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CHARACTERIZATION OF HUMAN HETEROPHIL HEMOLYSINS INDUCED BY *SCHISTOSOMA MANSONI* INFECTION

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Heterophil antibodies could be detected in sera from normal or from patient with chronic schistosomiasis. Their hemolytic activities depend on the integrity of the complement classic pathway. The heterophil antibodies from patient sera presented a higher specificity for Schistosoma mansoni *antigen preparations than those detected in normal sera. Most of the hemolytic activity observed in normal sera can be destroyed at 56°C for 4min. On the other hand, about 80% of the sera from infected patients are partially or totally resistant to this heat-treatment. The hemolytic activities of sera were eluted from a gel filtration column in different fractions of the first peak.*

Perez (1944) described in sera of patients with schistosomiasis, heterophil antibodies against sheep red blood cell (SRBC), and guinea pig kidney. Pautrizel et al (1964) also found antibodies against SRBC in African patients with *Schistosoma haematobium.* These findings at least concerning *Schistosoma mansoni* infection could not be corroborated by some other investigators (Dammin & Weller, 1945; Antunes & Pellegrino, 1967).

In the present paper, some characteristics of heterophil hemolysins detected in sera of patients with chronic *S. mansoni* infection are presented.

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MATERIAL AND METHODS

Serum samples

Sera collected from patients with chronic intestinal schistosomiasis (viable *S. mansoni* eggs in stool) (Katz, Chaves & Pellegrino, 1972) and from normal individual (without eggs in stool). The normal sera were checked for the absence of antibody to *S. mansoni* by negative skin test to adult worm antigen (Kagan & Pellegrino, 1961) lack of cytotoxicity to mechanically obtained schistosomula (Tavares, 1977) and negative complement fixation test (Kagan & Pellegrino, 1961). Blood was allowed to clot at room temperature, centrifuged and used immediately without freezing or storage. Unless stated, each sample was assayed unheated. Monospecific goat antiserum toward human IgM or IgG (Hyland Laboratories), IgA (Institute Pasteur) and IgE (Behring Institute) were used in certain experiments.

Complement source

Fresh guinea-pig serum was absorbed with 1/4 volume of packed SRBC in an ice-bath. The blood cells were discarded by centrifugation and the absorbed serum diluted 1:400 with cold isotonic buffer (Kent, Garcia-Otero & Harrigan, 1957) pH 7.4 containing 0.123 g $MgSO_4,7H_20$, 0.02g $CaCl_2$, $2H_20$, 7.5g NaCl, 2.8ml of triethanolamine, 18ml N HCl, and 0.5% gelatin in 1 liter of distilled water (TBS).

Treatment of sera

For absorption of sera 0.1ml of serum was added to 0.1ml of TBS and 0.1ml of different antigen suspension. After incubation in ice-box for 20 h (Antunes & Pellegrino, 1967), 3.7ml of TBS was added to a final dilution of 1:40. For control, saline was added to serum instead of antigen.

For mercaptoethanol (2-ME) treatment, nine volumes of pooled fresh human sera were treated with one volume of IM 2-ME in TBS without gelatin. The mixture was kept at room temperature for 2h and then exhaustively dialysed against saline at 4°C. Control samples without 2-ME received the same treatment. In order to investigate the complement pathway involved in the lysis, 0.1 ml of fresh human serum was individually treated with 1mg of boilled zymosan (Pillemer et al, 1956) and the mixture kept at 17°C of 37°C for 1h. After the addition of 9 ml of cold TBS the tubes were centrifuged in cold and the precipitated material was discarded. To study the effect of chelates (Fine et al, 1972), on complement activation, 0.1ml of six normal or six immune individual serum was mixed with either 0.9ml 8×10^{-3}M EGTA containing 10^{-4}M Mg SO_4 or 10^{-2}M EDTA in TBS buffer without Mg^{++} or Ca^{++}. To study the effect of heat treatment, 0.1ml of individual sample of human serum was kept at 56°C in a water bath for the time stated and then immediately colled. Control sera were kept at 4°C.

Antigen preparations

Cercariae, schistosomula or adult worms were disrupted by sonication in an ice-bath and dialyzed overnight against 0.15M sodium chloride solution as described by Mota-Santos et al (1977). The amount of protein in these extracts were determined by the Lowry method (Lowry et al, 1951) and their concentration ajusted to 1.2 – 1.5mg of protein per ml with cold 0.14M sodium chloride. These crude preparations were used as antigen source and possess fractions of membrane and several others structure of the *S. mansoni.*

Hemolytic assay

The hemolytic assay is a modification of that used by Hall, Rowlands & Nilsson (1972). One half mililiter in TBS of 1:40 diluted sera either absorbed by *S. mansoni*

extracts, 2-ME treated or heated sera during 60 min was added to 0.5ml 1% SRBC in TBS which was standardized spectrophotometrically (Mayer, 1961) plus 2ml of 1:400 absorbed guinea-pig sera. In the experiment of Table I, guinea-pig serum was omitted. For the kinetic experiment (Alves-Oliveira, 1982) (Fig. 3) 1ml of 1:20 diluted human sera was used in the hemolytic assay. The mixture was incubated at 37°C for 30 min. The cells were sedimented by centrifugation and the hemolysis in supernatant was estimated by measuring photometrically (412nm) the amount of hemoglobin released. For each experiment a control was performed replacing the complement source with TBS-EDTA or complement source previously heated for 60 min. at 56°C and containing 10^{-2}M EDTA. The optical density of hemoglobin liberated by treating 0.5ml of SRBC with distilled water was recorded and represented 100% lysis. The degree of hemolysis (hemolytic activity) was expressed as the percentage value calculated from the following formula:

$$100 \times \frac{\text{absorbance of sample}}{\text{absorbance of 100\% hemolysis}}$$

Preliminary experiments demonstrated that the complement from guinea-pig sera was present in excess in the assay and therefore not rate limiting. The hemolytic activity in chelated or zymosan treated sera was determined by mixing one mililiter of 1:10 chelated or zymosan treated sera was determined by mixing one mililiter of 1:10 chelated or zymosan treated sera with one tenth of SRBC five times concentrated and incubated as above. To determine the percent lysis, 2.0ml of cold saline were added and the hemoglobin liberated was read optically.

Hemolysin fractionation by gel filtration with Sephadex G-200

A 5ml sample pool of human serum was applied on the top of a column (80x3cm) and the elution carried out at 4°C with 0.15M sodium chloride solution buffered with 10% by volume of triethanolamine buffer (pH 7.2) without Ca^{++}, Mg^{++} or gelatin in 0.02% sodium azide. The average flow rate was 18ml per hour under hydrotastic pressure and the eluates were collected in 4 ± 0.5ml fractions. The protein concentration of the fractions were estimated by absorption at 280nm. The hemolysins in eluates were determined by taking one half volume of eluates just obtained from the column to exclude storage effects (Lee, Takahashi & Davidson, 1963; Yoshida & Nahmias, 1967) and used directly in hemolytic activity assay. The presence of certain immunoglobuline classes on Sephadex filtrated were measured by semi-quantitative Ouchterlony method (Barron, Friedman & Milgron, 1967).

Antiglobulin test

To one half mililiter of serial human serum dilution in TBS-EDTA, one half mililiter of 1% suspension of SRBC was added. The tubes were placed in an ice-box and incubated for 3h with occasional shaking. After incubation the SRBC was washed three times in cold TBS-EDTA and 1.0ml goat antiglobulin serum diluted 1:100 in TBS-EDTA was added. Following incubation over night in ice-box, the agglutination was recorded without centrifugation (Tonder, Natvig & Matre, 1967). No direct agglutination of SRBC was observed in any serum dilution (Dammin & Weller, 1945; Antunes & Pellegrino, 1967) (the goat antiserum were previously absorbed with SRBC and not agglutinated this cell).

Cytotoxic assays

Cytotoxic assays were carried out according to Tavares (1977).

RESULTS

SRBC hemolysin specificity in human sera

As can be seen in Fig. 1, nonabsorbed sera from both infected and normal patients presented about 80% of heterophil hemolytic activity. After absorption with

either cercaria, schistosomula or adult worm extracts, 86.4% (19/22) of normal sera and 74.1% (40/54) of immune sera showed a reduction of their original hemolytic activity.

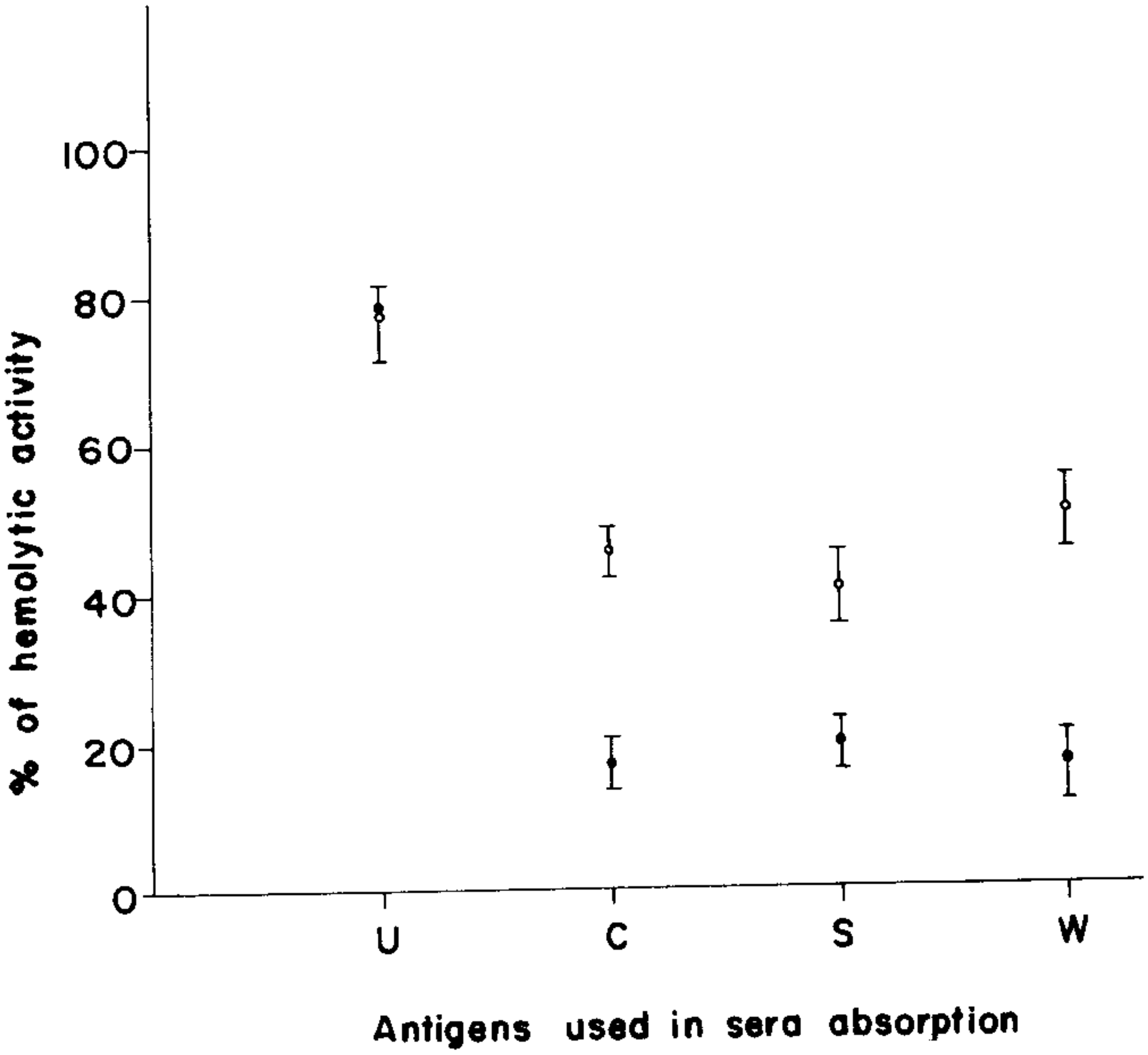

Fig. 1 – Mean percentual hemolytic activity of 19 normal (○) and 10 immune sera (●) nonabsorbed (U) and absorbed by cercariae (C), schistosomula (S) and adult worm (W) preparations.

The hemolytic activities of normal and immune sera after absorption with either cercaria, schistosomula or adult worm extracts were reduced, respectively, to 42.6 ± 4.0, 41.0 ± 5.4 and 50.0 ± 5.4 (normal sera) and to 10.6 ± 6.0, 19.6 ± 8.3 and 18.4 ± 5.8 (immune sera).

Effect of chemical and inhibitors on hemolytic activity of sera

The hemolytic activity was completely inhibited by EDTA, EGTA and zymosan (37°C). The treatment with zymosan at 17°C did not affect the hemolytic activity (Table I). These data indicate that the classical pathway of complement activation is involved in the hemolytic activity here described. The hemolytic antibodies in pool of 15 normal or 10 immune sera were destroyed by 2-ME treatment.

Temperature effect on SRBC hemolysin

When sera from normal individuals were heated at 56°C for 1h their hemolytic activity were completely destroyed. In the other hand, some sera from infected patients

TABLE I

Hemolytic activities of normal and immune serum pools treated by either EDTA, EGTA and zymosan

Sera treatment	*Hemolytic activity*	
	Normal serum	*Immune serum*
None	94.4	97.0
EDTA	<5	<5
EGTA	<5	<5
Zymosan, 17°C	92.7	94.7
Zymosan, 37°C	<5	<5

Each pool: six different sera.

are partially or totally resistant to this treatment (Fig. 2). The heterophil hemolytic activities of many sera treated at 56°C for different periods of time are presented in Fig. 3. It was possible by this treatment to distinguish three different groups among the sera from infected patients. In the first group, 20.0% (14/70) of those were very labile, loosing

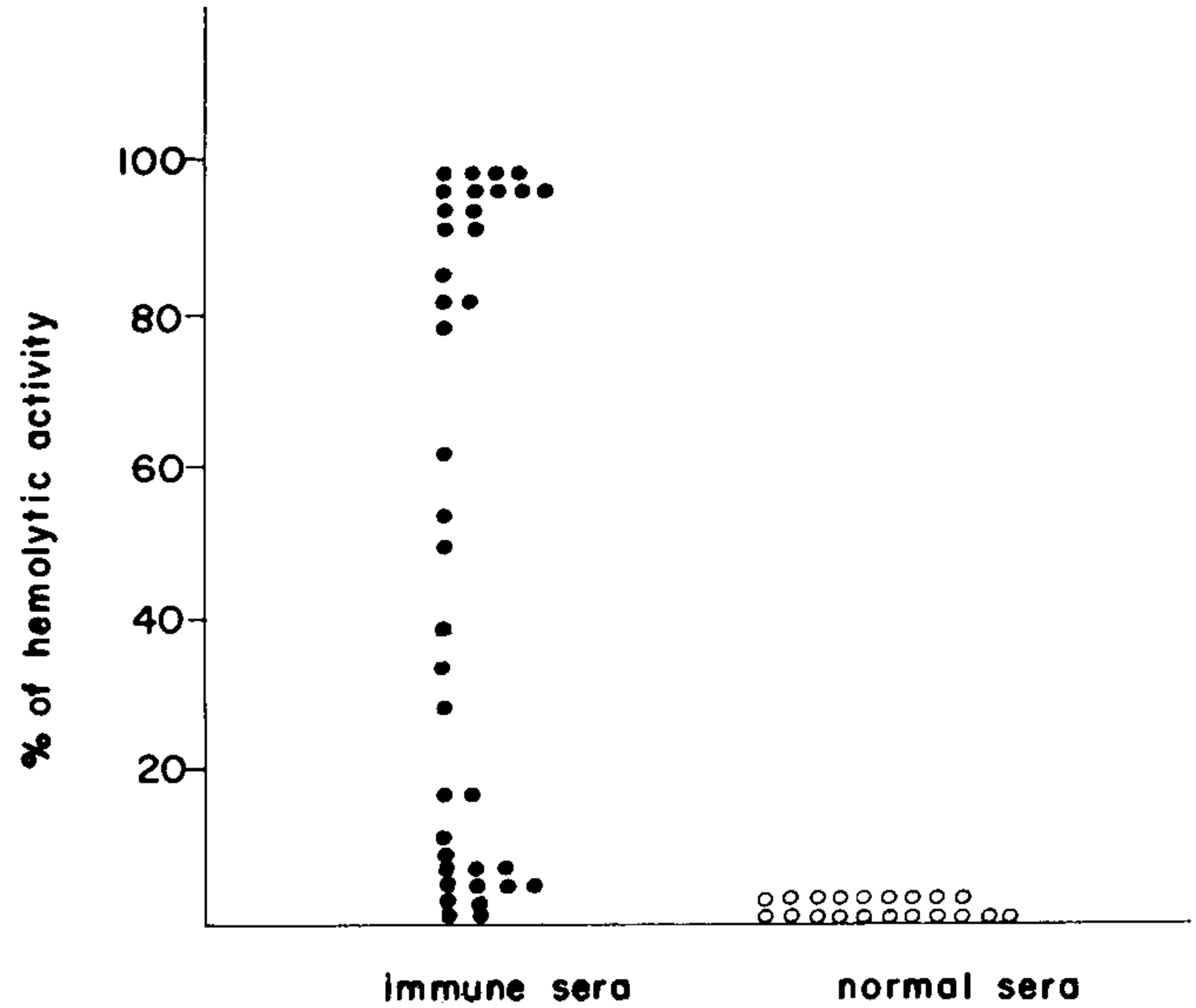

Fig. 2 – Hemolytic activity of 20 normal and 38 immune sera after incubation at 56°C, 60 min. Each ● or ○ = one serum.

about 80% of their original activity in about 4 min (L4 sera). A second group includes 47.1% (33/70), whose activity remained practically unchanged after heating for 60 min (R60 sera). Finally, an intermediary group of sera, 32.9% (23/70) was identified presenting a gradual loss of their hemolytic activity after heating at 56°C for more than 4 min (R4 sera).

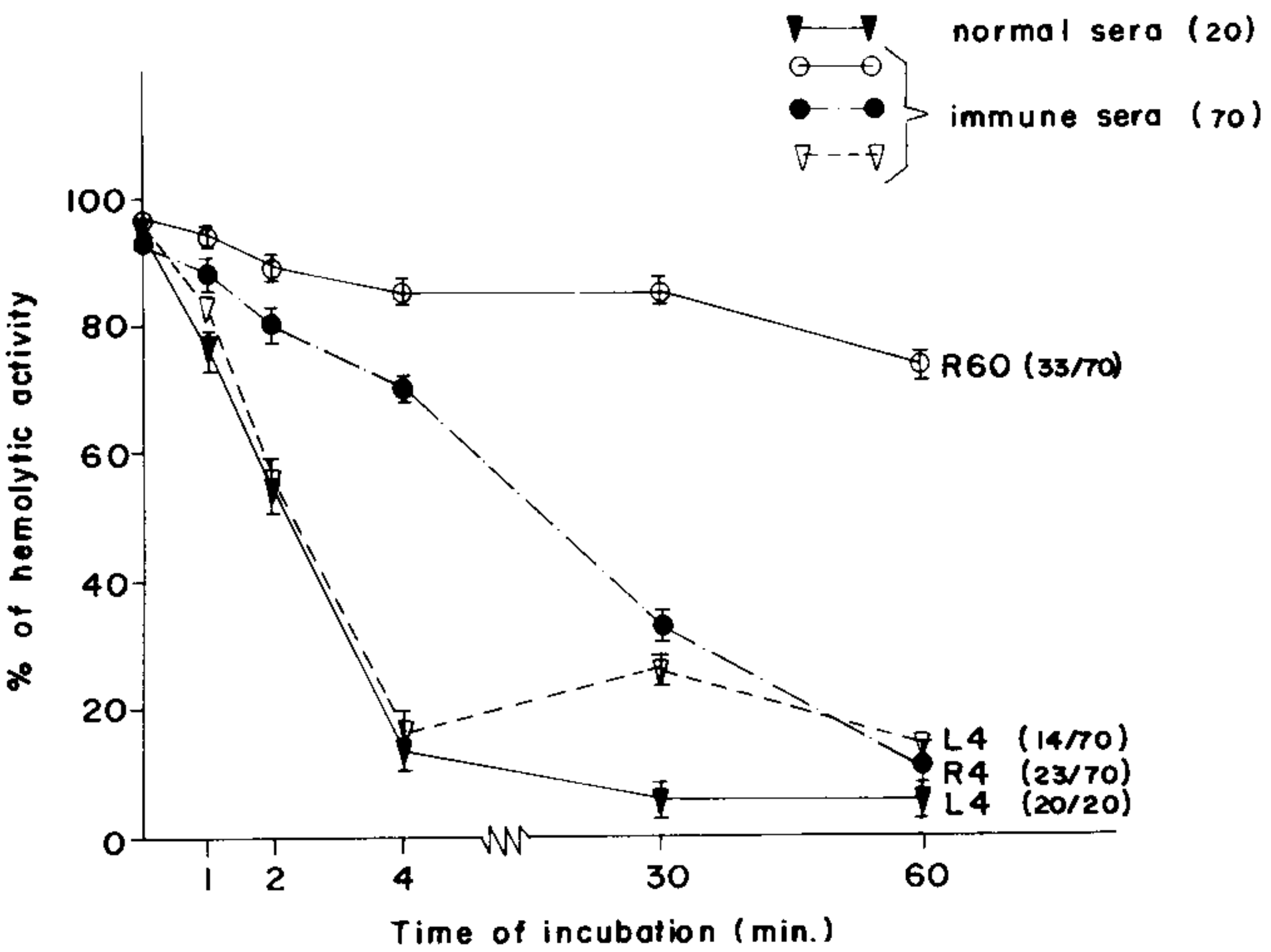

Fig. 3 – Mean percentual hemolytic activity of 20 normal and 70 immune sera after incubation at 56°C during different times.

Gel filtration chromatography of normal, L4, R4 and R60 sera

Further characterization of the heterophil hemolysins belonging to these different groups was attempted by molecular sierve filtration in Sephadex G-200. As shown in Table II, the hemolytic activities of sera from the three groups were eluted in first peak. Pool of ascendent or top fractions of the first peak filtrated showed one line of reaction, with an antiserum towards IgM by double diffusion whereas no reaction with anti-IgG was visible. Pool of descend fractions of this peak showed reaction with an antiserum towards IgM or IgG. Pool of top of the second peak filtrated showed one line of reaction with and antiserum to IgG.

TABLE II

Localization of heterophile hemolysin from normal sera and different types of immune sera (L_4, R_4, R_{60}) in first peak eluted from G-200 Sephadex column

Sera	*Distribution of heterophile hemolysin in first peak*	*Hemolytic Activity**
Normal (3)	Top	70
L_4 (21)	ascendent	19.4
R_4 (12)	ascendent	25
	and descendent	25.7
R_{60} (7)	ascendent	50
	and descendent	86.4

( ) Number of sera in pool.
*Percent of SRBC lysis due to 0.5ml of filtrate.

Antiglobulin test

Experiments were undertaken to detect the antibody class able to react against SRBC membrane after incubating cells with several amounts of heterophil human sera. After washing, specific anti-IgM, anti-IgG, anti-IgA, or anti-IgE antisera were added to the cells. Table III shows that IgG and IgM are detected in all groups of sera examined. In the R60 group IgM were detected at highest dilutions, IgA was barely detected and IgE was not detected. When the sera from infected patients were heated (56°C for 1h) no significant reduction of IgM or IgG titres were observed.

TABLE III

Antiglobulin test and distribution of heterophile antibodies obtained from normal and immune sera in different immunoglobulin classes

Sera pools	*Highest dilution of sera given positive agglutination*			
	Anti-IgM	*Anti-IgG*	*Anti-IgA*	*Anti-IgE*
Normal	40	40	20	0
L_4	20	20	0	0
R_4	80	40	0	0
R_{60}	160	20	20	0

Each pool: six different sera.

Lack of lethal activity against schistosoluma of the heterophil antibody

To investigate the lethal activity of these heterophil antibodies in presence of complement, the cytotoxic assay against schistosomula was performed with a pool of sera from infected patients before and after heating inactivation at 56°C for 1h. After this treatment the hemolytic activity of this pool of sera was completely destroyed. Its lethal activity, however, remained unchanged (Fig. 4).

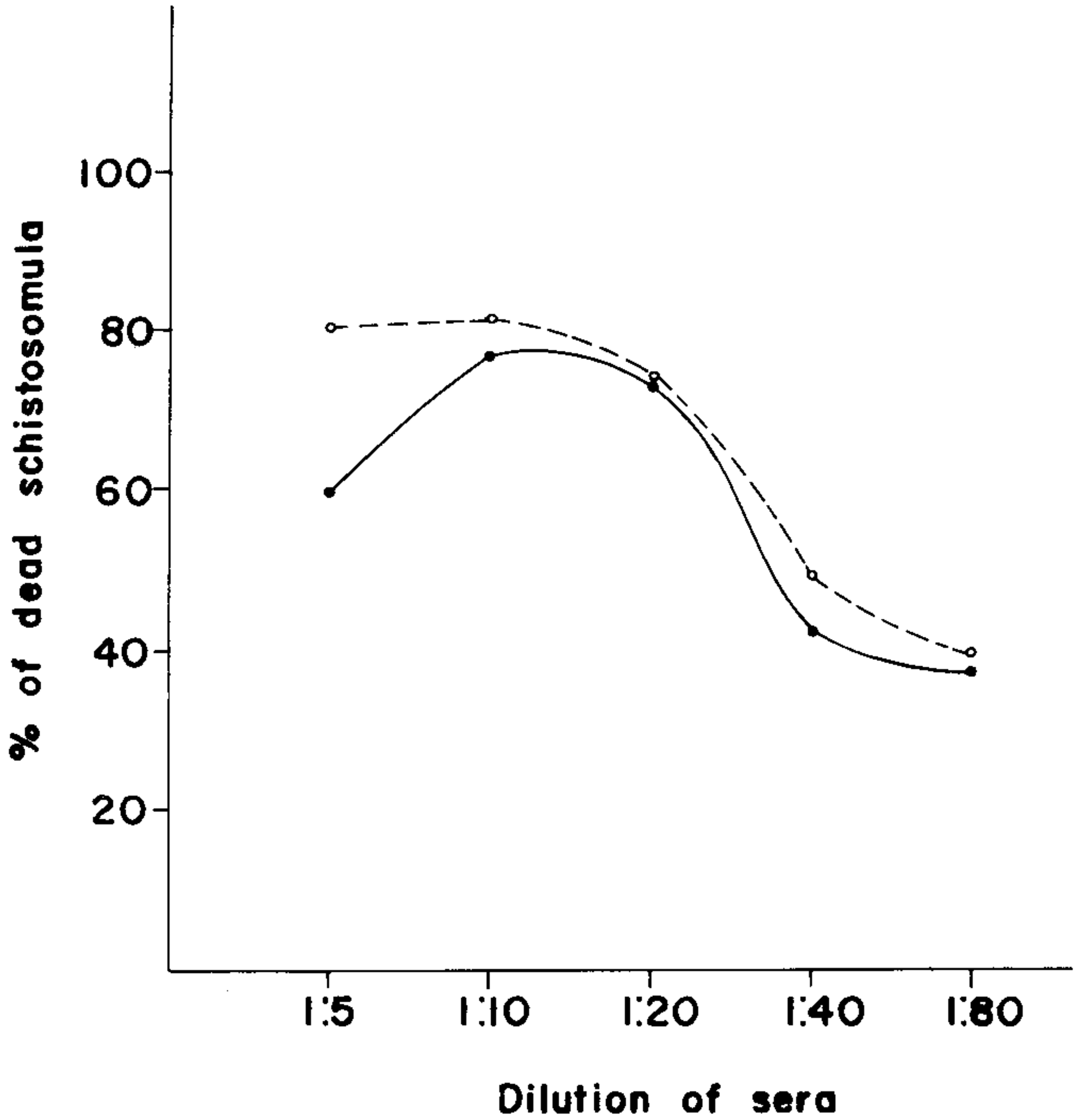

Fig. 4 – Lethal activity against schistosomula of unheated (○ – – ○) and heated (● – ●) pool of 8 immune sera at 56°C for 30 min.

DISCUSSION

The results here presented corroborated and extended those of Perez (1944) who described the presence of hemolysins in sera from patients with chronic schistosomiasis. High levels of hemolytic activities were also detected in sera from normal individuals. These activities, however, could be distinguished from those presented by sera from infected patients by absorption with antigens obtained from different evolutive stages of *S. mansoni*. It was observed, by absorption procedures, hemolysins with higher specificity for several antigen preparations in sera from patients than from normal. Since antibody absorption by antigens depends on the basic characteristic of the antibody molecule (that is, the ability to combine with antigens) (Altemeier, Belianti & Buescher, 1961), this finding suggests the induction of a qualitative fluctuation of heterophil hemolytic antibodies in human host after the parasite infection. It is important to point out that in the concentration used, the antigens showed no detectable anti-complementary effect in the hemolytic assay.

To our knowledge, no fluctuation of specificity of heterophile hemolysin has been described up to know concerning the reactions for SRBC, or evolutionary stages of *S. mansoni*. In *E. coli* strain infected patients is probable that there is a relation between the fluctuation titers of the heterophil antibodies and infection (Borstein, 1942).

The study of the specificity of the heterophil antibodies by absorption technic has been applied for human (Antunes & Pellegrino, 1977; Lee, Takahashi & Davidsohn, 1963; Perez, 1964; Yoshida & Nahmias, 1967) or experimental schistosomiasis (Dean & Sell, 1972) and for human trypanosomiasis, leishamaniasis (Houba & Allison, 1966) mononucleosis (Lee, Takahashi & Davidsohn, 1962; Yoshida & Nahmias, 1967), syphilis (Frei & Heming, 1927) and *Salmonella typhi* vaccination (Altemeier, Belianti & Bueschen, 1961). The human heterophil antibody was absorbed by *S. mansoni* (adult worm) homogenates, guinea pig homogenates by not ox red blood cells (Antunes & Pellegrino, 1967).

The hemolytic activity of control and infected patient sera disappeared under conditions in which the alternative pathway of complement remained intact, while the classic pathway was blocked (EGTA). The 2-mercapto-ethanol was able to inhibit the hemolytic antibodies in the immune and control sera group. The heterophil antibodies in mononucleosis (Lee et al, 1963) or *T. rhodesiense* (Houba & Allison, 1966) infected patients were also sensitive to treatment with sulphidryl reductors.

Heat stability was also used to distinguish between different types of heterophil antibodies from normal or infected sera. It is known that natural heterophil hemolysins of normal human sera against SRBC is heat-labile (Teale, 1934), and present results substantiate this finding. Nevertheless, the hemolytic antibodies of infected patients have a tendency to be more heat-resistant. Heat stability is generally considered to be one of the characteristics of immune antibody (Teale, 1934; Landsteiner, 1945). The antiglobulin test fails to show similar loss of lytic activity by heated sera, showing that the antibodies are different at least when thermal effect and Sephadex G-200 filtration are concerned. Similar results has been described in human mononucleosis infection (Wollheim & Williams, 1966).

The destruction of heterophil hemolysins by heating did not reduce the lethal activity of heterophil labil immune sera (L4) against schistosomula *in vitro*.

In sickness sera disease is not possible to exclude immune complexes formation with heterophil antigen from horse serum and the pre-existent heterophil antibody in the human sera (Ramsdell & Davidshon, 1930; Davidshon, 1930).

In *S. mansoni* infection the part played by the heterophil antibody in the formation of circulating immune complexes is unknown.

Summarizing, the data presented here indicate that heterophil hemolysins in sera from patients with chronic schistosomiasis are heterogeneous. The heterogeneity of these hemolysins was demonstrated not only by their different heat stability, but also by elution profile of gel filtration.

RESUMO

Anticorpos heterófilos foram detectados nos soros de pacientes normais ou com esquistossomose mansoni crônica. Suas atividades hemolíticas dependem da integridade da via clássica do sistema do complemento. Os anticorpos heterófilos dos pacientes esquistossomóticos apresentaram maior especificidade para antígenos de *Schistosoma mansoni* do que aqueles anticorpos detectados nos soros de pacientes normais. A atividade hemolítica do anticorpo nos soros normais podia ser destruída pelo aquecimento des-

tes soros a 56°C durante 4 minutos. Por outro lado, cerca de 80% dos soros de pacientes esquistossomóticos eram parcial ou totalmente resistentes ao mesmo tratamento. As atividades dos anticorpos heterófilos foram eluídas através da filtração em gel, em diferentes frações no primeiro pico.

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