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Circulating immune complexes in sera of patients infected with *Echinococcus granulosus*

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SUMMARY

Circulating immune complexes (CIC) were investigated by the C1q binding assay in sera of 23 patients infected with *Echinococcus granulosus*. For the 23 sera studied, nine were found to be positive in the test. When the samples were grouped according to the cyst localization, the highest rate of CIC positivity was found in the group of sera from patients with pulmonary cyst; in this group, double diffusion (DD) and indirect haemagglutination (IHA) tests gave a low rate of positivity for antibodies directed against parasitic antigens. Rheumatoid factor, anti-nuclear and anti-mitochondrial autoantibodies were not detectable in patients' sera by indirect immunofluorescence technique (IIF). Anti-smooth muscle autoantibodies, detectable by IIF, were present in 56% of the sera and this positivity was higher in the hepatic (83%) than in the pulmonary form (40%).

INTRODUCTION

Circulating immune complexes (CIC) have been detected in many infectious diseases using several techniques (Theofilopoulos & Dixon, 1980; Groupe Scientifique de l'O.M.S., 1977; Lambert & Houba, 1974). Immune complex formation during infection, in vertebrate animals as in man, sets in train processes that can neutralize and eliminate the infectious agent. In some instances, mostly in diseases with chronic course and/or caused by infectious agents of low pathogenicity, CIC may have a direct pathogenetic role in some manifestations of the diseases such as vasculitis, arthritis, erythema nodosum and nephritis (Theofilopoulos & Dixon, 1980; Valesini *et al.*, 1981).

Parasitic infections are generally accompanied by a chronic release of antigens which may contribute to CIC formation since antibody production is triggered. In fact, CIC have been detected in helmintiasis such as schistosomiasis (Bout *et al.*, 1977), trichinosis (Genitau *et al.*, 1977) and onchocerciasis (Lobayakawa *et al.*, 1979).

The presence of CIC in hydatidosis could be suspected from the fact that cold precipitable immune complexes (cryoglobulins) are a frequent finding in sera of patients suffering from this disease (Bombardieri, Teichner & Vicari, 1973). Moreover the recent report (Vialtel *et al.*, 1981) of a membranous nephropathy associated with hydatid disease, in which the parasitic antigen and the corresponding antibody were detected in glomeruly, further supports this view. Some authors, however, were able to find CIC in sera of patients with hydatidosis rarely, using the Raji cells radioimmunoassay (Richard-Lenoble *et al.*, 1978), the C1q binding assay (Bekhty *et al.*, 1977) and other techniques (La Ganga *et al.*, 1980).

The purpose of the present study is to investigate the presence of CIC in human hydatidosis and to evaluate a possible correlation between such complexes, cyst localization and the presence of antibodies to parasitic antigens detected by usual serological procedures.

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MATERIALS AND METHODS

Patients and sera. Sera from 23 patients with clinically diagnosed hydatidosis, confirmed in all cases by surgery, were kindly obtained from Professor G. Ferretti (Istituto di Parassitologia, Cagliari, Italy). When different bleedings from the same patient were available, only the first one (before any treatment) was used. All patients were living in the Sardinia area.

The cyst localization was hepatic (eight patients), pulmonary (12 patients), multiple (two patients) and bony (one patient).

After collection the sera were stored at −30°C until used.

^{125}I-C1q binding assay (C1qBA). CIC were detected in patients' sera using the ^{125}I-C1q binding assay as described by Zubler *et al.* (1976). The results are expressed as percentage of the C1q binding activity and samples were considered positive for CIC when displaying a ^{125}I-C1q binding activity of more than 4·4% (mean + 2 standard deviations of the ^{125}I-C1q binding activity of sera from 97 healthy blood donors).

Double diffusion (DD). Double diffusion tests were performed as previously described (Bombardieri *et al.*, 1974).

Indirect haemagglutination test (IHA). The indirect haemagglutination test was performed with a Takatsy microtitrator (Cooke Engineering Co., Alexandria, Virginia, USA) as previously reported with minor modifications (Iacona, Pini & Vicari, 1980). Briefly, sheep red blood cells, after repeated washings, were treated for 10 min at 37°C with a solution containing 5 mg tannic acid in 100 ml phosphate-buffered saline (PBS). The cells were coated with different amounts of sheep hydatid fluid precipitated according to the method of Oriol *et al.* (1971). The optimal sensitizing dose was empirically determined by a preliminary test, using a reference human serum positive for antibodies against echinococcus antigens 5(A) and B. All the serum specimens were absorbed before use with packed sheep red blood cells. Two-fold dilutions of the sera were performed in PBS with 1% normal rabbit serum pre-absorbed with sheep blood cells.

Titres below 1/200 were considered to be negative (Kagan, 1968).

Rabbits immunization. Antiserum against sheep hydatid fluid precipitated according to the method of Oriol *et al.* (1971) containing mainly Echinococcus antigens 5(A) and B was obtained by injecting a rabbit (No. 229) into footpads with 0·5 ml of the corresponding fraction incorporated into 0·5 ml of Freund's complete adjuvant. After ten days a second injection of the same antigen dose was given in Freund's incomplete adjuvant. Ten days later the animal received a third antigen dose intramuscularly and was subsequently bled after 2 weeks.

Antiserum against whole hydatid fluid was obtained by injecting another rabbit (No. 232) with crude concentrated hydatid fluid according to the above procedure.

Autoantibodies. Anti-nuclear (ANA), anti-smooth muscle (SMA) and anti-mitochondrial (AMA) autoantibodies were investigated in patients' sera by the indirect immunofluorescence (IIF) standard technique (Johnson & Holborow, 1973) on rat's liver and kidney cryostat sections, using fluoresceinated goat anti-human gammaglobulin (Behringwerke, Italy).

To detect some cross-reactions between antibodies directed against cyst fluid, against Echinococcus antigens 5(A) and B and anti-smooth muscle autoantibodies, IIF was performed with antisera No. 232 and 229 on rat's liver and Kidney cryostat sections using a fluoresceinated goat anti-rabbit gammaglobulins (kindly provided by Dr P.G. Natali).

RA latex test. RA latex test for detection of rheumatoid factor was performed using Behringwerke, Italy reagents according to the manufacturer.

Statistical analysis. For statistical analysis of the results Kendall rank correlation (Kendall, 1970) and chi-square Yates correction (Yates, 1934) tests were employed.

RESULTS

The results obtained in the C1qBA with the sera of patients infected with *E. granulosus* are shown in Fig. 1. Of the 23 sera, nine (39%) gave positive results. The positivity was very high in two cases only.

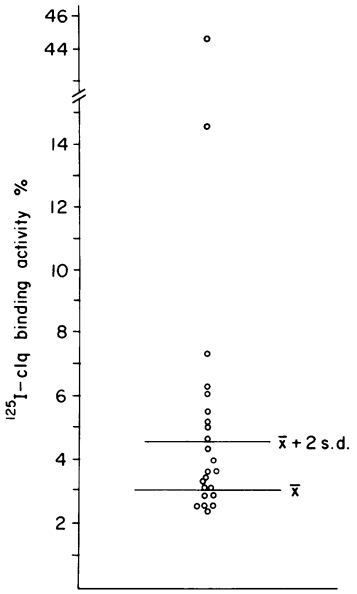

Fig. 1. Distribution of individual results obtained in the C1q binding assay performed with sera of 23 patients with hydatid disease. ($\bar{x}$ = mean of ^{125}I-C1q binding activity of 97 normal human sera from blood donors; s.d. = standard deviation).

The distribution of CIC positive sera according to their positivity in DD and IHA tests is shown in Table 1. Eight of 17 (47%) DD positive sera were positive for CIC. However, both in the DD positive and DD negative sera a number of specimens (53% and 83% respectively) was negative for CIC.

If we consider the distribution of CIC positive sera according to IHA reactivity, five of the 13 IHA positive sera and four of the IHA negative sera were positive for CIC.

Table 2 shows the preferential distribution of sera positive for CIC in the group of sera negative in the IHA test but positive in the DD test versus the groups positive or negative in both the tests.

A very interesting result was obtained when all the sera tested were grouped according to cyst localization. As shown in Table 3, only one serum in the hepatic group, for which we have high rate of positivity both in DD and IHA tests, was positive for CIC (12·5%). When we consider the group of sera from patients with pulmonary localization, six specimens out of 12 (50%) were found to be positive for CIC. Both DD and IHA tests gave a lower rate of positivity as compared with the hepatic group. Sera from patients with multiple and osseous cysts gave respectively 50% and 100% of positivity for CIC but the low number of samples (three) do not allow us to use these data. The

Table 1. Distribution of sera positive or negative for CIC in relation to their positivity or negativity in DD and IHA tests

	Number of sera	Number of sera positive for CIC	%	Number of sera negative for CIC	%
DD positive	17	8	47	9	52·9
DD negative	6	1	16·6	5	83·3
IHA positive	13	5	38·4	8	61·5
IHA negative	10	4	40	6	60

Table 2. Distribution of sera positive for CIC in relation to their reactivity both in DD and IHA tests

	Number of sera	Number of sera positive for CIC	%
IHA pos, DD pos	13	5	38·4
IHA neg, DD pos	4	3	75
IHA neg, DD neg*	6	1	16·6
Total	23	9	39·1

*None of the sera was IHA pos, DD neg.

Table 3. Distribution of positivity for CIC, DD and IHA tests according to cyst localization

Cyst localization	Number of sera	DD positive sera		IHA positive sera		CIC positive sera	
		No.	%	No.	%	No.	%
Hepatic	8	7	87·5	6	75	1	12·5*
Pulmonary	12	7	58·3	5	41·6	6	50*
Multiple	2	2	100	2	100	1	50
Osseous	1	1	100	0	0	1	100
Total	23	17	73·9	13	56·5	9	39·1

*The difference for CIC positive sera between hepatic and pulmonary group is statistically significative ($0{\cdot}025 < P < 0{\cdot}05$).

Table 4. SMA in sera of patients with hydatid disease

Cyst localization	Number of sera	Sera positive for SMA	
		No.	%
Hepatic	6	5	83·3
Pulmonary	10	4	40
Total	16	9	56·3

number of sera positive for CIC in the group of pulmonary cysts is significantly higher than in the hepatic group (chi-square = 4·8; $0{\cdot}025 < P < 0{\cdot}05$).

The RA latex test for detection of rheumatoid factor was negative in all sera. Sixteen sera, six from hepatic and 10 from pulmonary group were also tested for autoantibodies by IIF. ANA and AMA were not detectable in any sera, while SMA were present in more than 50% (nine sera out of 16). As shown in Table 4 the incidence of SMA in sera from patients with hepatic cyst was higher than the incidence in sera from pulmonary group (chi-square = 1·37; $0{\cdot}2 < P < 0{\cdot}3$).

Table 5 shows that a preferential distribution of sera positive for SMA can be found in the groups of specimens also positive for haemagglutinating (87%) and, at a less extend, precipitating (64%) antibodies.

Table 5. Correlation between SMA and serological positivity in IHA and DD tests

	Number of sera	Sera positive for SMA		Sera negative for SMA	
		No.	%	No.	%
Sera positive for IHA	8	7	87	1	13
Sera positive for DD	11	7	64	4	36

The IIF test with antisera from rabbit Nos 232 and 229 on rat liver and kidney cryostat sections did not show cross-reactions between rabbit antibodies directed against crude cyst fluid and echinococcus 5(A) and B antigens with antigens present on rat's tissues.

DISCUSSION

Recently, a few authors (Vialtel *et al.*, 1981; Ibarrola *et al.*, 1981) have reported cases of membranous glomerulonephritis probably secondary to hydatid disease. These are the first cases of hydatidosis in which a clear renal implication can be demonstrated as a consequence of immune complexes fixed on the glomerular basement membrane.

However, the presence of CIC in hydatid disease could be suspected on the basis of previous findings of cold precipitable immune complexes (cryoglobulins), which could be detected in 85% of such patients (Bombardieri *et al.*, 1973). More recently, the presence of CIC has been reported by other authors (Richard-Lenoble *et al.*, 1978; Bekhty *et al.*, 1977; La Ganga *et al.*, 1980).

In the present work we were able to find C1q binding CIC in 39% of the patients tested; the difference between this frequency and that of cryoglobulins may be due to the fact that only a fraction of cold precipitable immune complexes are able to fix complement. Moreover it cannot be assumed that all the cryoprecipitate is constituted by immune complexes. On the other hand, it is now commonly accepted that the C1q binding assay is a more specific and sensitive test for the detection of CIC than cryoprecipitation.

The demonstration of CIC in sera of patients with hydatidosis obtained by other authors (Richard-Lenoble *et al.*, 1978) using the Raji cells system gave different results. In such case, only about 11% of the sera tested were positive for CIC. This fact is possibly due to the relatively low sensitivity of the Raji cells assay in the detection of CIC in human echinococcosis.

An attempt to correlate serological negativity and presence of immune complexes has been considered. In fact it has been suggested that such complexes in the serum of patients with hydatid disease may cause false negative reactions for anti-hydatid antibodies in patients with clinically and surgically confirmed disease (Richard-Lenoble *et al.*, 1978). Our results indicate that the higher number of sera positive for CIC is distributed in the group of sera at least positive for one of the two techniques (DD and IHA) employed. Moreover a preferential distribution of sera positive for CIC can be registered in the group of sera negative in the IHA test but positive in the DD test (Table 2). This fact could be explained on the basis of the immunochemistry of the antigen–antibody reaction in agar gel as compared to a haemagglutination test. In the first case, immune complexes eventually present can dissociate in the presence of new antigen that can diffuse up to equivalence with antibodies. In the second case (IHA) the antigen is adsorbed on the red cell surface at a concentration that remains constant during the test.

The importance of immune complexes in serodiagnosis of hydatid disease is further supported if we consider cyst localization. In fact, the highest rate of positivity for CIC is detectable in the sera of patients with pulmonary localization (66% of all the sera positive for CIC). This is an indirect evidence that immune complexes could play a role in influencing serological tests, because pulmonary hydatidosis is more difficult to be detected by serological techniques, as shown in Table

3. In any case, no significant correlation could be demonstrated between IHA titres and CIC levels measured by the C1q binding assay, as evaluated by the Kendall correlation test (data not shown).

The fact that the pulmonary cyst have the highest rate of sera positive for CIC may suggest that there is a difference in complexes formation according to the cyst localization. At present we are not able to explain such finding.

Using an indirect immunofluorescence technique, high incidence of anti-smooth muscle autoantibodies was found in patients' sera, the rate of positive results being significantly higher in sera from hepatic (83%) than pulmonary (40%) localization. Moreover, no rheumatoid factor, neither ANA or AMA were detectable in all sera tested, and no correlation was found between SMA and CIC positivity.

On the contrary, there is a positive correlation between the presence of SMA and the positivity for haemagglutinating and, less significantly, precipitating antibodies; this correlation is also confirmed if we analyse the results according to cyst localization.

The explanation of such results is not obvious. The general hyporeactivity of sera from patients with pulmonary cyst (less positivity for DD, IHA and SMA) could be explained with the presence of CIC that are recognized to play a role in immune regulation (Theofilopoulos & Dixon, 1980). The concomitant lower CIC positivity in the hepatic localization and the high serological reactivity for both anti-echinococcus antibodies and SMA could be in agreement with such hypothesis.

Any way, many chronic infections due to bacteria, virus, fungi or parasites can lead to the production of autoantibodies (Talal, 1981). Such a production may be non-specifically triggered by a polyclonal B cell activation mediated by parasite antigens, but other mechanisms of autoimmunization may play a role, as cross-reactions between auto- and parasitic antigens.

The results obtained in our experiments, showing that rabbit antibodies against hydatid fluid and Echinococcus 5(A) and B antigens do not stain (on indirect immunofluorescence) smooth muscle, allow us to conclude that SMA positivity in patients' sera is not due to cross-reactions between *E. granulosus* antigens and host antigens.

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