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IMMUNOGLOBULIN STUDIES IN SERUM OF PATIENTS WITH AMERICAN TRYPANOSOMIASIS (CHAGAS' DISEASE)

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SUMMARY

Individuals with acute American trypanosomiasis had normal serum concentration of immunoglobulins-G, -A and -M. In the chronic stage they had normal levels of serum IgA and IgM, and a small but significant increment in IgG. Anti-trypanosome antibodies of the IgM class were regularly found in the acute stage but only occasionally in the chronic stage. IgG and IgA specific antibodies were present in both stages, but titres were higher during the chronic phase. No abnormalities were observed in the levels of immunoconglutinins or in the presence of rheumatoid factors and antinuclear factors. High titres of anti-sheep and anti-rat red blood cell (RBC) antibodies appeared early in the acute phase. Anti-sheep RBC agglutinins could be removed completely by absorption with rat RBC, but anti-rat RBC agglutinins could be absorbed only partially with sheep RBC. Antigens responsible for the heterophile reaction were localized to the external surface of culture forms of *Trypanosoma cruzi*.

INTRODUCTION

American trypanosomiasis (Chagas' disease) is endemic in many South and Central American countries (World Health Organization Study Group, 1960). Its clinical and pathological manifestations are well documented, and several useful procedures are available for its laboratory diagnosis. By contrast, very limited work has been performed on the possible existence of quantitative and qualitative abnormalities in the serum immunoglobulins of patients with Chagas' disease (Muniz, 1962; Masseyeff & Lamy, 1966). Such studies are of great interest, particularly in view of the immunoglobulin abnormalities that regularly accompany African trypanosomiasis. These consist of a very high level of serum immunoglobulin-M (Mattern *et al.*, 1961; Masseyeff & Lamy, 1966), and high titres of immunoconglutinins (Ingram & Soltys, 1960; Lachmann, 1966), heterophile antibodies

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(Henderson-Begg, 1946) and rheumatoid factors (Klein & Mattern, 1965; Houba & Allison, 1966). A series of studies was therefore conducted to assess the following parameters in individuals infected with *Trypanosoma cruzi*: serum concentration of immunoglobulins-G, -A and -M; specific antibody activity in the three main immunoglobulin classes; and levels of immunoconglutinins, rheumatoid factor, antinuclear factors, and anti-red blood cell antibodies. Sera were also analysed by cellulose acetate electrophoresis and by immunoelectrophoresis. Part of these studies have been reported previously in abstract form (Lelchuk *et al.*, 1968).

MATERIALS AND METHODS

Sera

Blood from persons, generally adults, with Chagas' infection, both in the acute and in the chronic stage, was collected in several areas of Argentina. Samples from cases in the initial stage were procured during the first 2 months of infection, following demonstration of the parasite in the blood by direct examination, differential centrifugation or xenodiagnosis (Maekelt, 1964). The diagnosis in the chronic stage was based in the positivity of the three serological reactions described below. All chronic cases had an immunofluorescence titre of 1 : 32 or greater, and 67% of them yielded a positive xenodiagnosis test. Most chronic cases were free of clinical abnormalities and were detected at the time of routine serological examination of blood donor candidates. After collection, blood was allowed to clot at room temperature for 2 hr, the serum was separated by centrifugation and samples were stored at −20°C or at −70°C from few days to 6 months. Control sera were obtained from laboratory personnel and blood donors which had negative serological reactions for Chagas' disease.

Serological reactions for Chagas' disease

Sera were analysed by complement fixation, haemagglutination and immunofluorescence. The antigens used were obtained from culture forms of *T. cruzi* derived from parasites that were isolated from the blood of acute patients from several areas of Argentina and maintained in biphasic media (Gonzalez Cappa *et al.*, 1968). The complement fixation test (Cerisola & Rosenbaum, 1958) was carried out with an antigen obtained from 7- to 10-day-old cultures. The parasites were filtered through cotton and washed three times with 0·15 M NaCl solution. The cells were collected by centrifugation at 2000 ***g*** for 10 min. One volume of cells was thoroughly mixed with 30 volumes of distilled water and left at 4°C for 30 min. Then the mixture was subjected to 20,000 rev/min for 15 sec in an Ultra-Turrax homogenizer (Janke and Kunkel, K.B., Stanfen, Germany). After adding sufficient NaCl to obtain isotonicity and sodium azide 1 : 1,000, the preparation was stored at 4°C, remaining stable for 3 months. A similar procedure was used to prepare the antigen for haemagglutination, except that the material was not homogenized after lysis with water. At this step the lysate was centrifuged and the supernatant reconstituted to isotonicity and used in a proportion of 4 volumes per volume of packed tanned sheep erythrocytes (Boyden, 1951; Cerisola, Fatala Chaben & Lazzari, 1962). Haemagglutination was performed on glass test slides. Immunofluorescence (Fife & Muschel, 1959) was carried out using 4-day-old cultures washed in 0·15 M NaCl solution buffered with 0·01 M phosphate, pH 7·2. The trypanosomes were treated at 4°C with 1% formalin in the same buffer. In most experi-

ments a fluorescein-conjugated γ-globulin prepared from a rabbit polyvalent antiserum reacting with the immunoglobulins-G, -A and -M (Inmunoquemia, Argentina) was used at a dilution of 1 : 40. In certain experiments labelled immunological reagents specific for IgG and IgA (Behringwerke, Germany) were employed at a dilution of 1 : 15. All cases studied in this paper had serum immunofluorescence titres of 1 : 32 or greater as determined with conjugated polyvalent anti-immunoglobulins. The incidence of positive tests in serum of 120 normal controls was 6·7%, with titres of 1 : 10 or less (Cerisola, Alvarez, Böck & Wegner, unpublished observations).

Electrophoresis

This was carried out for 1½ hr on cellulose acetate using veronal buffer, pH 8·2, $T_{\frac{1}{4}}$ 0·05. Total serum protein was determined by a modification of the biuret method (Gornall, Bardawill & David, 1949). Immunoelectrophoresis was performed according to Scheidegger (1955).

Quantitation of immunoglobulins-G, -A and -M

This was performed by the radial immunodiffusion technique using monospecific rabbit antisera (Fahey & McKelvey, 1965).

Fractionation of serum

Serum was filtered on Sephadex G-200 using a 100 × 2·5 cm column and 0·1 M NaCl solution buffered with 10% by volume of 0·1 M borate buffer, pH 8·0. Four millilitres of serum was applied to the column and 5-ml samples were collected in an automatic fraction collector. The samples were evaluated for protein at 280 mμ in a Beckman DU spectrophotometer and then combined in three pools essentially as described by Lehrich, Kasel & Rossen (1966), which were designated fractions 1, 2 and 3. To preclude contamination with IgM, fraction 2 was formed with the samples comprising only the intermediate third of the ascending branch of the second protein component. Immunoglobulins-G, -A and -M were evaluated in each fraction after concentration by ultrafiltration to 1·5–2·0 ml. Recovery of immunoglobulins varied from 20 to 75%. In the various experiments fraction 1 contained 0·65–1·8 mg IgM/ml, 0 to trace amounts of IgA and no IgG; fraction 2 contained 2·4–4·0 mg IgA/ml, 4·7–10·0 mg IgG/ml and no IgM; fraction 3 contained 4·0–7·2 mg IgG/ml, trace to 0·7 mg IgA/ml and was free of IgM.

Immunoconglutinins

This was determined using cell-bound human and guinea-pig complement. Complexes of sensitized erythrocytes and the guinea-pig complement components C1, C2, C3 and C4 were prepared as described by Lachmann & Liske (1966a). Similar complexes of sensitized erythrocytes and human complement were prepared by incubation for 20 min at 37°C of 5×10^9 sensitized erythrocytes with 1 ml of fresh human serum in the presence of 2×10^{-4} M ZnCl (Lachmann & Liske, 1966b) in a volume of 20 ml obtained with veronal buffer. This buffer contained $1{\cdot}5 \times 10^{-4}$ M $CaCl_2$, 5×10^{-4} M $MgCl_2$ and 0·1% gelatin, and was prepared according to Kabat & Mayer (1961). The intermediate complexes were washed once in cold veronal buffer containing 0·01 M EDTA and twice in the same buffer without EDTA. They were then suspended in veronal buffer at a concentration of 0·6%. Sera to

be tested for immunoconglutinins were incubated for 30 min at 56°C to inactivate complement and absorbed twice for 30 min at 4°C with 0·5 volume of packed sensitized erythrocytes. Immunoconglutinin titration was carried out by a sedimentation pattern technique (Lachmann, 1966).

Other methods

Titres of anti-red blood cell (RBC) antibodies were determined by agglutination of 1% sheep and 1% Lewis rat RBC suspensions in veronal buffer after 1 hr at 22°C. To study the effect of various absorptions on the anti-RBC antibodies, 1-ml serum samples were incubated for 30 min at 56°C and then absorbed three times during 30 min at 37°C with one of the following cell types: 1 ml packed sheep RBC, 1 ml Lewis rat RBC and 0·2 ml packed culture forms of *T. cruzi*, either intact or disrupted by three cycles of freezing in a mixture of dry-ice and acetone, and thawing in a 37°C water bath. The presence of anti-γ-globulins was ascertained by the latex technique (Singer & Plotz, 1956) and the Waaler-Rose method (Valkenburg, 1963). Antinuclear factors were investigated by immunofluorescence using calf thymus imprints (Holborow, Weir & Johnson, 1957).

RESULTS

Table 1 shows the results of quantitation of immunoglobulins-G, -A and -M. The differences between controls and patients with acute and chronic Chagas' infection were not significant, except with IgG in the chronic cases which had a mean value significantly higher than the control group ($P<0·02$). The values of total serum protein concentration and of the various fractions obtained by separation on cellulose acetate of serum from groups of patients

TABLE 1. Serum concentration of immunoglobulins-G, -A and -M in human American trypanosomiasis

Stage of infection	No. of cases	Immunoglobulin concentration (mg/ml) (mean ± SE)		
		IgG	IgA	IgM
Acute	17	14·4 ± 1·0	2·5 ± 0·4	2·2 ± 0·3
Chronic	12	16·2 ± 1·0	2·8 ± 0·4	1·2 ± 0·1
Normal controls	17	13·1 ± 0·7	2·7 ± 0·4	1·6 ± 0·2

with acute and chronic Chagas' infection were compared with a normal control group. Significant changes were not observed in the acute stage of the infection. Protein fractions of individuals in the chronic stage showed a small but significant increment in gamma globulin concentration, as previously reported (Kortsarz, Strizic & Castillo, 1965). All sera studied by radial immunodiffusion were also examined by immunoelectrophoresis using a potent rabbit anti-human serum. No significant abnormalities were observed.

Anti-trypanosome antibody activity was evaluated in the fractions obtained by filtration of serum on Sephadex G-200. A summary of the results obtained by immunofluorescence is presented in Table 2. Haemagglutination and complement fixation analysis yielded similar results. Most of the acute sera had low antibody activity in the three fractions. By

contrast, only a small proportion of the chronic cases had antibody activity in fraction 1 and titres in fractions 2 and 3 were considerably higher than in the acute cases. Since fraction 1 usually consisted exclusively of gamma M, it may be concluded that anti-trypanosome antibodies of the IgM class are regularly present in the acute stage of the Chagas' infection

TABLE 2. Antibody activity of Sephadex G-200 fractions from sera of patients with American trypanosomiasis

Acute cases					Chronic cases				
	Antibody titres* (reciprocal)					Antibody titres* (reciprocal)			
Serum No.	Unfractionated serum	Fraction†			Serum No.	Unfractionated serum	Fraction†		
		1	2	3			1	2	3
1	128	32	64	16	11	512	2	64	32
2	32	4	8	8	12	256	0	64	64
3	64	2	64	16	13	128	0	32	16
4	64	0	16	16	14	256	0	64	64
5	128	8	8	16	15	512	4	128	256
6	64	2	16	16	16	256	0	32	16
7	64	8	8	4	17	128	0	16	16
8	32	8	0	0	18	256	0	64	256
9	32	16	2	2	19	64	0	8	32
10	64	0	16	16	20	64	0	16	32
					21	128	0	64	8

* Antibody titres were determined by immunofluorescence using rabbit anti-human immunoglobulins-G, -A and -M.

† The procedure by which these fractions were obtained and their immunoglobulin content are described in 'Materials and methods'.

TABLE 3. Immunoconglutinin titres in serum of patients with American trypanosomiasis

Stage of infection	No. of cases	$\log_3$ of serum dilution (mean and range)	
		Human complement	Guinea-pig complement
Acute	11	0·8 (0–2)	2·0 (1–4)
Chronic	17	1·1 (0–4)	2·7 (2–4)
Normal controls	9	0·8 (0–1)	2·7 (2–4)

but only occasionally in the chronic stage. To establish whether specific antibodies are present in the IgG and IgA γ-globulin classes, immunofluorescence studies were performed on sera from acute and chronic cases and on Sephadex fractions 2 and 3 employing rabbit anti-human IgG and anti-human IgA. It was found that most sera and fractions from both stages of the infection contain IgG and IgA specific antibodies. Titres were generally higher for IgG than for IgA.

Table 3 shows the results of immunoconglutinin titres. In the acute and in the chronic stages they were similar to the controls. Titres were higher using guinea-pig complement than using human complement. Anti-γ-globulin factors and antinuclear factors were found positive in few cases, with titres and incidence similar to those found in the general population (Alexander, Bremner & Duthie, 1960; Seligman *et al.*, 1967).

TABLE 4. Serum red blood cell agglutinins in American trypanosomiasis

Stage of infection	No. of sera studied	Reciprocal of agglutination titres against red blood cells of: Sheep: Range	Sheep: No. of cases with titre of: <40	Sheep: 40–320	Sheep: >320	Rat: Range	Rat: No. of cases with titre of: <320	Rat: 320–1280	Rat: >1280
Acute	10	40–160	0	10	0	40–2560	2	8	1
Chronic	10	80–2560	0	6	4	320–10,240	0	4	6
Normal controls	14	2–20	14	0	0	20–160	14	0	0

TABLE 5. Effect of various absorptions on serum red blood cell agglutinins in human American trypanosomiasis

Serum No.	Reciprocal of agglutination titre against red blood cells of: Sheep: Un-absorbed	Sheep: Absorbed with: Sheep RBC	Rat RBC	Intact trypanosomes	Disrupted trypanosomes	Rat: Un-absorbed	Rat: Absorbed with: Sheep RBC	Rat RBC	Intact trypanosomes	Disrupted trypanosomes
1	80	<5	<5	10	10	640	40	<5	40	40
2	320	<5	<5	80	40	1280	160	<5	320	320
3	320	<5	<5	20	20	2560	320	<5	320	320
4	80	<5	<5	40	40	2560	320	<5	640	160

Anti-sheep and anti-rat RBC agglutinins were observed both in acute and in chronic cases (Table 4). The highest values were obtained with rat erythrocytes and in the chronic cases. In individuals that were studied at monthly intervals during the first 6 months of infection the titres were observed to rise progressively. The increased levels of anti-RBC antibodies appeared very early in the course of the infection, at the time of or before demonstration of specific antibodies by immunofluorescence, which was the most sensitive of the three methods used in this study to detect specific antibody in the initial phase of the infection. Representative examples of absorption experiments are presented in Table 5.

Absorption with rat erythrocytes abolished the agglutinating capacity against rat and sheep RBC. Absorption with sheep RBC removed the agglutinating capacity of sera against sheep RBC but caused an incomplete reduction of the titre against rat RBC. Absorption with culture forms of *T. cruzi* was carried out in all cases but never resulted in the total removal of anti-sheep and anti-rat RBC antibodies. Entirely similar results were obtained in absorption experiments using a cell-free preparation from culture forms of *T. cruzi* disrupted by three cycles of freeze-thawing.

DISCUSSION

The results presented in this paper indicate a small but significant increment of serum IgG concentration in chronic American trypanosomiasis as the only change in serum proteins that could be revealed by cellulose acetate electrophoresis, immunoelectrophoresis and immunochemical quantitation of immunoglobulins. No significant changes were observed during the acute stage of the infection. These findings stand in frank contrast to African trypanosomiasis, which is characterized by a pronounced increment in the level of IgM (Mattern *et al.*, 1961; Masseyeff & Lamy, 1966). Other immunological abnormalities that also constitute constant features of African trypanosomiasis, such as very high levels of immunoconglutinins (Ingram & Soltys, 1960) and rheumatoid factors (Houba & Allison, 1966) are not present in American trypanosomiasis. Thus it is clear that these two diseases are dissimilar with regard to serum immunoglobulin abnormalities, despite the large degree of immunological relationship of *T. cruzi*, *T. gambiense* and *T. rhodesiense* (Sadun *et al.*, 1963). Therefore the distinct immunological response of each form of human trypanosomiasis must be sought in relation to the biological properties which are peculiar to each aetiologic agent (Muniz, 1962; Vickerman, 1965).

The studies to determine the presence of anti-trypanosome antibody activity in the three main immunoglobulin classes in serum of infected persons regularly showed specific antibody of the IgM class in the initial phase but only occasionally in the chronic phase. Fractions containing IgG and IgA had titres appreciably higher in the chronic phase than in the acute phase. Since the immunoglobulins contained in fraction 3 consisted almost exclusively of IgG, there was little doubt that it carried antibody activity. Immunofluorescence studies using labelled anti-IgG and anti-IgA demonstrated the presence of antibody activity in both immunoglobulin classes in the acute and in the chronic stages.

The presence of anti-RBC antibodies in the serum of persons infected with *T. cruzi* had been known for many years (Muniz & Dos Santos, 1950; Neto & Da Silva, 1954). In contrast to what was generally reported, we observed higher titres in chronic than in acute cases. It seems of interest that the anti-RBC agglutinins are of very early appearance in the course of the infection. High titres of agglutinins were found against rat RBC, which could be absorbed only partially by sheep RBC. This suggests that the immunological reaction responsible for the anti-RBC antibodies in American trypanosomiasis might be of a similar type to that observed in transplantation immunity (Rapaport, Kano & Milgrom, 1968). A significant immunological cross-reaction could be demonstrated between *T. cruzi* and sheep and rat RBC, indicating that the anti-RBC antibody response might result from the direct antigenic stimulation provoked by the parasite itself rather than from antigens arising from host tissue destruction. The results of absorption experiments using intact *T. cruzi* and disrupted parasites showed that the *T. cruzi* antigens which are related to sheep and

rat RBC antigens are located on the external surface of the parasite. The possibility that *T. cruzi* elicits specific antibodies which cross-react even with host tissues, particularly heart, has been considered previously (Kozma, 1962; Ejden & Lanari, 1967) and appears worthy of further exploration in connection with pathogenic mechanisms involved in the lesions of Chagas' disease.

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