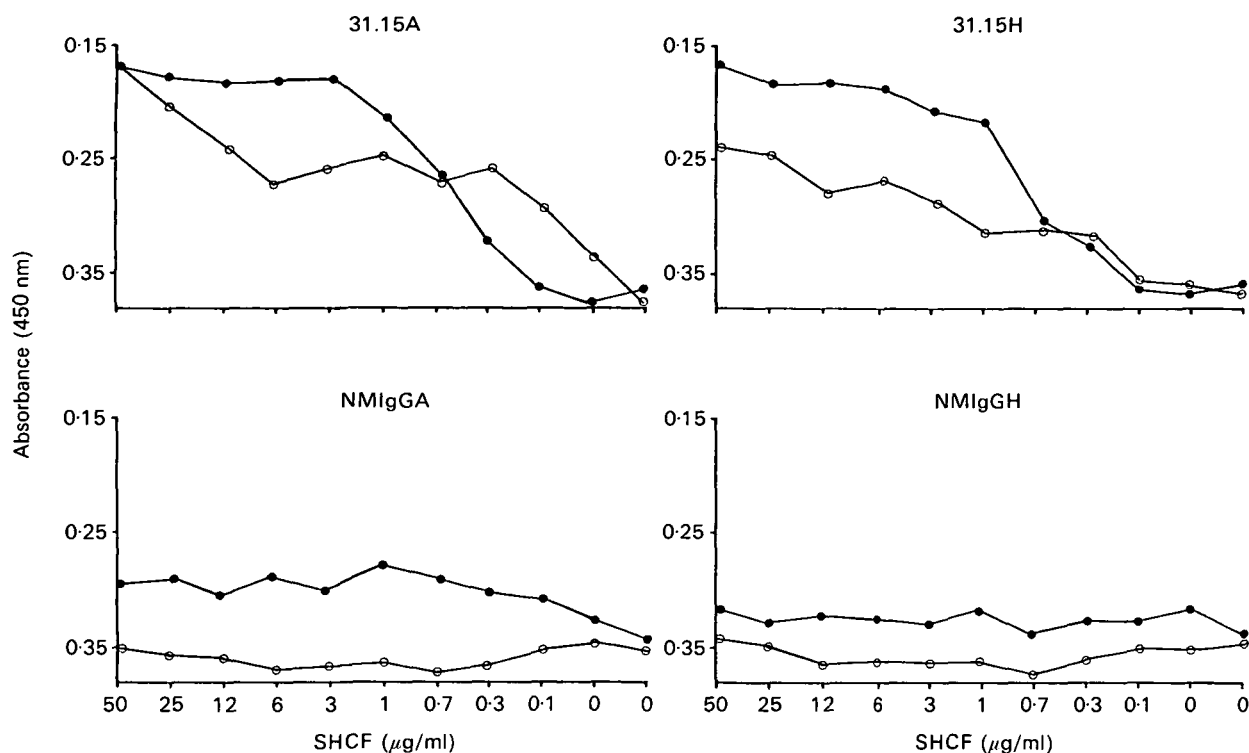


Fig. 2. Examination of acid and heat treatments of NHS. Plates were coated with 50–0 $\mu\text{g/ml}$ SHCF (acid or heat treated) in 1/10 NHS (also acid or heat treated) in BCB, the last two wells (0, 0) being no antigen controls, one with 1/10 NHS (treated as above) in BCB and the other BCB only. All further steps were the same as in Fig. 1. The heading 31.15A indicates that NHS and SHCF (○), or SHCF alone (●) were acid-treated before being examined by MAb 31.15. The heading 31.15H indicates that NHS and SHCF, or SHCF alone were heat-treated before being examined by MAb 31.15. The same also applies to NMigGA and NMigGH.

Examination of heat and acid treatments of NHS

As shown in Fig. 2, acid treatment of NHS together with SHCF increased the non-specific binding of MAb 31.15 and NMigG with NHS. Heat treatment of SHCF alone did not alter much the reactivity of SHCF with MAb 31.15, while heating SHCF together with NHS somewhat reduced the reactivity of SHCF with MAb 31.15. The combination of heat and acid treatments seemed to produce effects comparable to two treatments alone, namely non-specific binding of MAb 31.15 with NHS increased in wells with little or no SHCF while at the same time the reaction of MAb 31.15 with NHS reduced in wells with relatively large amounts of SHCF (data not shown). Similar results were also obtained with the other three MAb (data not shown).

Detection of circulating antigens in sera of hydatid-infected patients

Of the 200 sera from 42 hydatid patients examined, 11 (5.5%) were shown to have CAg detectable by MAb 24.14, 16 (8%) by MAb 61A12, 15 (7.5%) by MAb 31.15 and 15 (7.5%) by MAb 39B3. The combined detection rate using these four MAb was 19% (38/200). If the duration of serum samples taken before or after the current operation or the difference of primary and recurrent infections was

disregarded, 21 of 42 (50%) patients were shown to be CAg positive in at least one serum sample by one of the four MAb.

Although there was no correlation between serum antibody titres in ELISA and their absorbance values in the CAg-ELISA ($P > 10\%$), a significant correlation was observed between the lower IEP titres and higher absorbance values in the antigen assay using MAb ($P < 1\%$). That is, sera with IEP bands of 1–2 (0) or less were more likely to have higher absorbance values than those with IEP > 3 (0) or Arc 5 bands in the CAg-ELISA using 24.14, 61A12, 31.15 and 39B3.

Among 21 patients that had no detectable CAg in any of their serum samples by MAb (Table 1, Group A), eight (Nos. 3, 5, 6, 21, 25, 40, 41 and 42) were known to have hydatid cysts present in various organs, and two of them (Nos 6 and 25) actually had a second operation for hydatid cysts 34 and 44 months after the current surgery. Of the 21 patients in Group B (Table 1) that had CAg detected by MAb, fourteen (Nos 1, 4, 8, 10, 12, 16, 18, 23, 24, 27, 28, 29, 30 and 39) had CAg detectable in the later serum samples rather than in earlier samples, three of them (Nos 10, 27 and 29) were clearly shown to have hydatid cysts present. Patients No. 19 and 20 were CAg positive in their pre-operative sera, but became negative after the current surgery. A third patient (No. 17) also had reduced amounts of CAg in the serum samples about

Table 1. Data from 42 hydatid patients examined by MAb for CAg in the serum samples

Patient*		Post-operative (months)†					Hydatid history‡
Group	No.	0	≤ 6	≤ 12	≤ 18	> 18	
A	2	0/1	0/1	—	—	—	Liver cysts removed
	3	0/1	0/1	0/1	—	—	Cysts drained
	5	0/1	0/1	—	0/2	0/4	Liver cysts not removed
	6	—	—	—	0/1	0/2	Op 34 months after this one (lung)
	7	—	0/2	0/1	—	—	Liver cysts removed
	9	—	—	0/2	0/2	—	Op for fertile liver cysts
	11	—	—	0/1	0/1	0/2	Op for hydatid cysts
	13	—	0/1	0/1	—	0/1	Liver and pelvic cysts removed
	15	0/1	—	0/1	—	—	Fertile cysts removed
	21	0/1	0/1	—	—	—	Fertile cysts in pelvis drained
	25	—	0/3	—	0/1	0/10	Op (lung) 44 months after this
	26	—	0/1	—	0/1	0/6	Fertile liver cysts removed
	32	0/1	0/1	—	0/2	0/5	Op 8 y before this (liver)
	34	0/1	—	0/2	—	0/2	Op twice a few years before this
	35	0/1	0/1	0/1	—	0/2	Op 3 times before this
	36	—	—	0/1	—	0/4	Op many times before this (abdomen)
	37	—	0/1	—	0/1	0/1	Liver cysts removed 3 y before this
	38	—	0/1	0/3	—	0/1	Op 1 month before this, on MBZ
	40	—	0/2	0/2	0/1	0/2	Op 20 y before, has 3 cysts, on MBZ
	41	—	—	—	—	0/5	Still has 2 cysts after this, on MBZ
	42	—	—	—	—	0/5	Still has 3 cysts in liver
B	1	0/1	—	0/1	1/1	—	Op for liver cysts
	4	0/1	0/1	—	1/1	—	Op for tibial cyst
	8	—	—	—	0/1	1/4	Liver cysts removed
	10	—	0/1	0/1	1/1	—	Fertile liver cysts drained
	12	—	0/1	—	—	3/4	Calcified liver cysts removed
	14	1/1	1/1	1/1	—	—	Op for abdominal cysts
	16	0/1	—	1/2	—	—	Cardiac cysts excised, on MBZ
	17	1/1	0/2	—	1/1	1/3	Fertile cysts removed
	18	0/1	1/1	—	—	—	Liver cysts removed
	19	1/1	0/1	—	—	0/1	Fertile cysts removed
	20	1/2	—	—	—	0/1	Asymptomatic cyst removed
	22	1/1	2/3	—	—	—	Large liver cyst removed
	23	0/1	1/3	—	—	—	Liver cysts removed
	24	—	—	0/1	—	2/4	Fertile liver cysts removed
	27	—	—	0/2	1/2	1/4	Liver cysts drained
	28	0/1	0/1	2/2	—	—	Op 2 × before liver cysts removed
	29	0/1	0/1	—	1/1	1/3	Op 30 y before lung cysts drained
	30	—	0/1	—	—	2/3	Cysts ruptured 2 y before this
	31	0/1	1/1	1/2	1/1	0/4	Op 3 × before, 2 × after cyst rupture
	33	1/1	1/1	—	—	0/1	Op 5 times before cysts ruptured
	39	—	1/1	0/1	0/1	1/5	Op 32 y (lung) before this (liver)

* The 42 hydatid patients were divided into two groups on the basis of the absence (Group A) or presence (Group B) of circulating antigens (CAg) in at least one of the serum samples as examined by MAb.

† Sera from patients were grouped into 5 categories (0, ≤ 6, ≤ 12, ≤ 18 and > 18) according to the time (months) at which serum sample was taken after the current operation, with 0 being sera taken at 0 month (or pre-operative sera), and ≤ 6, ≤ 12, ≤ 18 and > 18 being sera taken at less or equal to 6, 12, 18 and more than 18 months after the current operation. The results of CAg-ELISA using MAb are shown as 0/1, indicating that 0 out of 1 serum contains CAg detectable by one of the four MAb. —, Serum samples at these times were not available for the study.

‡ The reference points for hydatid patients' history are based on the current surgical operation. All patients had been operated on these points. Any further operations that have taken place before or after these points were stated. op, Operation; y, years; 3 ×, 3 times; MBZ, mebendazole treatment.

6 months after operation, but this patient became CAg positive again 18 months later. Both patients No. 31 and 33 had increasing amounts of CAg after cyst ruptures (the main reason for the current operation), one (No. 31) went from negative to positive and the other (No. 33) from lower positive to higher positive.

DISCUSSION

The presence of CAg in patients is a clear reflection of the state of activity of hydatid cysts. By using four MAb to *Echinococcus granulosus* Ag5 and AgB in the CAg-ELISA, it was shown that circulating antigens could be detected in 11–16 of 200 serum samples

from 42 hydatid patients of Australian origin by individual MAb. When combining results from these four MAb, the overall detection rate for hydatid CAg in human patient sera increased from a maximum of 8% to 19%. Some patients (e.g. No. 19 and 20) had reduced amounts of CAg after surgery. Patients (No. 31 and 33) with ruptured cysts showed an increase of CAg in post-operative sera. However, more often, CAg levels in many patients did not change much after removal of hydatid cysts in that while some remained negative throughout (e.g. Nos 2, 15, 32, 34 and 35), others had detectable CAg from beginning to end (e.g. No. 14 and 22). The absence of CAg in sera of hydatid patients does, however, not rule out the possibility that hydatid infection is progressing. This was clearly demonstrated by the fact that eight patients with known hydatid cysts present were negative in the assay throughout, two of which even had another operation after the current surgery.

Craig (1986) found that the sensitivity of CAg-ELISA for Turkana patients was 90% and for UK patients 50%. The enhanced antigen detection for Turkana patients in CAg-ELISA was possibly due to the fact that hydatid cysts of Turkana patients tended to be fast growing, highly fertile and active, thus producing larger amounts of CAg. Not all sera from hydatid patients contained detectable CAg, for UK patients, IgM-CIC detection by ELISA would be more suitable for hydatid diagnosis than CAg detection. In this study, regardless of the serum samples being pre- or post-operative, 21 out of 42 (50%) patients had no detectable CAg. Perhaps this is an indication that hydatid patients of Australian origin, while similar to those of UK, are clearly different from those in Turkana in that the latter tend to have more CAg in the serum samples. In light of this finding, it appears that antibody detection rather than CAg detection should be the focus point of primary diagnosis of hydatid infection in general, as anti-hydatid circulating antibodies are usually more abundant and more readily detected than hydatid CAg. Antigen detection will only be valuable to those patients with detectable CAg in sera, and to those with clearly false negative results using antibody assays such as the hydatid patients from Turkana (Chemtai *et al.* 1981).

Analysis of the antigen detection data in relation to the serum antibody titres showed no correlation between antibody titres in ELISA and absorbance values in the MAb-based CAg assay. However, there was a significant correlation between lower IEP titres and higher absorbance values in the CAg test using MAb 61A12, 31.15 and 39B3. Perhaps sera with higher titres in IEP contained large amounts of precipitating antibodies which might react with CAg in the sera, resulting in the formation of antigen-antibody complexes (or CIC), thus reducing the amounts of CAg available for detection.

Nevertheless, the potential of the CAg-ELISA using MAb was limited by the fact that it had a relatively low sensitivity (detecting 0.39 µg/ml SHCF at best) compared with those (0.05–0.27 µg/ml SHCF) using polyclonal antisera (Craig & Nelson, 1984; Gottstein, 1984). Naturally, polyclonal antisera tend to provide better sensitivity than MAb because they can react with multiple epitopes of the antigen. Another major reason for the low sensitivity observed in this study was probably due to the interfering effect of normal human serum components on the binding of MAb or NMIGG with SHCF. Indeed the interference with immunoassays by human serum factors has been noted previously (Kato *et al.* 1979; Lew *et al.* 1982). Craig & Nelson (1984) were able to reduce the background binding in CAg assay through acid treatment of patient sera and use of rabbit antisera affinity-depleted of components binding to normal human serum. Attempts to minimize the interference of NHS with MAb by acid treatment of NHS and SHCF in this study, however, resulted in an increase in non-specific binding of MAb and NMIGG to NHS, possibly due to exposure by acid treatment of previously unavailable non-specific binding sites in NHS.

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