

Echinococcus granulosus: specific quantification of the two most immunoreactive antigens in hydatid fluids

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SUMMARY Preparations of the two most immunoreactive *Echinococcus granulosus* antigens (antigens 4 and 5) from sheep hydatid fluid, purified by a simplified method, and monospecific antisera against antigens 4 and 5, prepared by a new procedure, were used to measure the antigenic concentrations of antigens 4 and 5 in swine, sheep, and human hydatid fluids from pulmonary or hepatic cysts. Two bovine samples and two commercial preparations were also tested. The concentration of both antigens was significantly higher in sheep and human hydatid fluids than in swine hydatid fluid. The antigenic content of the two bovine samples and of the two commercial preparations was below the sensitivity level of the method employed. Independently of the species tested, the amount of *Echinococcus* antigens was greater in hepatic than in pulmonary cysts. The ratio between the concentrations of antigens 4 and 5 was constant at about 1:10 in the samples from various organs and from different species. When there were enough samples for statistical analysis a linear correlation was found between the contents of these two antigenic components but there was none between the amounts of proteins and the antigenic concentrations in the single cysts. Sheep hydatid fluid must therefore be considered the best source of antigenic material for diagnostic purposes even though in human cysts the antigenic fraction is less contaminated by serum proteins. We describe a reliable method of standardising antigenic material for the immunodiagnosis of hydatid disease.

Serological tests for hydatidosis in man are hampered by the lack of specific standardised parasitic antigenic material. Recently *Echinococcus granulosus* antigens have been isolated (Pozzuoli *et al.*, 1972) and two of them (antigens 4 and 5) have the highest immunoreactivity (Pozzuoli *et al.*, 1974, 1975). These purified antigens, apart from their direct use in serological tests, can be used to standardise the most often used and commercially available antigenic material—namely, hydatid fluid obtained from different sources.

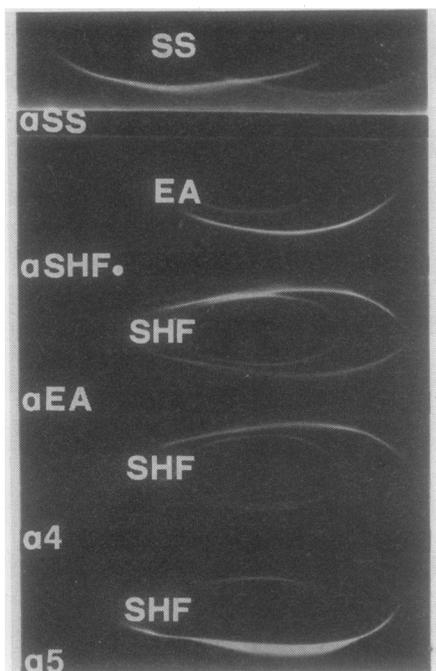
This paper reports the concentrations of antigens 4 and 5 in hydatid cyst fluids from various organs and various animal species. These concentrations were measured by a radial immunodiffusion technique using monospecific anti-antigen 4 and anti-antigen 5 antisera obtained by a simple procedure. Our goal was to identify the best material for immunodiagnostic tests in order to standardise the methods and the materials used in various laboratories.

Material and methods

ECHINOCOCCUS ANTIGENS

Echinococcus antigens 4 and 5 (EA) were purified from sheep hydatid fluid (SHF). Crude SHF was collected, concentrated, and cleared as previously reported (Pozzuoli *et al.*, 1972) without lyophilisation. The fluid concentrated to 3-4 g protein/100 ml was dialysed against 0.05 M acetate buffer, pH 5.0, according to the procedure of Oriol *et al.* (1971). The precipitated proteins containing antigens 4 and 5 were then solubilised in 0.1 M phosphate buffer (pH 7.6), 0.5 M NaCl, and applied to an immunoadsorbent column containing rabbit anti-sheep serum antibodies covalently coupled to Sepharose 4B (Cuatrecasas *et al.*, 1968). The material thus obtained when injected into rabbits elicits antibody formation only against *Echinococcus* antigens 4 and 5 (aEA) (see Figure).

Antigen 5 was prepared from the purified material containing both antigens 4 and 5. Proteins were dialysed overnight at 4°C against a 0.2 M glycine-



Immunoelectrophoretic patterns of sheep serum (SS), purified preparation of *Echinococcus granulosus* antigens 4 and 5 (EA), and sheep hydatid fluid (SHF) tested against rabbit antisera anti-sheep serum (aSS), adsorbed anti-sheep hydatid fluid (aSHF), anti-*Echinococcus* antigens 4 and 5 (aEA), anti-antigen 4 (a4), and anti-antigen 5 (a5). The immunoelectrophoretic pattern of the purified preparation of *Echinococcus* antigens 4 and 5 (EA) tested against aSHF, shows only two precipitation lines, identifiable as antigens 4 and 5. No precipitation lines are present when the same material is tested against aSS. The serum against antigen 4 (a4) and the serum against antigen 5 (a5) when tested against SHF give a single precipitation line, which is that determined by antigen 4 and by antigen 5 respectively.

HCl buffer (pH 2.8) and then heated for five minutes at 100°C. The precipitate thus formed was removed by centrifugation at 20 000 rpm for 30 minutes. The recovered supernatant was neutralised by adding 1 N NaOH. Sera from rabbits immunised with this final preparation contained antibodies only against antigen 5 (a5).

Echinococcus antigens 4 and 5 were also separately obtained from sheep hydatid fluid as previously reported (Pozzuoli *et al.*, 1975).

ANTISERA

Anti-sheep serum (aSS) and adsorbed anti-SHF

(aSHF) were prepared as previously reported (Pozzuoli *et al.*, 1972).

Anti-*Echinococcus* antigens 4 and 5 rabbit serum (aEA) and anti-antigen 5 rabbit serum (a5) were obtained by injecting 1 mg protein plus 0.5 ml Freund's complete adjuvant into foot pads of adult New Zealand animals followed by booster injections after 20 and 30 days.

A monospecific antiserum against antigen 4 (a4) was prepared by adsorption of the anti-antigen 5 antibodies present in the anti-antigen 4 and 5 serum (aEA). 200 mg protein of the purified antigen 5 preparation was dialysed against 0.01 M phosphate, pH 7.4, 0.5 M NaCl, and then coupled to 4 g silica supports (Biomaterial GAO-3940 supports, Corning Biological Products, Medfield, Mass, USA) using a 2.5% aqueous glutaraldehyde solution. Immuno-adsorption was done by a batch procedure for three hours at room temperature. On each occasion 20 ml anti-EA antiserum was incubated with the immobilised antigen 5 preparation. Regeneration of the immuno-adsorbent was performed with 1 M K thiocyanate solution in 0.01 M phosphate buffer (pH 7.4), 0.5 M NaCl.

Polyacrylamide gel disc electrophoresis was carried out on 5% gels following the procedure described by Laemmli (1970). Microdensitometry, using a Kipp and Zonen (Delft, Holland) integrating microdensitometer, was performed on the gel, which contained 100 µg protein of *Echinococcus* purified antigens 4 and 5 stained with Coomassie Brilliant Blue.

Radial-immunodiffusion was performed by the procedure described by Mancini *et al.* (1965) using monospecific rabbit antisera against antigen 4 and against antigen 5. As standards, purified antigen 4, purified antigen 5 (Pozzuoli *et al.*, 1975), and appropriate dilutions of EA preparation (containing both antigens) were used.

Hydatid fluid (HF) samples aspirated by syringe directly from parasitic fertile cysts were filtered and stored at -25°C until used: 83 samples were collected—15 from swine cysts (six from liver and nine from lung), 58 from sheep cysts (26 from liver and 32 from lung), two from bovine cysts (one from liver and one from lung), and six from human cysts (three from liver and three from lung). All these samples except human HF were collected in many abattoirs of Northern Sardinia (Italy). One commercial sample of swine or bovine hydatid fluid from Behringwerke AG (Marburg-Lahn, West Germany, preparation 65 B) and one of sheep hydatid fluid from Istituto Sieroterapico Milanese (Milan, Italy, preparation 61) were purchased.

Protein determination was performed by Folin's method (Lowry *et al.*, 1951) using as a reference

purified sheep albumin (Fraction V, Pentex, Miles Laboratories, Kankakee, Ill, USA).

Results

RELATIVE CONCENTRATION OF ANTIGEN 4 AND 5 IN EA PREPARATION

Disc electrophoresis of EA on 5% polyacrylamide gel showed two bands. Microdensitometric evaluation indicated that the more anodic band, corresponding to antigen 5 (Piantelli *et al.*, 1977), accounted for about 89-91% and the more cathodic, identifiable as antigen 4 (Piantelli *et al.*, 1977), for most of the remaining applied proteins (8-10%).

CONCENTRATION OF ANTIGEN 4 AND ANTIGEN 5 IN HF SAMPLES

Concentrations of the two antigens were determined by radialimmunodiffusion in agarose gels containing anti-antigen 4 or anti-antigen 5 antisera. As standard we used suitable amounts of antigen 4 and antigen 5 and appropriate dilutions of EA preparation containing 5 mg/ml of protein. Therefore concentrations of 4.5 mg/ml for antigen 5 and 0.5 mg/ml for antigen 4 were calculated from densitometric analysis. The relative concentrations of proteins, antigen 4, and antigen 5 in HF samples from swine, sheep, and human are shown in the Table. Neither antigen 5 nor antigen 4 were determinable by immunodiffusion technique in the two bovine samples and the two diagnostic HF from commercial sources, while the protein concentrations were 0.39 and 1.2 mg/ml for bovine fluids and 0.58 and 0.56 mg/ml for commercial preparations.

Similar results were obtained using isolated 4 or 5 antigen as standard instead of EA preparation. As shown in the Table, the lowest protein concentration was found in human HF while the highest was observed in sheep HF, particularly in liver SHF (protein concentration: liver SHF versus lung SHF $P < 0.01$).

The concentrations of antigen 4 and 5 were higher in sheep and human HF than in swine. The highest content of both antigen 4 and 5 was found

in sheep hepatic cysts. Furthermore, the concentrations of antigens 4 and 5 were significantly higher (respectively $P < 0.01$ and $P < 0.001$) in hepatic SHF than in pulmonary SHF. Expressed as a proportion of the total protein concentration the highest content was in human lung HF, the lowest in swine fluids. The data in the Table also indicate that the ratio between antigen 4 and antigen 5 concentration was roughly 1:10 to 1:11. Moreover, a linear correlation was found between antigen 4 and antigen 5 concentrations in both pulmonary and hepatic SHF ($r = 0.98$; $P < 0.01$).

Discussion

Hydatid fluid contains at least 10 parasitic antigens. Two of them, antigen 4 and 5, are of most diagnostic interest. We have found antigen 4 in particular to be most immunoreactive and specific (Capron *et al.*, 1968, 1970; Pozzuoli *et al.*, 1975) while antigen 5 has been regarded as a lipoprotein common to several parasites (Bout *et al.*, 1974). However, whereas antigen 5 could be easily purified antigen 4 could be prepared only in small amounts and by rather complex procedures (Pozzuoli *et al.*, 1975).

We have described in this paper an easy method of preparing monospecific anti-antigen 4 and anti-antigen 5 antisera by two key steps: firstly, denaturation of antigen 4 in EA preparation and, secondly, adsorption of anti-antigen 5 antibodies, contained in anti-EA antiserum, using immobilised antigen 5 still present after EA denaturation. This method does not require isolation of antigen 4. We used the monospecific antisera prepared in this way to quantify antigens 4 and 5 in hydatid fluids by the radialimmunodiffusion technique.

Our quantitative results show that sheep and human HF contain significantly higher concentrations of both *Echinococcus* antigens 4 and 5 than swine HF. The antigenic content of the two bovine HF samples was very low since it was not determinable by our procedure. The different EA concentrations in the cysts of the animal species tested may reflect, at least in Italy, a variable degree of

Table Concentrations of protein and antigens 4 and 5 in hydatid fluids

Source	No. of samples	Antigen 4 (mg/ml \pm SD)	Antigen 5 (mg/ml \pm SD)	Protein (mg/ml \pm SD)	Antigen 4 + 5 % total protein
Swine lung	9	ND	0.021 \pm 0.004	1.017 \pm 0.170	2.1
„ liver	6	ND	0.024 \pm 0.011	1.006 \pm 0.285	2.4
Sheep lung	32	0.0064 \pm 0.0034	0.054 \pm 0.011	1.432 \pm 0.306	4.2
„ liver	26	0.0089 \pm 0.0037	0.088 \pm 0.031	1.667 \pm 0.252	5.8
Human lung	3	0.0051 \pm 0.0029	0.053 \pm 0.013	0.615 \pm 0.098	9.4
„ liver	3	0.0069 \pm 0.0033	0.059 \pm 0.018	0.884 \pm 0.113	7.4

ND = not determinable.

host-parasite adaptation. Moreover, independently of the species tested, there were more EA in hepatic than in pulmonary cysts. Thus sheep liver seems to provide the best environment for cystic growth.

The ratio between the concentrations of antigens 4 and 5 is constant (about 1:10) in the HF samples from various organs of different species. But despite the greater amount of antigen 5 in HF—particularly when the molecular weights of antigen 5 (150 000) and antigen 4 (> 400000) (Oriol *et al.*, 1971; Pozzuoli *et al.*, 1972) are considered—it is important to note that only antibodies against antigen 4 are regularly found in patients with hydatid infection while anti-antigen 5 antibodies are less often detected (Pozzuoli *et al.*, 1975).

No linear correlation was found between the amount of proteins and the antigenic concentrations in the single cysts. Hydatid fluid contains a mixture of host serum proteins with a minor fraction of parasitic proteins almost completely represented by antigens 4 and 5. In human cysts the antigenic material is less contaminated by serum proteins than in other species. However, obviously human HF cannot be considered a suitable source of antigenic material for diagnostic purposes. Therefore, according to our findings, sheep HF should be the best source of antigenic material for serological diagnostic tests. No antigenic material was detectable in the two commercial preparations we tested.

We conclude that to obtain a reliable standardisation of the reagents for the immunodiagnosis of hydatid disease the amount of antigen 4 and antigen 5 in HF preparations must be determined.

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References

- Bout, D., Fruit, J., and Capron, A. (1974). Purification d'un antigène spécifique de liquide hydatique. *Annales d'Immunologie*, **125C**, 775-778.
- Capron, A., Biguet, J., Vernes, A., and Afchain, D. (1968). Structure antigénique des helminthes. Aspect immunologiques des relations hôte-parasite. *Pathologie et Biologie*, **16**, 121-138.
- Capron, A., Yarzabal, L., Vernes, A., and Fruit, J. (1970). Le diagnostic immunologique de l'échinococcose humaine (bilan personnel à propos de 400 observations). *Pathologie et Biologie*, **18**, 357-365.
- Cuatrecasas, P., Wilchek, M., and Anfinsen, C. B. (1968). Selective enzyme purification by affinity chromatography. *Proceedings of the National Academy of Sciences*, **61**, 636-643.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, **193**, 265-275.
- Mancini, G., Carbonara, A. O., and Heremans, J. F. (1965). Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry*, **2**, 235-254.
- Oriol, R., Williams, J. F., Pérez Esandi, M. V. and Oriol, C. (1971). Purification of lipoprotein antigens of *Echinococcus granulosus* from sheep hydatid fluid. *American Journal of Tropical Medicine and Hygiene*, **20**, 569-574.
- Piantelli, M., Pozzuoli, R., Arru, E., and Musiani, P. (1977). *Echinococcus granulosus*: identification of major antigen subunits. *Journal of Immunology*, **119**, 1382-1386.
- Pozzuoli, R., Musiani, P., Arru, E., Patrono, C., and Piantelli, M. (1974). *Echinococcus granulosus*: evaluation of purified antigens immunoreactivity. *Experimental Parasitology*, **35**, 52-60.
- Pozzuoli, R., Musiani, P., Arru, E., Piantelli, M., and Mazzarella, R. (1972). *Echinococcus granulosus*: isolation and characterization of sheep hydatid fluid antigens. *Experimental Parasitology*, **32**, 45-55.
- Pozzuoli, R., Piantelli, M., Perucci, C., Arru, E., and Musiani, P. (1975). Isolation of the most immunoreactive antigens of *Echinococcus granulosus* from sheep hydatid fluid. *Journal of Immunology*, **115**, 1459-1463.